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Determination of tetracationic zinc(II) phthalocyanine derivative RLP068 in rabbit serum by liquid chromatography–tandem mass spectrometry

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Abstract

The development and validation of a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of the tetracationic zinc(II) phthalocyanine derivative RLP068 in rabbit serum is described. The dodecadeuterated product (RLP068-D_{12}) was used as co-eluting internal standard. RLP068 was isolated from serum samples by solid-phase extraction using weak cationic exchange cartridges (WCX). An oxidative derivatisation was used in order to simplify the peculiar HPLC and MS behaviour of the analyte and thus increasing sensitivity. Liquid Chromatography was carried out on a Polaris C18 Ether column (50 mm × 2.0 mm) with an isocratic run of 0.5% aqueous TFA/methanol. Detection was achieved by means of a Bruker Esquire 3000+ Ion Trap Mass Spectrometer equipped with an ESI source working in positive mode. A Multiple Reaction Monitoring method following the transitions 297.1 → 282.1 for the analyte and 300.1 → 282.1 + 285.1 for the internal standard was used. The analytical method was validated over the concentration range 2–65 ng/mL. lower limits of detection (LLOD) and quantification (LLOQ) were respectively 1 and 2 ng/mL. The method is innovative and applicable to pharmacokinetic studies.

Keywords: Zinc(II) phthalocyanine; RLP068

1. Introduction

Since their synthesis early this century, phthalocyanines have attracted chemists' attention for their particular properties. Synthesised as dyes, they have been investigated in several application fields, including chemical sensors, liquid crystals, nonlinear optics and Langmuir–Blodgett films [1,2]. In the last 20 years, research on phthalocyanine derivatives for photodynamic therapy (PDT) and other light driven processes has been increased notably [1–4].

Photodynamic therapy is a bimodal therapeutic strategy based on a drug (called a *photosensitizer*) activated by visible light; it has been proposed as an alternative or as a complement to conventional protocols in the treatment of malignant tumours. To date, PDT based on hematoporphyrin or synthetic porphyrin derivatives as photosensitizers is in an advanced experimental phase for specific clinical applications [5].

The use of porphyrin and phthalocyanine dyes as selective photosensitizers in the photodynamic therapy for selective local inactivation of microorganisms also has a remarkable medical potential, but is not yet fully developed. The improvement of selectivity and efficiency against microorganisms proliferation has led to the proposal of several phthalocyanines as potential photosensitizers to develop into clinical use [6,7].

Despite the growth of knowledge in phthalocyanine derivatives synthesis and medical applications, there's a disappointing inactivity in the evolution of analytical methods for their characterisation and determination in biological samples.

In fact very few papers about HPLC separation of phthalocyanine derivatives have been published and mostly of them are referred to the separation of positional isomers of tetra-substituted phthalocyanine derivatives [8–12]. Phthalocyanine derivatives are difficult HPLC analytes. Because of

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the presence of an extended π region they are prone to adsorption and π-stacking phenomena. In addition to this, derivatives bearing polar peripheral substituent groups, such as amino or ammonium groups, often show an amphiphilic nature (presence of polar and non-polar region in the same molecule) that causes dramatic retention changes with low variation in the chromatographic phases. Finally, for metal coordinated phthalocyanine derivatives, the central metal ion can contribute to peculiar LC behaviour. In fact, coordinative unsaturated metal complexes (as Zn(II) phthalocyanines) are weakly retained in reverse phase HPLC due to mobile phase coordination [11,12].

Methods for determination of phthalocyanine derivatives in low concentration in biological samples are even less reported. Most of them are based on fluorimetric determinations to measure the uptake of photosensitizer in cell, blood or tissues [13,14]. These methods do not guarantee the required selectivity due to the lack of analyte separation from the biological matrix and often the analyte recovery is very scarce at low concentrations; in addition to this the use of an internal standard to control it is not feasible.

To our knowledge only the determination of a sulfonated aluminium phthalocyanine ($AlPcS_4$) in plasma, and of an axially substituted silicon phthalocyanine (Pc4) in plasma and red blood cell concentrated (RBCC), using HPLC methods with visible spectrophotometric or fluorimetric detection have been published [15,16]. The reported methods show excellent recoveries, but only the μM concentration range (μg/mL) was investigated. Often for safety pre-clinical or clinical trials much lower concentrations have to be monitored. In addition there are no publications concerning HPLC methods for the determination of cationic phthalocyanine derivatives.

Mass spectrometric analysis with ESI, APCI or MALDI sources is widely used for the characterisation of newly synthesised metal phthalocyanine derivatives. The isotopic cluster of the coordinated metal is very useful for qualitative purposes, but causes a lowering in sensitivity when diluted solutions are analysed. For example Zn(II) phthalocyanine derivatives show the typical Zn distribution pattern (five most abundant isotopes), causing a strong reduction in signal height versus baseline noise if compared with a non-zincated mass/charge (*m*/*z*) distribution.

We now describe an innovative LC–MS/MS procedure for the determination of a tetrasubstituted Zn(II) phthalocyanine derivative (RLP068) in rabbit serum. This substance is currently under toxicological investigation as photosensitizer intended for topical microbial inactivation. The method is based on a SPE extraction from the serum using weak cationic exchange cartridges ($–COO^-$ groups), followed by an oxidative derivatisation. The procedure affords four phthalimide derivative molecules from each phthalocyanine derivative molecule (a factor 4 increase in the molar concentration of the analyte), simplifies the LC separation by converting the tetracationic into a monocationic analyte and enhances the MS sensitivity by eliminating the zinc atom.

The method was validated, according to FDA requirements for bioanalytical methods [17], over the concentration range 2–65 ng/mL. A deuterated analogue was used as internal standard providing a very strong control of the recovery from the biological matrix.

2. Experimental

2.1. Zn(II) phthalocyanine derivative RLP068, internal standard and chemicals

Tetracationic Zn(II) phthalocyanine derivatives RLP068/Cl ({1(4),8(11),15(18),22(25)-Tetrakis[3-(*N*,*N*,*N*-trimethylammonium)phenoxy]phthalocyaninato} zinc(II) chloride, $M_W = 1320.5$ g/mol) and RLP068-D_{12}/Cl (deuterated internal standard, $M_W = 1332.5$ g/mol) were synthesised by Molteni Organic Synthesis Department as mixtures of three positional isomers and used without further purification [8,18]. Structures are shown in Fig. 1. Batch purities were respectively 99.5% (water content 9.1%, w/w) and 99.9% (water content 19.2%, w/w).

Methanol Hipersolv®, Acetonitrile Hipersolv®, Sulfuric Acid 96% Suprapur®, ammonium acetate, potassium dichromate and sodium hydroxide 1N solution were purchased from Merck (Milan, Italy).

Trifluoroacetic acid Spectranal® was purchased from Riedel-de Haën (Seelze, Germany).

Drug-free rabbit serum was collected from SPF New Zealand rabbits (Charles River, Italy), stored at −40 °C and thawed daily.

Isolation of drugs was performed by SPE using Phenomenex STRATA WCX (55 μm, 70A) cartridges, 100 mg/1 mL (Chemtek Analytica, Bologna, Italy) positioned on a Supelco Visiprep™ DL SPE-device (Milan, Italy) working under vacuum. SPE solutions were dried by means of a Supelco Visidry™ DL device (Milan, Italy) working with nitrogen.

2.2. Stock solutions and calibration samples

Stock solutions of RLP068/Cl and internal standard RLP068-D_{12}/Cl (1 mg/mL in water) were prepared daily from powders stored at −40 °C. Spiking solutions were prepared by further dilutions in water using class A glassware. For the preparation of rabbit serum calibration solutions 30 μL of the appropriate spiking solution were added to 270 μL of drug-free serum to yield the mentioned range of calibration concentrations (1.0, 2.1, 4.1, 8.2, 16.4, 32.8, 49.2, 65.6 ng/mL for analyte, fixed concentration of 28.8 ng/mL for the internal standard). For recovery evaluation standard solutions in water were prepared at 2.5 times higher concentration in order to account for concentration factor of the SPE step.

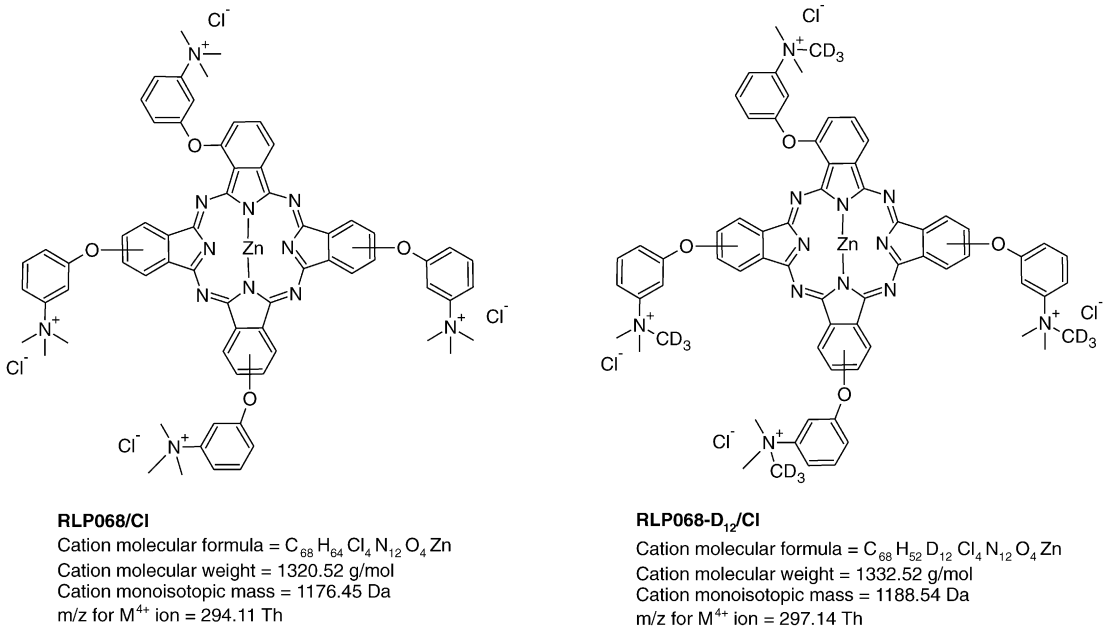

Fig. 1. Chemical structures, molecular weights and monoisotopic masses of the tetracationic Zn(II) phthalocyanine photosensitizer RLP068/Cl and the deuterated internal standard RLP068-D_{12}/Cl.

2.3. Samples preparation

Rabbit serum samples were extracted as follows: 30 μL of 0.5 M NaOH solution were added to 300 μL of spiked serum in order to ensure that albumin bore a net negative charge. After priming the SPE column with 1 mL of methanol and 1 mL of water, 250 μL of sample were allowed to pass through the cartridge. It was rinsed successively with 0.5 mL of 1 mM NaOH solution, 1 mL of water (for salts elimination) and finally 1 mL of methanol (to eliminate other serum components). Elution was achieved by protonation of the WCX polymer with 0.5 mL of an acidic methanol/water solution (methanol/water 9:1, v/v, with 2% TFA). The eluent was dried under a gentle stream of nitrogen and the residue was derivatised adding 50 μL of the derivatisation reagent solution ($K_2Cr_2O_7$ 100 μM in 1% H_2SO_4). After 2 h the pH was adjusted adding 50 μL of ammonium acetate 0.2 M in water.

2.4. Instrumentation

2.4.1. HPLC system

An Agilent HP1100 system (Agilent Italia, Milan, Italy) equipped with vacuum degasser device, binary pump, autosampler and column heater was used. Separation was performed on a Varian Polaris C18 Ether column (50 mm × 2.0 mm, 5 μm particles) at 25 ± 1 °C with a mobile phase of 0.5% (v/v) aqueous TFA/methanol (81:19, v/v). Mobile phase was prepared by mixing 805 mL of water with 4 mL of trifluoroacetic acid and then adding 190 mL of methanol. Flow rate was 0.2 mL/min and 20 μL injection were performed (the needle was washed with methanol). Run time was 8 min. Back-pressure was 40 bar and a slight increase for long analytical sessions was observed. A precautionary counter-flow washing (20 min) with water (0.5% TFA)/methanol (1:1, v/v) was used every 50 injections.

2.4.2. ESI-MS ion trap system

All mass spectrometric measurements were performed on a Bruker Esquire 3000+ Ion Trap Mass Spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray source working in positive ion mode. The instrument was connected with the HPLC system outlet via peek tubing and the divert valve was programmed to waste

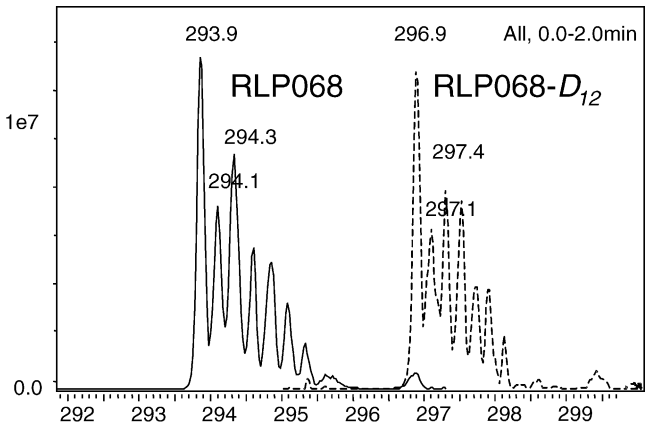

Fig. 2. MS spectra of the M^{4+} ions of RLP068 and the internal standard RLP068-D_{12}. Recorded from 10 μg/mL solutions in acetonitrile/water (1:1, v/v) with maximum scan resolution.

the first 2.5 min of the chromatographic run. MS parameters were the following: scan range $m/z = 250$–320, scan speed 13.000 $m/z\,s^{-1}$ with unit resolution (if not else specified), Nebulizer flow 35 psi, dry gas flow 8.0 L/min, dry temperature 300 °C, capillary 4 kV, skimmer 40 V, ion charge control target 20,000, maximum accumulation time 200 ms, spectra averages 5, rolling averages 2.

Quantification was performed by multiple reaction monitoring (MRM) of the derivatisation products of the analyte and of the internal standard. Data were processed by means of QuantAnalysis™ (Bruker Daltonics, Bremen, Germany) using the internal standard method plotting peak area ratios versus relative concentration analyte/IS without weighting factor. The transitions 297.1 → 282.1 for the analyte and 300.1 → 282.1 + 285.1 for the internal standard were monitored using an isolation width of 3.0 Da and a fragmentation amplitude of 1.10 V.

To determine recovery values from the biological matrix for the analyte (at every calibration concentration in the investigated range) and for the internal standard, transition peak areas obtained from serum calibrators were divided by transition peak areas from standard solutions in water.

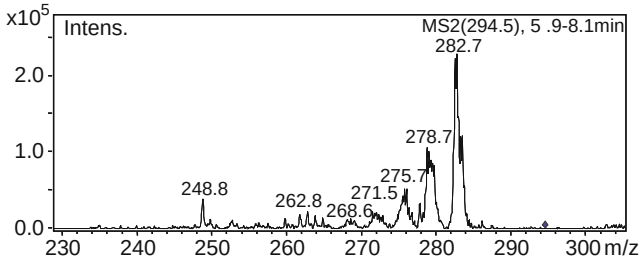

Fig. 3. MS/MS spectra of RLP068 (M^{4+}). Recorded from 10 μg/mL solution in acetonitrile/water (1:1, v/v) with normal scan resolution. (Isolation width 3 Da, fragmentation amplitude 1.2 V.)

3. Results and discussion

3.1. Derivatisation procedure

As described in the introduction, HPLC analysis of Zn(II) phthalocyanine derivatives is a very challenging task; the photosensitizer RLP068 is a mixture of three positional isomers of a Zn(II) phthalocyanine derivative carrying four peripheral quaternary ammonium groups. All these features contribute to the complicated LC behaviour. In fact both

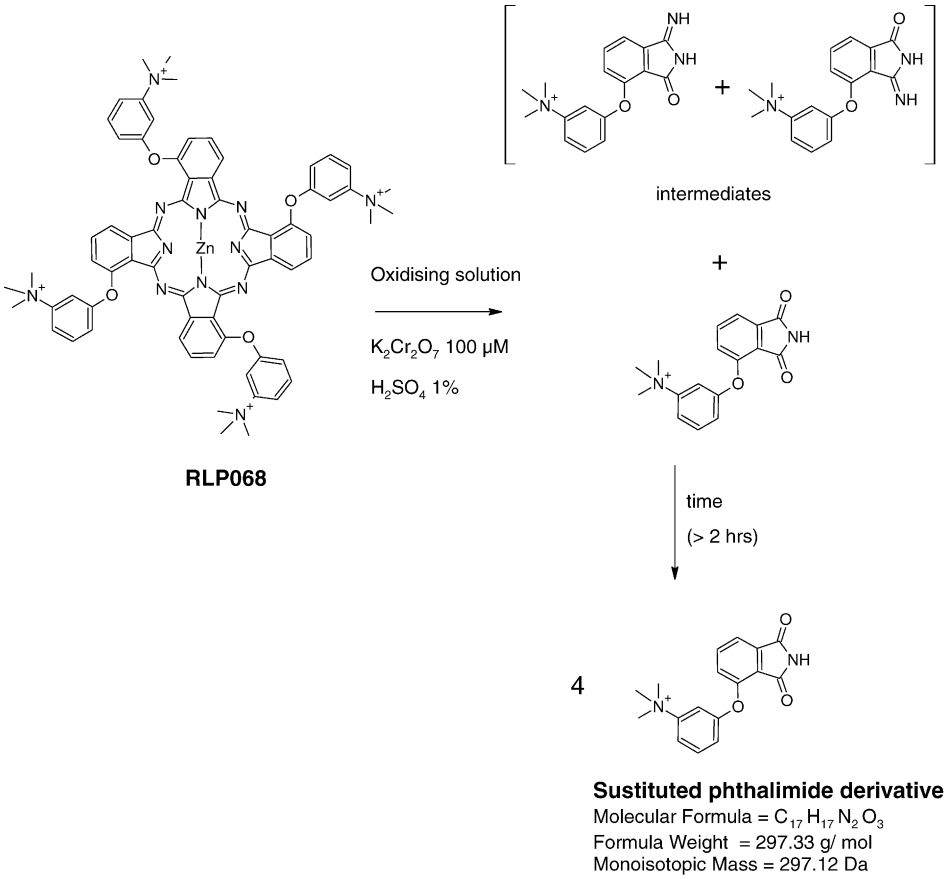

Fig. 4. Oxidative derivatisation of RLP068 to a substituted phthalimide derivative (four times increase in molar concentration). Analogously, the internal standard RLP068-D_{12} gives a D_3 substituted phthalimide derivative.

the zinc atom as well as ammonium groups result in a great affinity of the analyte for residual silanol groups even on encapped reverse phase materials. In addition to this the macrocycle π region causes adsorption phenomena on HPLC column walls and frits. This led to an irreversible retention at analyte concentrations lower than 4 μg/mL (data not shown). This disappointing behaviour has been observed on a wide variety of reverse phase packings (i.e. Phenomenex LUNA C18(2) and C8(2), Phenomenex Synergi Hydro-RP and Max-RP, Varian Polaris C18 Ether and C18 Amide). It is also worth mentioning that RP separation of phthalocyanine derivatives requires gradient elution (long run times) or ion pair reagents (MS incompatible).

The presence of the central zinc atom in RLP068 affects also mass spectrometric sensitivity. In fact the ions are distributed according to the isotopic pattern of the metal atom (see Fig. 2), that has five most abundant isotopes. Therefore relatively high concentrations of RLP068 and internal standard are required in order to discriminate their MS signals from the baseline noise.

Finally the presence of four peripheral substituent groups led to complex MS/MS spectra (see Fig. 3). The phthalocyanine aromatic ring is not sensitive to fragmentation, so only loss of the substituents occurs. The lowest energy pathway is the loss of the methyl groups, thus in the MS/MS spectra ions with a different number of methyl groups are observed. Even though the ion trap fragmentation amplitude was optimized in order to maximise one specific daughter ion (loss of three methyl groups, $m/z = 282.8$ for M^{4+}), only a 20% fragmentation yield is obtained.

All these problems have been solved by a derivatisation procedure. The cleavage of the macrocycle region for phthalocyanine derivatives is a well-known process, often named as bleaching (or photobleaching if it is driven by light). Zn(II) phthalocyanine derivatives are very sensitive to oxidants and we found that treatment of RLP068 with strong oxygen donors (dichromate or nitric acid) led to phthalimide-like products shown in Fig. 4. The kinetic of oxidation of a 10 μg/mL RLP068 aqueous solution in 1% H_2SO_4 containing $K_2Cr_2O_7$ 100 μM was monitored by LC–MS. After 30 min from the preparation there was no RLP068 left and a mixture composed by 96% substituted phthalimide derivative and 4% intermediates was observed. After 90 min only the substituted phthalimide derivative was present. Longer reaction times did not show the formation of other products. Using 6.5% HNO_3 in water as oxidising agent the reaction time must be increased to 240 min. The derivatisation of RLP068 aqueous solutions at different concentration levels (in the range 0.020–20 μg/mL), afforded solutions of the substituted phthalimide derivative of proportional concentration. This ensured that the procedure was linear and reproducible over a wide range of concentration.

In conclusion, letting the derivatisation reaction go to completion, a quantitative conversion of one RLP068 molecule into four substituted phthalimide derivative molecules was obtained. Thus a four times increase in molar concentration of the analyte is achieved. Moreover the derivatisation product is a much better LC–MS/MS analyte compared to the precursor.

3.2. HPLC–MS/MS

After the derivatisation step a low molecular weight, monocationic and not amphiphilic analyte, corresponding to the substituted phthalimide derivative is obtained. These characteristics made the HPLC step much easier, permitting an isocratic run without ion pairing reagents. It is worth to be mentioned that while RLP068 is composed by several positional isomers, only one derivative product is obtained. This process increases four times the molar concentration of the analyte and therefore contributes to the overall sensitivity of the method.

An enhancement in MS/MS sensitivity is also obtained because of the loss of the zinc atom with its broad isotopic pattern and the simplification of the fragmentation pathway.

In Fig. 5, MS and MS/MS spectra of the derivatisation products of RLP068 and the internal standard are shown. The lowest energy fragmentation is again the loss of a

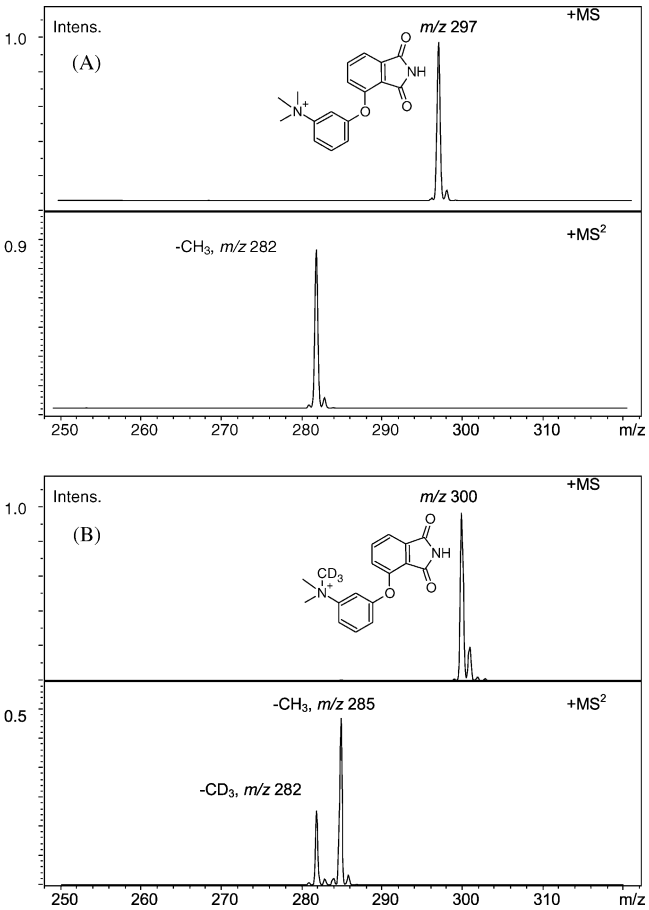

Fig. 5. MS/MS spectra of substituted phthalimide derivatives obtained by oxidizing derivatisation of RLP068 (A) and its internal standard RLP068-D_{12} (B). Recorded from 10 μg/mL solutions in acetonitrile/water (9:1, v/v).

methyl group, but now only one daughter ion is observed for RLP068, with an 85% fragmentation yield.

Obviously, in the derivatisation step the internal standard RLP068-D_{12} is converted into the corresponding D_3 substituted phthalimide derivative. Therefore the MS/MS spectrum of the internal standard derivative shows two abundant signals with a relative ratio of 2:1, due to the loss of CH_3 ($m/z = 285.1$) or CD_3 ($m/z = 282.1$).

Fig. 6 presents an HPLC–MRM chromatogram obtained by injecting standard solutions of the derivatisation products in 0.1 M ammonium acetate and following the transitions 297.1 → 282.1 for the analyte and 300.1 → 282.1 + 285.1 for the internal standard. Co-elution of analyte and internal standard was observed; the retention time was 6.3 ± 0.1 min ($n = 24$) and the run time was set at 8 min.

A mobile phase containing 0.5% aqueous trifluoroacetic acid and methanol was preferred to aqueous acetic acid and methanol mixtures because of the better performance when used for the rabbit serum samples.

3.3. Rabbit serum calibrators—SPE

As described in the introduction, to date no extraction methods compatible with HPLC–MS/MS analyses are available for the determination of phthalocyanine derivatives in biological fluids or tissues. This represents a huge limiting factor for toxicological studies required for biomedical applications of such a product. The typical extraction procedure from serum, plasma and tissues relies on a digestion with 2% sodium dodecylsulfate solution in water, followed by protein precipitation with a chloroform/methanol mixture (1:2, v/v). According to the experiments performed in our laboratories, such a procedure gives a 35–50% recovery for RLP068 from rabbit serum in the concentration range

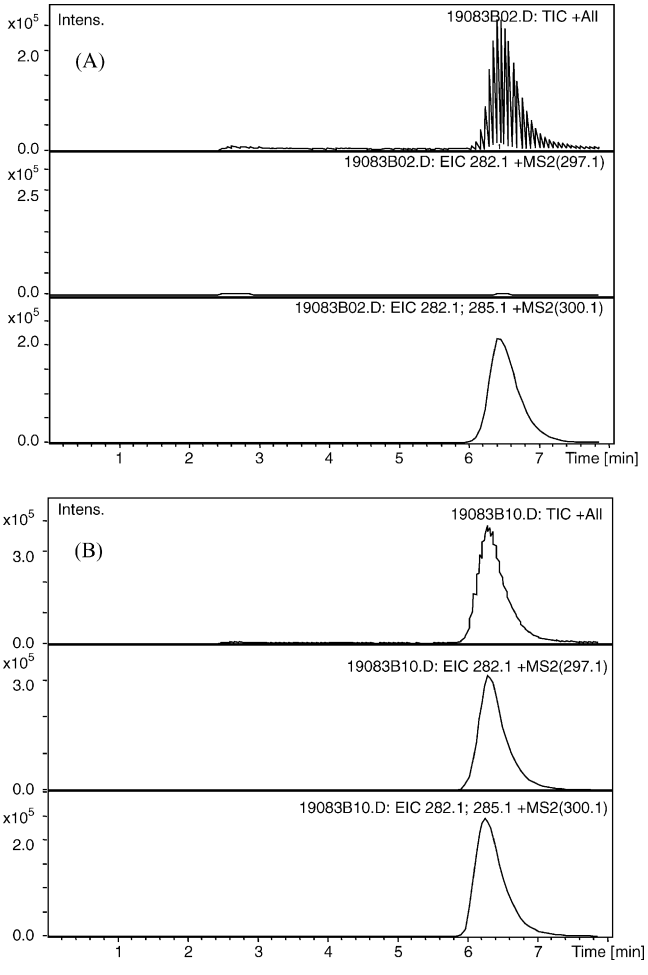

Fig. 6. HPLC–MRM chromatograms (multiple reaction monitoring) obtained by injecting standard solutions of the derivatisation products in 0.1 M ammonium acetate. Concentration levels: (A) RLP068 0.0 ng/mL, IS 72 ng/mL (blank); (B) RLP068 82 ng/mL, IS 72 ng/mL. Transitions followed: 297.1 → 282.1 for the analyte and 300.1 → 282.1 + 285.1 for the internal standard. MS/MS parameters: isolation width 3.0 Da, fragmentation amplitude 1.10 V.

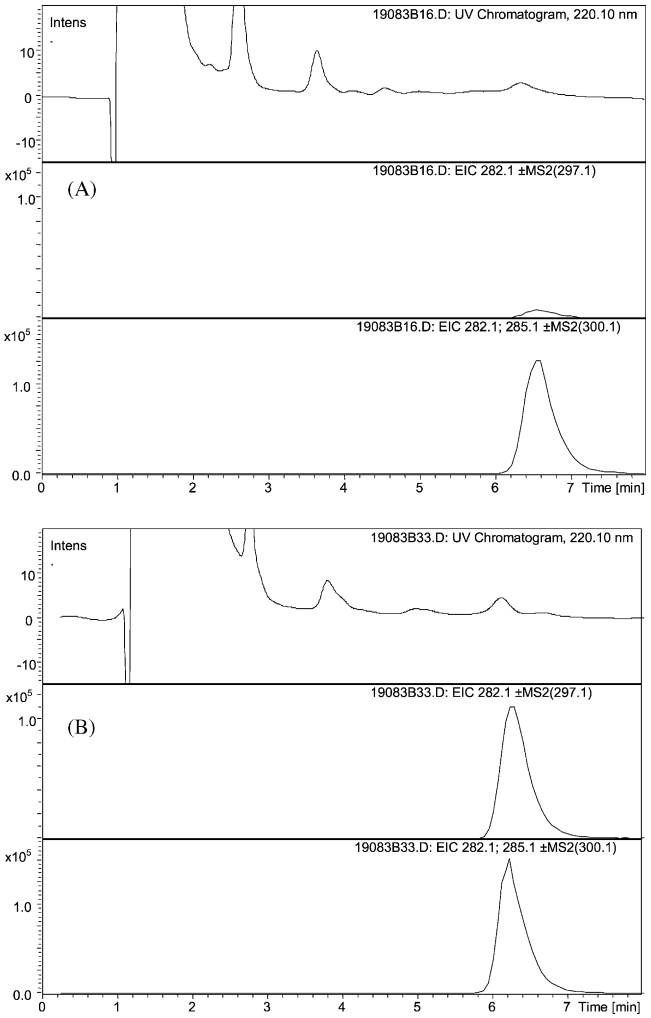

Fig. 7. HPLC–MRM chromatograms (multiple reaction monitoring) obtained by injecting rabbit serum calibration solutions. Concentration levels: (A) RLP068 0.0 ng/mL, IS 28.8 ng/mL (blank); (B) RLP068 32.8 ng/mL, IS 28.8 ng/mL. Transitions followed: 297.1 → 282.1 for the analyte and 300.1 → 282.1 + 285.1 for the internal standard. MS/MS parameters: isolation width 3.0 Da, fragmentation amplitude 1.10 V.

Table 1
Between-day validation data in the concentration range 2.05–65.7 ng/mL of RLP068 in rabbit serum (IS concentration 28.8 ng/mL)

Between-day[a]									
Nominal conc. (ng/mL)	0.00	2.05	4.10	6.16	8.21	16.4	32.8	49.3	65.7
Calculated conc. (ng/mL)	–	1.91	3.91	4.64	8.94	17.6	34.7	48.9	64.8
Accuracy (%)[b]	–	92.9	95.2	75.4	108.9	107.0	105.8	99.3	98.6
Precision (R.S.D., %)[c]	5.4	8.8	4.5	5.6	7.4	4.8	7.4	4.3	2.4
Extraction recovery (%)[d]	–	40	41	35	35	42	42	41	40
IS extraction recovery (%)[d]	44 ($n = 50$)								
Regression analysis	$y = (0.07 \pm 0.01) + (0.53 \pm 0.01)x$ $R = 0.9990$ $N = 10$ S.D. $= 0.0194$ $P < 0.0001$								

Data elaborated by means of QuantAnalysis™ (Bruker Daltonics, Bremen, Germany) using the internal standard method plotting peak area ratios vs. relative concentration analyte/IS.

[a] Calculated in five series.

[b] Found/nominal × 100.

[c] Relative standard deviation.

[d] In comparison with not extracted standard solutions in water.

50–150 ng/mL. The recovery decreases strongly below 50 ng/mL of analyte and is negligible at a 15 ng/mL level, thus not permitting pharmacokinetic measurements when low amounts of drug are administered or a low adsorption is expected, as in topical applications. The main reason for the low recovery of RLP068 from serum is the high affinity of the drug for albumins. The constant of binding with bovine serum albumin (BSA), measured via fluorimetric titration, was found to be $9 \times 10^3\ M^{-1}$ (data not shown).

We solved the problem using solid-phase extraction with STRATA WCX (Weak Cationic eXchange) cartridges carrying carboxylate groups. The retention of the analyte on the stationary phase was achieved by the interaction of the quaternary ammonium groups of the analyte with $–COO^-$ of the stationary phase. The rabbit serum was basified adding a NaOH solution prior to the SPE step in order to exceed isoelectric point of serum albumins. After the sample loading, the SPE cartridges were rinsed with 1 mM NaOH solution, water and then methanol to eliminate salts and lipoproteins, respectively. The analyte and the internal standard were eluted with acidic methanol/water (methanol/water, 9:1, v/v, with 2% TFA) leading to a protonation of the carboxyl groups of the solid phase. In this way a very clean final solution was obtained.

Table 1 shows that the recovery from serum was 35–42% for RLP068 in the range of 2–65 ng/mL and 44% for RLP068-D_{12} internal standard at 28.8 ng/mL concentration ($n = 50$). The recovery was calculated against unextracted standards in water (as suggested by FDA guideline) and was found to be consistent, precise and reproducible for concentration levels in serum as low as 2 ng/mL. A mean recovery close to 40% in the investigated range that is a good result considering the strong affinity of the analyte to serum albumins.

It is worth noting that the use of a deuterated internal standard guarantees a very good control on analyte recovery.

Fig. 7 presents the HPLC–MRM chromatograms obtained from a blank injection and from rabbit serum spiked with RLP068 (32.8 ng/mL) calibrators. Both of the samples contained 28.8 ng/mL of the internal standard. A very low baseline noise can be observed.

Also RP-C18 SPE cartridges have been investigated for RLP068 extraction. After the priming of the SPE cartridge and the serum sample loading, it was washed with water (0.1% AcOH)/methanol (8:2, v/v) and finally eluted with acidic methanol (0.1% TFA). Silica based phases (Phenomenex STRATA-U and STRATA-E) gave very low recoveries (lower than 2%), while polymer based phases (Phenomenex STRATA-X and Waters OASIS HLB) worked with a mean recovery of 12–15% in the range 8–65 ng/mL.

3.4. Validation

The validation data for the rabbit serum samples are presented in Table 1. Linear correlation was verified for RLP068 over the range of 2–65 ng/mL with a fixed concentration of the internal standard (28.8 ng/mL), using nine calibration levels. The linear fit equation was $y = (0.07 \pm 0.01) + (0.53 \pm 0.01)x$, with a correlation coefficient $R = 0.9990$. The method precision was excellent. Between-day precision (CV, %) was lower than 10% in the whole range. Accuracy was also very good and only one point exceeded the ± 15% interval. It is worth mentioning that the quantitative data are obtained by means of an ion trap mass spectrometer.

LLOQ and LLOD were found to be as low as 2 and 1 ng/mL. At the LLOD concentration level the signal to noise ratio was notably greater than 10, but it was not possible to improve the LLOD because of the not negligible interference found in blank serum calibrators; method validation was performed in the same facility were RLP068 was synthesised and Active Pharmaceutical Ingredient (API) specifications were checked. For this reason drug free matrices

and samples were difficult to maintain, especially during the SPE step. So the interference is likely from RLP068 contamination. When the calibration was performed with standard solutions prepared in water (without extraction) a negligible analyte peak was observed in the chromatogram of the blank (containing only the internal standard). The *y*-axis intercept from the linear regression analysis was not statistically different from zero. With the unprocessed calibration samples in water the LLOQ was as low as 0.2 ng/mL RLP068.

Detection and quantitation limits from serum samples can be lowered by increasing the sample volume applied onto the SPE cartridges. Currently, only 250 μL of serum have been used. This amount can be increased by a factor of 4 without changing the size of the SPE cartridges.

4. Conclusion

We described an innovative HPLC–MS/MS method for the determination of a tetracationic Zn(II) phthalocyanine photosensitizer in serum. Although phthalocyanine derivatives are currently under investigation as drug substances for cancer or antimicrobial photodynamic therapy, to our knowledge no reliable analytical method was available in order to follow pre-clinical or clinical toxicology studies.

We described a very reproducible method that is based on solid-phase extraction (SPE) from serum without using surfactants or other MS incompatible substances. Only 250 μL of serum were required, allowing pharmacokinetic studies even on small size animals. We introduced a derivatisation procedure that enhances MS–MS sensitivity. Besides, the chromatography of the resulting derivatives is greatly simplified compared to the phthalocyanine macrocycle itself. The derivatisation step has proved to be fast, stoichiometric and quantitative. We demonstrated linearity, accuracy, precision and good recoveries from serum over the range from 2 to 65 ng/mL of analyte with LLOQ and LLOD values respectively of 2 and 1 ng/mL. In conclusion, considering the overall performances of the reported method, we believe it constitutes a good improvement if compared with those based on fluorimetric detection.

Acknowledgements

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