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LC–MS/MS determination in rabbit plasma of the main photoproduct of RLP068/Cl, a cationic sensitizer proposed for photodynamic therapy (PDT) of microbial infections

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Abstract

The clinical development of a sensitizer for photodynamic therapy (PDT) requires the structural identification of the photoproducts and their quantification in biological fluids and tissues. We describe the LC–MS identification of the most important photoproducts of a cationic phthalocyanine sensitizer (RLP068/Cl) and a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of the main photoproduct (the cationic phthalimide derivative 3-[(1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl)oxy]-*N*,*N*,*N*-trimethylbenzenaminium chloride) in rabbit plasma. The tri-deuterated product was used as co-eluting internal standard. The cationic photoproduct was isolated from plasma samples by protein precipitation with perchloric acid in methanol (7%, v/v). HPLC step was performed on a Phenomenex Synergi Hydro-RP column (20 mm × 2.0 mm, 2 μm particles) with a mobile phase of 0.5% (v/v) aqueous TFA/methanol (85:15, v/v). Flow rate was 0.2 mL/min and 40 μL injection were performed. Run time was 10 min. 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 0.46–91.2 ng/mL and lower limits of detection (LLOD) and quantification (LLOQ) respectively of 0.2 and 0.5 ng/mL were found.

Keywords: Phthalocyanine; Photodynamic therapy (PDT); Photoproduct; Ion trap mass spectrometer

1. Introduction

Photodynamic therapy (PDT) is a bimodal therapeutic technique based on the action of a drug (called photosensitizer) activated by UV or visible light. It was originally proposed for the treatment of malignant tumours [1], but in the last years the interest in the use of PDT for selective inactivation of microorganisms has grown notably [2,3]. A role in this renewed interest has been played by the concern about the large variety of pathogens and the rapid increase in antibiotic resistance phenomena [4,5].

The possibility of using synthetic porphyrin or phthalocyanine dyes with tuneable chemical-physical properties, coupled with the development and the miniaturisation of dedicated light sources has led to the proposal of several photosensitizers to develop into clinic. The preclinical and clinical toxicological trials of a newly synthesised active pharmaceutical ingredients (API) require reliable methods for the determination of the administered product and its metabolites in biological fluids or tissues [6]. Obviously, the methods should provide sufficient specificity and sensitivity to ensure the safety of the treatment. The toxicological investigation of a PDT sensitizer is a bit more complicated. Usually, the activation performed by the light leads to a partial degradation of the drug: the extent of this process, known as photobleaching, depends on both the experimental protocol (irradiation

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time and fluence rate in mW/cm^2) and the properties of the sensitizer. For sure, photoproducts originated during the irradiation of the drug have to be identified and their toxicity have to be investigated.

As we reported in a previous publication [7], phthalocyanine derivatives are difficult HPLC analytes and a very few papers about their chromatographic separation have been reported [8–12]. The same occurs for bioanalytical methods for the determination of such products in biological matrices [7,12–14].

Until now the process of photobleaching of phthalocyanine derivatives is not fully understood; the formation of products related to phthalimide and the influence of the solvent have been observed [15–17], but a lot of work have to be done to reach a satisfactory knowledge of the degradation pathways activated by light. However, the investigation of the main photoproducts of a PDT sensitizer and the development of methods for their measurement in biological fluid or tissues are fundamental tools to ensure the safety of a PDT clinical protocol.

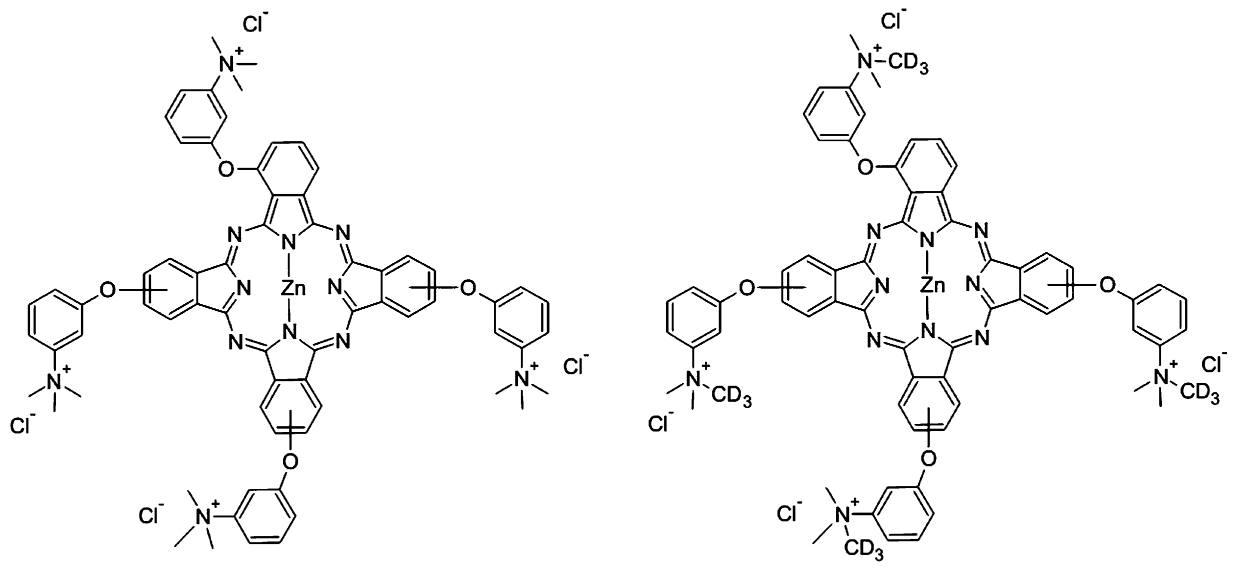
We now report a LC–tandem-MS method for the determination in rabbit plasma of the main photoproduct of a cationic Zn(II)-phthalocyanine derivative (RLP068/Cl). The sensitizer is currently under toxicological trials and a LC–tandem-MS method for its determination has been already published [7].

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*-trimethylamonium)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 [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®, sulfuric acid 96% Suprapur®, ammonium acetate, acetic acid (for HPLC, 50% solution in water), potassium dichromate and perchloric acid 70% Suprapur® solution were purchased from Merck (Milan, Italy). Trifluoroacetic acid Spectranal® was purchased from Riedel-de Haën (Seelze, Germany). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (Milan, Italy). Drug-free rabbit plasma was obtained from SPF New Zealand rabbits (Charles River, Italy) using Alsever solution purchased from Sigma–Aldrich (Milan, Italy) as anticoagulant, stored at −40 °C and thawed daily.



RLP068/Cl
Molecular formula = $C_{68}H_{64}Cl_4N_{12}O_4Zn$
Molecular Weight = 1320.52 g/mol
Cation monoisotopic mass = 1176.45 Da
m/z for M^{4+} ion = 294.11 Th

RLP068-D$_{12}$/Cl
Molecular formula = $C_{68}H_{52}D_{12}Cl_4N_{12}O_4Zn$
Molecular Weight = 1332.52 g/mol
Cation monoisotopic mass = 1188.54 Da
m/z for M^{4+} ion = 297.14 Th

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.

2.2. LC–MS identification of the photoproducts of the Zn(II)-phthalocyanine derivative RLP068/Cl

Photobleaching experiments were conducted on solutions of the cationic Zn(II)-phthalocyanine derivative RLP068/Cl prepared in water and in aqueous solution of bovine serum albumin (BSA). The solvents were chosen in order to investigate the main photodegradation pathways in aqueous formulations for topical application. BSA concentration was set to 0.2 μM (13.2 mg/mL) to simulate the protein content of exudates of localised infections. The effect of the red light used in the available clinical protocols (wavelength range 630–730 nm) was evaluated.

2.2.1. Stock solutions of Zn(II)-phthalocyanine derivative RLP068/Cl

Stock solutions of RLP068/Cl (1 mg/mL in water or 0.5 mg/mL in BSA 0.2 μM) to be used for photobleaching experiments were prepared daily from powders stored at −40 °C.

2.2.2. Irradiation of the RLP068/Cl solution in water

The irradiation experiments were performed using an halogen light source (Waldmann PDT1200, Germany), equipped with a set of bandpass filters to isolate the 630–730 nm spectral window. The lamp fluence rate was 100 mW/cm^2. Two milliliters of RLP068/Cl solution (concentration 1 mg/mL in water or 0.5 mg/mL in BSA 0.2 μM) were placed in a 20 mL glass becher (obtaining a liquid thickness of approximately 0.5 cm) and irradiated for a fixed time (20 or 60 min). To avoid thermal degradation of the product, the sample was cooled with a water/ice bath. Irradiated solutions prepared in water were directly injected in the LC–MS system and compared with the not irradiated one. Samples containing bovine serum albumin were processed as follows: 125 μL of a 7% (v/v) solution of $HClO_4$ in methanol were added to 500 μL of sample to achieve protein precipitation. The sample was kept at −20 °C for 10 min and centrifuged for 10 min at 4000 rpm. Finally, 400 μL of surnatant were separated and buffered adding 100 μL of a 1 M ammonium acetate solution before LC–MS analysis.

2.2.3. Instrumentation

2.2.3.1. 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). Temperature was set at 25 ± 1 °C and a gradient elution of acidic water (acetic acid 0.1%, v/v) and methanol was used. Mobile phase composition was varied from 15% to 100% methanol in 20 min. Flow rate was 0.2 mL/min and injection volume was set at 5 μL. UV–vis detection was performed with diode array detector G1315B (Agilent, Milano, Italy) operating at 220 ± 20 nm for UV components and at 690 ± 20 nm for phthalocyanine derivatives.

2.2.3.2. ESI-MS ion trap system. Mass spectrometry investigation of the photoproducts formed from RLP068 irradiation were performed with 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. MS parameters were the following: scan range $m/z = 100–1000$, scan speed 13.000 m/z s^{-1}, nebulizer flow 35 psi, dry gas flow 8.0 L/min, dry temperature 300 °C, capillary −4 kV, skimmer 40 V, ion charge control (ICC) target 20,000, maximum accumulation time 200 ms, spectra averages 5, rolling averages 2.

2.3. LC–tandem-MS determination of 3-[(1,3-dioxo-2,3-dihydro-1H-isoindol-4-yl)oxy]-N,N,N-trimethylbenzenaminium chloride (the main photoproduct of RLP068/Cl) in rabbit plasma

2.3.1. Stock solutions and calibration samples of the photoproduct

It is not easy to obtain a reference standard of the cationic phthalimide derivative 3-[(1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl)oxy]-*N*,*N*,*N*-trimethylbenzenaminium chloride; the only way is to synthesize it alkylating (with methyl iodide, for example) the corresponding dimethylaminophenoxy compound. However, this reaction affords mixtures of mono- and di-alkylated phthalimide derivatives, because the phthalimide nitrogen atom is more prone to alkylation than the desired aminic nitrogen atom. We found useful and very reproducible to prepare the standard solutions of the analyte and internal standard by oxidation respectively of the Zn(II)-phthalocyanine derivative RLP068/Cl and the dodecadeuterated analogue (RLP068-D_{12}/Cl) with 100 μM K_2CrO_7 in 1% H_2SO_4 [7].

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. Stock solutions of the analyte and of the internal standard were prepared by adding 100 μL of the phthalocyanine stock solution to 900 μL of 100 μM K_2CrO_7 in 1% H_2SO_4. After 2 h, the oxidation reaction is completed and the reaction was quenched diluting 1:10 (v/v) the derivatised solution in ammonium acetate 0.1 mM. The obtained 10 μg/mL solutions were stored in glass flask at room temperature and were proved to be stable for 1 week. Spiking solutions were prepared by further dilutions in water using class A glassware. For the preparation of rabbit plasma calibration solutions 50 μL of the appropriate spiking solution were added to 450 μL of drug-free plasma to yield the mentioned range of calibration concentrations (0.2, 0.5, 0.9, 2.3, 4.6, 6.8, 9.1, 22.8, 68.4, 91.2 ng/mL for analyte, fixed concentration of 9.1 ng/mL for the internal standard).

2.3.2. Samples preparation

Rabbit plasma samples were processed with a protein precipitation technique optimised for polar analytes [19]. One hundred and twenty-five microliters of a 7% (v/v) solution of

$HClO_4$ in methanol were added to 500 μL of spiked plasma. The sample was mixed by means of a vortex, kept at −20 °C for 10 min and finally centrifuged for 10 min at 4000 rpm. Four hundred microliters of surnatant were separated and buffered adding 100 μL of a 1 M ammonium acetate solution before LC–MS analysis.

2.3.3. Instrumentation

2.3.3.1. 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 Phenomenex Synergi Hydro-RP column (20 mm × 2.0 mm, 2 μm particles) placed in a Mercury MS^{TM} holder. Temperature was set at 25 ± 1 °C and a mobile phase of 0.5% (v/v) aqueous TFA/methanol (85:15, v/v) was used. Mobile phase was prepared by mixing 845 mL of water with 4.5 mL of trifluoroacetic acid and then adding 150 mL of methanol. Flow rate was 0.2 mL/min and 40 μL injection were performed (the needle was washed with methanol). Run time was 10 min. Back-pressure was 27 bar and a no increase was observed, even for big size analytical batches. A counter-flow washing (20 min) with water/methanol (1:1, v/v) was used after each analytical session.

2.3.3.2. 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 the first 2.0 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, nebulizer flow 35 psi, dry gas flow 8.0 L/min, dry temperature 300 °C, capillary −4 kV, skimmer 40 V, ion charge control (ICC) target 20,000, maximum accumulation time 200 ms, spectra averages 5, rolling averages 2.

Quantification was performed by multiple reaction monitoring (MRM) of the analyte and of the internal standard. 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. Data were processed by means of $\text{QuantAnalysis}^{TM}$ (Bruker Daltonics, Bremen, Germany) using the internal standard method plotting peak area ratios versus relative concentration analyte/IS. A quadratic curve fitting with $1/x$ weighting factor was used.

To determine recovery values from the biological matrix for the analyte (at three concentration levels in the investigated range) and for the internal standard, transition peak areas obtained from plasma calibrators were divided by transition peak areas from not extracted standard solutions in water.

3. Results and discussion

3.1. Irradiation of RLP068/Cl and LC–MS identification of the photoproducts

The exposure of the aqueous solutions of RLP068/Cl to red light in the used experimental conditions caused the degradation of the phthalocyanine backbone with the formation of UV products. This phenomenon, known as photobleaching, is solvent dependent in rate and nature of the products. Usually, a mixture of oxidation products with structure related to phthalimide is obtained. In some manuscripts the formation of dinitrile compounds via depolymerization is supposed [15,20,21], but in our opinion this mechanism is highly unlike.

The presence of proteins able to bind the sensitizer can produce strong variations in the photobleaching process, usually leading to an increase of its extent [22]. The photosensitizer RLP068/Cl is proposed for antimicrobial topical applications, so a localised infection had to be simulated in order to mimic its environment of action. Characteristic clinical signs of infections or infected wounds are granulation of the tissues and increased level of exudates [23], whose protein content usually is in the range 10–20 mg/mL. For this reason, photobleaching experiments in solution of bovine serum albumin were performed. It worth to remark that the presence of proteins cause a series of practical limitations in the LC–MS identification of the photoproducts. First of all BSA has to be removed with a protein precipitation technique before LC–MS analysis. Because of the high affinity of RLP068/Cl for BSA (the binding constant measured spectrofluorimetrically was found to be $9 \times 10^3\ M^{-1}$ in phosphate buffered saline solution at pH 7.4 and 298 K), the recovery of the sensitizer was negligible, and we have reason to believe that the same occurs for closely related structures. In this way, it was not possible to assess if the photoprocess caused peripheral modifications in the RLP068/Cl structure, leading to similar phthalocyanine derivatives.

In addition, the sensitizer concentration has to be reduced to avoid protein precipitation: we found that 0.5 mg/mL was the maximum allowed RLP068/Cl concentration. Reduced sample concentration, in conjunction with the dilution due to the required sample pretreatment, made the identification of the formed photoproducts more complicated, especially for minor photoproducts.

Therefore, we began the LC–MS identification of the photoproducts formed after irradiation of not protein containing aqueous solutions; in this way the main photoxidation pathways were investigated and the results obtained for protein containing solutions were of easier interpretation.

In Fig. 2 is reported the LC–DAD–MS chromatogram of a non-irradiated RLP068/Cl aqueous solution. As described in Section 2.1, the active pharmaceutical ingredient RLP068/Cl was composed by three isomers (with symmetry D_{2h}, C_s and C_{2v}) and, in the used chromatographic conditions, gave three not resolved peaks with retention times of 12.0, 12.3 and

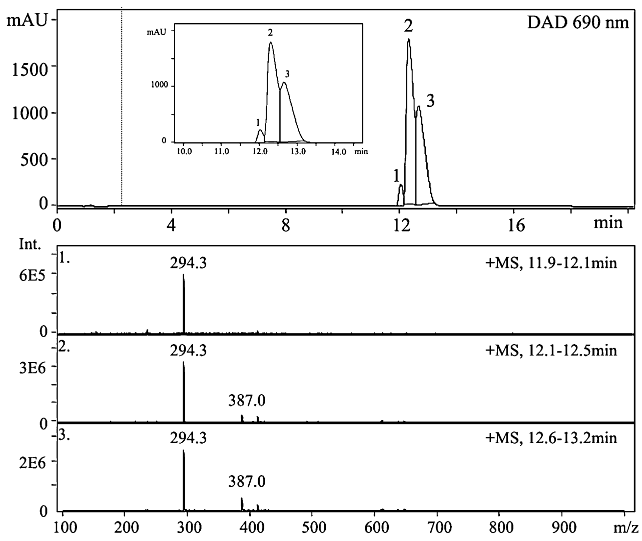


Fig. 2. LC–DAD–MS chromatogram of a not irradiated RLP068/Cl solution in water. Injection 5 μL of a 0.25 mg/mL solution in water. DAD detection: 690 ± 20 nm. ESI-MS trap detection: scan range 100–1000 *m*/*z*, scan rate 13000 *m*/*z* s^{-1}.

12.7 min and MS spectra dominated by the M^{4+} ions with *m*/*z* 294. Also, the fragment ions $[M–CH_3]^{3+}$ with *m*/*z* 387 were detected.

After irradiation with red light (20 or 60 min at 100 mW/cm^2), the formation of three major UV degradants, with retention times, respectively, of 0.90, 1.05 and 2.70 min, was observed. The MS analysis of them is shown in Fig. 3; MS spectra indicated for these UV degradants the molecular weight respectively of 295, 296 and 297 Da. The photoproduct with retention time 2.70 min and *m*/*z* 297 was consistent with a well know molecule, the cationic derivative 3-[(1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl)oxy]-*N*,*N*,*N*-trimethylbenzenaminium chloride, briefly named as trimethylamoniumphenoxyphthalimide. The assignation was confirmed by LC–MS analysis of a solution of the synthesized compound. There was an identity in retention time and mass spectrum.

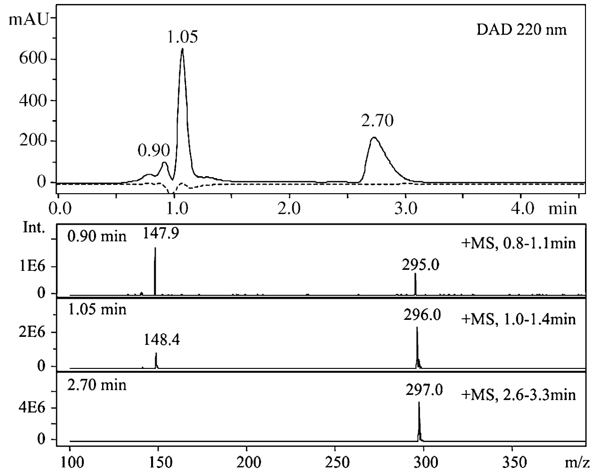


Fig. 3. LC–DAD–MS chromatograms (RT range: 0–5 min) of an irradiated (solid line) and a not irradiated (dash line) RLP068/Cl solution in water (1.0 mg/mL). Irradiation conditions: 60 min at 100 mW/cm^2. DAD detection: 220 ± 20 nm. ESI-MS trap detection: scan range 100–1000 *m*/*z*, scan rate 13000 *m*/*z* s^{-1}.

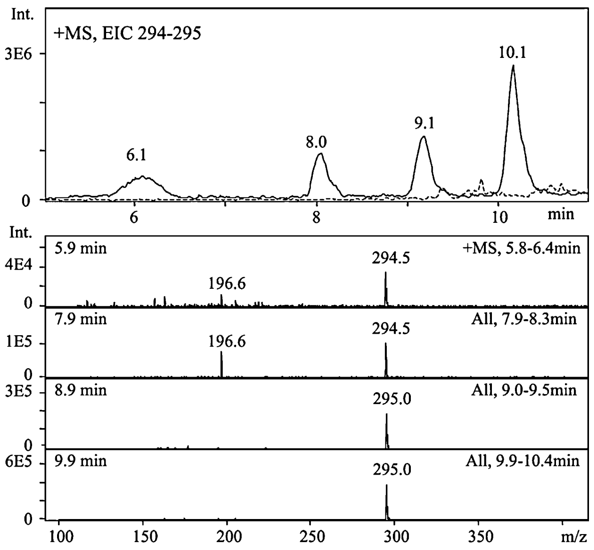


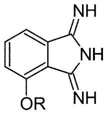
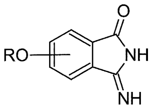
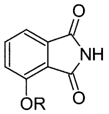
Fig. 4. Extracted ion current (*m*/*z* 294–295) LC–MS chromatograms (RT range: 5–11 min) of an irradiated (solid line) and a not irradiated (dash line) RLP068/Cl solution in water (1.0 mg/mL). Irradiation conditions: 60 min at 100 mW/cm^2. ESI-MS trap detection: scan range 100–1000 *m*/*z*, scan rate 13000 *m*/*z* s^{-1}.

So it was possible to deduce that the photoproducts with molecular weight of 295 and 296 Da were compounds more hydrophilic and polar than the substituted phthalimide (retention times close to column dead time). In addition they should have a different number of nitrogen atoms respect to the substituted phthalimide. It was interesting to observe that in the MS spectra of these products also doubly charged ions are present (with *m*/*z*, respectively, of 148 and 148.5), while this did not happen for trimethylamoniumphenoxyphthalimide. The formation of doubly charged ions required the presence of protonable groups in the structures.

The photoproduct with *m*/*z* 296 was also observed oxidising the phthalocyanine derivative RLP068/Cl with oxygen donor oxidants ($K_2Cr_2O_7$ or HNO_3). In a previous manuscript, we reported it as an intermediate (existing as two positional isomers) of the oxidative derivatisation of the phthalocyanine to the substituted phthalimide derivative [7]. So we concluded that the UV degradants with retention times, respectively, of 0.90 and 1.05 min were the diimino-isoindoline derivative and the inimo-oxo-isoindoline derivatives (two isomers) reported in Table 1. The presence of slightly protonable C=NH groups was considered responsible for the formation of the doubly charged ions in ESI-MS.

Looking for minor photoproducts, a group of four components with retention times respectively of 5.9, 7.9, 8.9 and 9.9 min was observed (Fig. 4). Two of them showed mass spectra of doubly charged ions M^{2+} with *m*/*z* 294.5 and triply charged ions MH^{3+} with *m*/*z* 196.6, while the others showed only doubly charged ions M^{2+} with *m*/*z* 295.0. The presence

Table 1
Retention times (RT), *m/z* values, calculated molecular masses and tentative structural assignations for the main photoproducts formed during irradiation of a RLP068/Cl solution in water

RT (min)	*m/z* values	Molecular mass (Da)	Structural hypothesis
0.90	M^+ *m/z* 295.0, $[M+H]^{2+}$ *m/z* 148.0	295	
1.05	M^+ *m/z* 296.0, $[M+H]^{2+}$ *m/z* 148.5	296	
2.70	M^+ *m/z* 297.0	297	
5.9	M^{2+} *m/z* 294.5, $[M+H]^{3+}$ *m/z* 196.6	589	Two isoindoline unit derivatives cation molecular formula $C_{34}H_{35}N_7O_3$
7.9	M^{2+} *m/z* 294.5, $[M+H]^{3+}$ *m/z* 196.6	589	
8.9	M^{2+} *m/z* 295.0	590	Two isoindoline unit derivatives cation molecular formula $C_{34}H_{34}N_6O_4$
9.9	M^{2+} *m/z* 295.0	590	

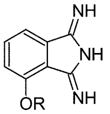
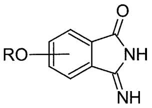
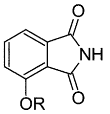
R = *m*-trimethylamoniumphenyl group.

of two fixed charges suggested the presence of two trimethylamoniumphenoxy substituents. Thus, structures formed by two isoindoline units were supposed; the photoproducts that gave MH^{3+} ions in the ESI source should have kept a C=NH group in their structures, while those that gave only M^{2+} ions should have only C=O groups. The structural hypothesis done for the observed photoproducts of RLP068 are summarised in Table 1.

It worth remarking that all of the degradation products described until now are UV products; none of them was detectable at 690 ± 20 nm, so all of them have lost the typical phthalocyanine π region. A stronger proof of their UV nature was the loss of the typical isotopic pattern of the Zn atom (five most abundant isotopes) in their MS spectra. The product RLP068/Cl did not give phthalocyanine-related photoproducts probably because of the stability of its peripheral substituent groups to oxidation.

Quantitative HPLC-UV analysis for photoproducts observed with abundance greater than 0.1% (w/w), were performed using an external standard solution of trimethylamoniumphenoxyphthalimide (concentration 1 μg/mL). Results are shown in Table 2. After 60 min of irradiation at 100 mW/cm^2 of a 1 mg/mL solution of RLP068/Cl in water, about 5% of the sensitizer was photobleached. The main degradation pathway was found to be the oxidative cleavage of the phthalocyanine ring with the formation of phthalimide related products. Performing the HPLC analysis exactly at the end of the photodynamic process, mixtures close to the 1:1 ratio of inimo-oxo-isoindoline and phthalimide derivatives were found. The extent of the photobleaching was quite low,

Table 2
Quantitative HPLC-UV data for the main photoproducts formed during irradiation of a RLP068/Cl solution in water

RT (min)	Proposed structure	Quantitative analysis 20 min irradiation (100 mW/cm^2)	Quantitative analysis 60 min irradiation (100 mW/cm^2)
0.90		<0.1%	0.3%
1.05		0.6%	2.4%
2.70		0.5%	1.9%
11.8–13.0	RLP068/Cl isomers	98.8%	95.1%

R = *m*-trimethylamoniumphenyl group.

considering the used irradiation time and fluence rate. The photoxidative nature of the observed reactions was confirmed by experiments conducted without the admission of oxygen. Samples were prepared in gas chromatography headspace vials and insufflated for 15 min with N_2 to remove the dissolved oxygen. After irradiation, less than 0.4% of the sensitizer was bleached and no phthalimide derivatives were observed.

We observed that the photobleaching was strongly solvent dependent. We found that a 60 min irradiation of a 1 mg/mL RLP068/Cl solution prepared in transcutol (common name of 2-(2-ethoxyethoxy)ethanol) instead of water, showed a 60% of photobleaching (data not shown). Also the binding with proteins made the phthalocyanine macrocycle more prone to photobleaching. In fact, the irradiation of RLP068/Cl in BSA solution afforded a much higher degradation of the sensitizer, if compared with the water solution. After 60 min of irradiation approximately, the 14.2% of the sensitizer was oxidated to substituted phthalimide and the 5.5% to inimo-oxo-isoindoline derivatives (respectively against 1.9% and 2.4% observed in water).

The collected information strengthen the importance of the development of a reliable method for the determination of the phthalimide-like photoproduct in plasma. In fact, the monitoring of the plasmatic levels of the main photoproduct of the sensitizer RLP068/Cl could represent a powerful tool during the preclinical and clinical toxicological trials of the product.

3.2. LC–tandem-MS determination of 3-[(1,3-dioxo-2,3-dihydro-1H-isoindol-4-yl)oxy]-N,N,N-trimethylbenzenaminium chloride (the main photoproduct of RLP068/Cl) in rabbit plasma

3.2.1. HPLC–MS/MS

The cationic analyte is a very polar compound. Its retention on a classic C18 reverse phase column requires the use of an high percentage of water in the mobile phase; so a polar modified C18 stationary phase was required. In addition trifluoroacetic acid was preferred to acetic or formic acid for its ion pair characteristics that improved the retention. In a previous publication a Polaris C18-ether column was used for the same task [7]. However, a Phenomenex Synergy Hydro RP was used, because we found it more resistant to the fouling caused by rabbit plasma endogenous components for long analytical sessions. No difference in retention time was observed between analyte and internal standard: the retention time was 6.95 ± 0.05 min ($n = 24$) and the run time was set at 10 min.

The high water content in the mobile phase (85%) did not compromise ESI-MS sensitivity; the analyte was already a cation (a IV ammonium compound), so did not suffered of the low ionisation efficiency due to the high water content of the mobile phase.

In order to increase the selectivity and the sensitivity of MS detection, the molecular reaction monitoring (MRM) technique was chosen. The lowest energy fragmentation for the analyte ions was the loss of a methyl group ($[M\text{–}CH_3]^+$ with $m/z = 282.1$). Obviously, the internal standard was able to lose a methyl or a tri-deuteromethyl group, so its MS/MS spectrum showed two abundant signals with a relative ratio of 2:1, due to the loss of CH_3 ($m/z = 285.1$) or CD_3 ($m/z = 282.1$).

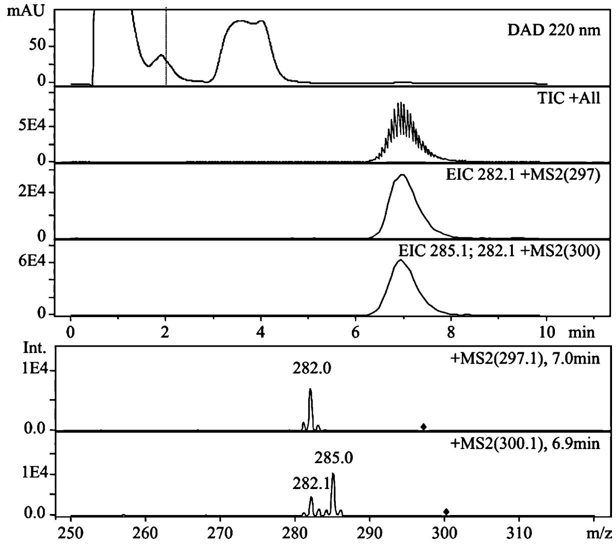


Fig. 5. HPLC-MRM chromatogram (multiple reaction monitoring) of a rabbit plasma calibration solution (analyte concentration 2.3 ng/mL, IS 9.1 ng/mL). Transitions followed: 297.1 → 282.1 for the analyte and 300.1 → 282.1 + 285.1 for the internal standard. MS/MS parameters: isolation width 3.0 Da, fragmentation amplitude 1.10 V.

Fig. 5 presents an HPLC-MRM chromatogram of a processed rabbit plasma containing 2.3 ng/mL of analyte and 9.1 ng/mL of Internal Standard: the transitions 297.1 → 282.1 for the analyte and 300.1 → 282.1 + 285.1 for the IS were followed.

3.2.2. Rabbit plasma calibrators

The described LC-MRM method afforded the required specificity for the analyte (the phthalimide-like photoproduct) against the Active Pharmaceutical Ingredient RLP068/Cl; in fact chromatographic and mass spectrometric properties of the photoproduct and of the main drug are totally different. A lot of care was taken in the assessment of the specificity of the method, because the analyte of interest is also the major degradation product of RLP068/Cl under oxidative conditions. In order to prevent phthalocyanine positive interference, the plasma samples had to be processed under mild conditions and avoiding oxidising agents. A rapid protein precipitation technique, optimised for polar analytes, was chosen. The used procedure was totally inadequate for the recovery of the phthalocyanine RLP068/Cl, because of its high affinity for albumins (the constant of binding of the drug with bovine serum albumin, measured via fluorimetric titration, was found to be $9 \times 10^3\,M^{-1}$). We demonstrated that, under the used conditions, no interference was observed up to 50 ng/mL of the main drug in plasma samples.

Table 3
Within-day validation data in the concentration range 0.23–91.2 ng/mL of analyte in rabbit serum (IS concentration 9.12 ng/mL)

Within-day[a]												
Nominal concentration (ng/mL)	0.00	0.23	0.46	0.91	2.28	4.56	6.84	9.12	22.8	45.6	68.4	91.2
Calculated concentration (ng/mL)	0.00	0.20	0.51	0.96	2.38	4.46	6.59	9.06	21.4	44.9	66.9	89.1
Accuracy (%)[b]	–	86.6	112.1	105.1	104.3	98	96.4	99.4	94.0	98.5	97.9	97.8
Precision (R.S.D., %)[c]	–	5.2	1.6	6.1	4.9	3.0	0.3	1.1	3.1	1.3	0.6	0.6
Regression analysis												
$y = 0.003 + 1.151x - 0.043x^2$ ($N = 12$)												
$R = 0.9997$												

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 three series.

[b] (Found/nominal) × 100.

[c] Relative standard deviation.

Table 4
Recovery values for analyte (low, medium and high quality control samples, respectively, 0.91, 9.12 and 68.4 ng/mL) and internal standard (9.12 ng/mL)

Analyte recovery[a]	Peak area in plasma	Peak area standard solution	Recovery value (%)
LQC (0.91 ng/mL)	$(1.26 \pm 0.08) \times 10^5$	$(1.67 \pm 0.09) \times 10^5$	74.9 ± 6.3
MQC (9.12 ng/mL)	$(31.60 \pm 1.30) \times 10^5$	$(36.75 \pm 0.84) \times 10^5$	86.0 ± 4.1
HQC (68.4 ng/mL)	$(92.43 \pm 1.46) \times 10^5$	$(100.5 \pm 1.89) \times 10^5$	92.0 ± 1.6
Internal standard recovery[b]	$(26.3 \pm 2.34) \times 10^5$	$(32.79 \pm 1.23) \times 10^5$	80.3 ± 8.8

[a] Calculated in five series.

[b] Calculated in 15 series.

The method was fully validated in the range of concentrations spanning from 0.2 to 91.2 ng/mL (with a fixed concentration of 9.1 ng/mL of the internal standard), according the current FDA guideline for bioanalytical methods [24]. The results are reported in Table 3. The zero sample and 10 concentration levels were analysed and a quadratic fitting with 1/x weighting was used. The regression curve equation was $y = +0.003 + 1.151x - 0.043x^2$, with a correlation factor R of 0.9996 ($n = 12$). LLOD and LLOQ were set, respectively, to 0.2 ng/mL and 0.5 ng/mL. In Fig. 6 are overlaid the LC-MRM chromatograms of four rabbit plasma zero samples and four rabbit plasma calibrators at the LLOD level.

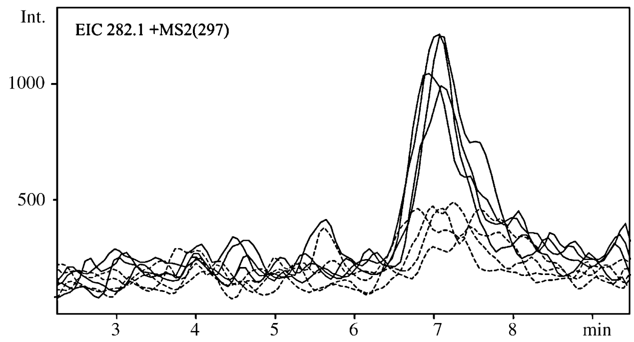


Fig. 6. HPLC-MRM chromatograms (multiple reaction monitoring) of four zero samples (dash lines) and four rabbit plasma calibrators at the LLOD level (solid line). Analyte concentration 0.2 ng/mL, IS 22.8 ng/mL. Transitions followed: 297.1 → 282.1 for the analyte and 300.1 → 282.1 + 285.1 for the internal standard. MS/MS parameters: isolation width 3.0 Da, fragmentation amplitude 1.10 V.

Accuracy was found to be in the ±15% interval for every investigated concentration in the range 0.46–91.2 ng/mL. Only one point at the 0.23 ng/mL concentration level was found with accuracy 82.9%. Intra-day precision, expressed as relative standard deviation (R.S.D. (%), with $n = 3$), was less than 6.0% at every concentration level.

Recoveries were determined for quality control samples at three concentration levels (HCQ 68.4 ng/mL, MCQ 9.12 ng/mL and LQC 0.91 ng/mL) against not extracted standard solutions in water. Recovery values are reported in Table 4. A slight decrease in the recovery was observed for diluted plasma samples, but the even at the 0.91 ng/mL level, the 75% of the analyte was extracted from the biological matrix. The use of a deuterated internal standard afforded a strong control on the recovery. However, because the method relies on a relatively 'dirty' sample preparation as the protein precipitation technique, the matrix effect was assessed preparing a series of processed blank samples and spiking them with a known amount of analyte (9.12 ng/mL). We found that ion suppression due to the biological matrix was quite low, probably because of the quaternary ammonium nature of the analyte. From a practical point of view, the overall recovery observed at the MCQ level (86.0%) was due to a 91% of matrix effect and, consequently, to a 94.5% of extraction efficacy.

Short-term temperature stability (24 h at room temperature) and post-preparative stability (24 h at room temperature in the HPLC autosampler plate) were checked and results are reported in Table 5. The room temperature stability in plasma was found to be not satisfactory. After 24 h, we recovered about the 40% of the starting analyte; so a maximum storage time of 6–8 h was suggested for plasma samples.

Table 5
Post-preparative and short-term room temperature stability values for analyte (low, medium and high quality control samples, respectively, 0.91, 9.12 and 68.4 ng/mL) and internal standard (9.12 ng/mL)

Post-preparative stability	Peak area 0 h	Peak area 24 h	Stability (%)[c,d]
Short-term r.t. stability			
LQC (0.91 ng/mL)[a]	$(1.26 \pm 0.08) \times 10^5$	$(1.43 \pm 0.14) \times 10^5$	114.0 ± 16.1
MQC (9.12 ng/mL)[a]	$(31.60 \pm 1.30) \times 10^5$	$(32.36 \pm 1.23) \times 10^5$	102.4 ± 7.9
HQC (68.4 ng/mL)[a]	$(92.43 \pm 1.46) \times 10^5$	$(89.89 \pm 3.36) \times 10^5$	97.3 ± 5.3
Internal standard (9.12 ng/mL)[b]	$(26.32 \pm 2.34) \times 10^5$	$(25.41 \pm 0.93) \times 10^5$	96.5 ± 12.6
Short-term r.t. stability			
LQC (0.91 ng/mL)[a]	$(1.26 \pm 0.08) \times 10^5$	$(0.60 \pm 0.12) \times 10^5$	48.0 ± 26.3
MQC (9.12 ng/mL)[a]	$(31.60 \pm 1.30) \times 10^5$	$(12.12 \pm 0.26) \times 10^5$	38.4 ± 6.3
HQC (68.4 ng/mL)[a]	$(92.43 \pm 1.46) \times 10^5$	$(38.21 \pm 0.35) \times 10^5$	41.3 ± 2.5
Internal standard (9.12 ng/mL)[b]	$(26.32 \pm 2.34) \times 10^5$	$(10.61 \pm 1.21) \times 10^5$	40.3 ± 20.3

Analysis performed after 0 and 24 h.

[a] Calculated in five series.

[b] Calculated in 15 series.

[c] Peak area after 24 h/peak area after 0 h.

[d] Relative standard deviation calculated using error propagation theory.

4. Conclusion

We described the identification and the LC-MRM determination in rabbit plasma of a photoproduct of the PDT sensitizer RLP068/Cl. The method allows the measurement of plasma level of the photoproduct after the complete PDT protocol (administration of the sensitizer and irradiation of the interested area with light) and can be applied to preclinical toxicological studies on small size animals. In this way, a better understanding of risks connected to a real PDT topic treatment is achieved: not only the permeation of the active pharmaceutical ingredient, but also the permeation of the main photoproduct can be monitored. The method proved to be rapid, specific (against both endogenous rabbit plasma components and the intact sensitizer), accurate, precise and sensitive (LLOD and LLOQ were, respectively, 0.2 and 0.5 ng/mL).

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