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Sensitive isocratic liquid chromatographic assay for the determination of 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin in plasma and tissue with electrochemical detection

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Abstract

A simple extraction procedure and a sensitive high-performance liquid chromatographic (HPLC) method are described for the determination of the photodynamic therapeutic agent 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (*m*THPC) in plasma and tumour tissue. Reversed-phase high-performance liquid chromatography was performed on a C_{18} column (70×4.6 mm I.D.) with a mobile phase of 0.01 *M* potassium dihydrogenphosphate buffer, pH 2.5–acetonitrile (55:45, v/v) and a coulometric detection (+0.80 V). The mean recoveries of *m*THPC in the concentration ranges (5–2000 and 10–1000 ng/ml) were 90 and 89% for plasma and tumour samples, respectively. The procedure for plasma and tissue preparation involved solvent precipitation using methanol combined with ammonia solution and dimethyl sulphoxide (4, 0.2, 0.1, v/v/v) and (2, 0.1, 0.1, v/v/v) for plasma and tissue, respectively. For mTHPC at concentrations ranging from 5 to 2000 ng/ml, the within-day relative standard deviations, based on triplicate determinations were less than 8% and the between-day relative standard deviations calculated by performing extraction procedure of plasma samples on three different days ranged from 3 to 18%. This highly sensitive method, 5 and 10 ng/ml for plasma and tissue respectively, was applied successfully to the determination of *m*THPC in mouse tumours for pharmacokinetic studies.

Keywords: 5,10,15,20-Tetra(*m*-hydroxyphenyl)chlorin

1. Introduction

A phototherapy for cancer treatment is currently being developed worldwide [1]. This photodynamic therapy of cancer is based on the use of photosensitizing agents that concentrate selectively in tumours and become cytotoxic when activated by light at the appropriate wavelength. An essential aspect of this effort entails the design, synthesis and in vivo biological assay of new photosensitizers. Most of experimental and clinical data have been obtained with the first generation photosensitizers, such as hematoporphyrin derivative and its partially purified form, photofrin II. These drugs have certain limitations: they are mixtures of compounds and have a modest absorption in the 600–900 nm wavelength region, in which tissue penetration is maximal. The *ortho*, *meta* and *para* isomers of 5,10,15,20-tetra(hydroxyphenyl)chlorin (*m*THPC, Fig. 1) were recently developed and tested as

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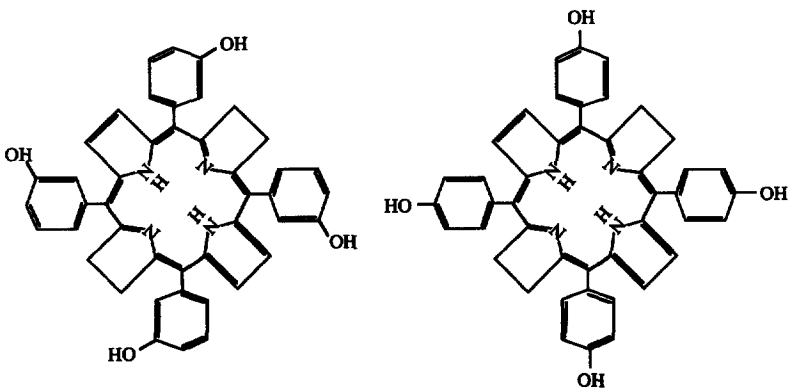

Fig. 1. Structure of 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (*m*THPC) and 5,10,15,20-tetra(*p*-hydroxyphenyl)chlorin (*p*THPC).

second-generation photosensitizers [2] with respect to different criteria: a high therapeutic ratio (extent of tumour vs. normal tissue photonecrosis), and little or no toxic side-effect in the dark. Moreover, *m*THPC absorbs light in the red part of the visible spectrum with a high triplet-state quantum yield. MesoTHPC has already been used in a pilot clinical study in patients with diffuse malignant mesothelioma and information on its pharmacokinetics is sparse [3]. A detailed analysis of its tissue distribution would allow a rationale determination of the time after administration when the therapeutic ratio is maximal, i.e., when significant tumour necrosis can be achieved without major detrimental effects due to lesion of healthy tissue. Moreover, clinical trials for a wide variety of cancers are expected to begin, and only a sensitive and specific assay for monitoring plasma and tissue concentrations would prove to be useful to understand the pharmacokinetic–pharmacodynamic relationships. According to previous studies [4,5], concentrations lower than 15 ng/ml could not be correctly quantified in plasma and tissues.

This paper describes a simple, rapid and sensitive (5 ng/ml) HPLC method using a reversed-phase column and coulometric detection (+0.8 V) and involving a simple protein solubilization. This method has been applied to measure plasma *m*THPC level 4 days after intravenous administration (0.15 mg/kg) to a patient entered into a clinical trial and it has also been validated for quantitation of mTHPC in mouse tumour tissues 4, 8, 48, 72, 96 and 120 h after intraperitoneal administration (0.30 mg/kg).

2. Experimental

2.1. Apparatus

The HPLC system was a computer-monitored GOLD PC apparatus [Model 126 pumps, Model 406 interface (Beckman, Gagny, France)] and an Ultrasphere XL ODS 3 μm column (70×4.6 mm I.D.), an ESA Coulochem 5100A dual-electrode coulometric detector equipped with a guard cell (Eurosep, Cergy Saint-Christophe, France) and a WISP 512 autosampler (Waters, St. Quentin-Yvelines, France). A micro tissue grinder (1 ml) with a teflon pestle was used to homogenize tumour tissue (Poly Labo, Strasbourg, France).

2.2. Reagents, standard solutions and internal standard

*m*THPC and its *p*-hydroxy isomer (*p*THPC) were synthesized at Queen Mary and Westfield College (London, UK) and were gifts from Scotia Pharmaceuticals Ltd. (Guilford, UK). Both compounds were dissolved in ethanol–poly(ethylene glycol) 400 (PEG 400, Sigma, Saint Quentin Fallavier, France)–water (2:3:5, v/v/v). Hemin (1,3,5,8-tetramethyl-2,4,-divinylporphine-6,7-dipropionic acid ferrichloride) was

obtained from Aldrich. Other reagents were of analytical reagent quality. Acetonitrile, methanol and glycerol were HPLC grade and were obtained from Prolabo (Paris, France). Dimethyl sulphoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), sodium molybdate solution, DL-dithiotreitol were purchased from Sigma. This solution was stored to ensure adequate conservation of the photosensitizer as already shown [6]. Tris–EDTA molybdate buffer was prepared as a stock solution, stored at 4°C no longer than 15 days and contained Tris buffer (10 m*M*), ethylenediaminetetraacetic acid (1.66 *M*), sodium molybdate solution (1.25%, 5 m*M*), glycerol (purity 99%, 10% v/v), DL-dithiotreitol (0.5 m*M*) in H_2O. pH was adjusted to 7.4 (±0.1) by adding 1 *M* HCl solution.

2.3. HPLC Conditions

Isocratic elution conditions were adopted. The eluting solvent was 0.01 *M* potassium dihydrogen phosphate buffer (pH 2.5)–acetonitrile (55:45, v/v). pH was adjusted by adding orthophosphoric acid.

Room temperature and a flow-rate of 0.5 ml/min were maintained throughout the analyses. A pressure of 100 bars (1500 p.s.i.) was used. Sample volume was 30 μl.

2.4. Electrochemical detection

The potentials corresponding to the limiting current wave for *m*THPC and *p*THPC were determined by generation of hydrodynamic voltammogram and the potential of the upstream electrode was set to 0.05 V being lower than the rising portion of the voltammogram of the analyte (Fig. 2). This stabilized the baseline and allowed a better reproducibility of the chromatographic results. The second electrode was used to detect the analytes (*m*THPC and *p*THPC) at their appropriate potential corresponding to the limiting current wave. Amounts of *m*THPC and *p*THPC were quantified by comparison of their peak area with that of standard samples.

2.5. Extraction of mTHPC from plasma

All samples were protected from light. Blood was collected in heparinized tubes and centrifuged at 1000 *g* for 10 min at 4°C. The separated plasma was frozen (−20°C) in polypropylene tubes. Plasma aliquots (500 μl) were spiked with *m*THPC (5–2000 ng/ml). The mixture was incubated at 37°C for 1 h then mixed for 10 s using a rotating mixer. *p*THPC (100 μl, 1 μg/ml) was added as internal standard. Ammonia solution (100 μl) was added to adjust the pH to ca. 10 and in order to dissolve protein without

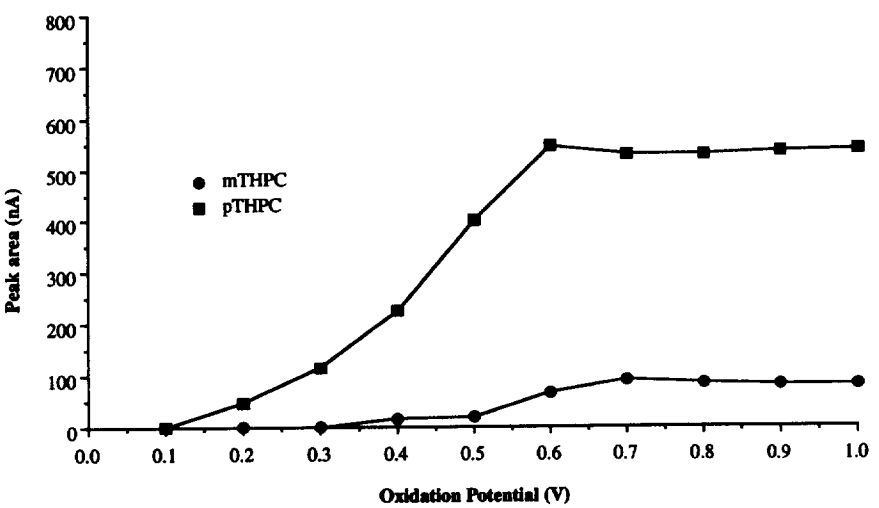

Fig. 2. Voltammograms of *m*THPC and *p*THPC. The mobile phase was 0.01 *M* potassium dihydrogen phosphate buffer (pH 2.5)–acetonitrile (55:45, v/v). For chromatographic conditions, refer to Section 2.3.

coprecipitation of *m*THPC, 50 μl of DMSO was finally added. The mixture was vortex-mixed with methanol (2 ml) and centrifuged at 3000 *g* for 10 min. The extract was concentrated by evaporation under a gentle stream of nitrogen at 30°C. The residue was reconstituted in acetonitrile (200 μl) by placing the tubes in an ultrasonic water-bath for 10 min and transferred into an autosampler microvial.

The mean recoveries from plasma samples of *m*THPC were determined by comparing the peak area measured from the final extracts of plasma containing known concentrations with the peak area measured from standard solutions of *m*THPC. Calibration standards covering the anticipated range of 5–2000 ng/ml were used.

2.6. Animals, tumour tissue collection and assessment of the mTHPC concentration in tumours

Female Swiss nude/nude mice (three weeks, weight range 20–25 g) were obtained from Iffa-Credo (L'Arbresle, France). Animals were injected subcutaneously into the flank with malignant tumour cells ($8 \cdot 10^6$ cells/mouse) of human colon adenocarcinoma cell line (HT-29). Approximately 20 days after transplantation, when the tumour diameter reached 8–10 mm, *m*THPC (0.30 mg/kg) was injected intraperitonally in ethanol–PEG 400–water (2:3:5, v/v/v). The mice were killed 4, 8, 48, 72, 96 and 120 h after drug administration and all tumours were stored in liquid nitrogen until analysis. After being injected until their sacrifice, the animals were kept in the dark.

2.7. Extraction of mTHPC from tumours

To remove surface blood, tissues were rinsed in distilled water, blotted dry in filter paper and weighed. Tissue samples (100 mg) were homogenized in Tris–EDTA molybdate buffer (1 ml) by using a potter for approximately 3 min. The homogenates were spiked with *m*THPC in acetonitrile (10–1000 ng/ml). The mixture was incubated at 37°C for 1 h in the dark. ParaTHPC (1 μg/ml) was added as internal standard. Ammonia solution (100 μl) then DMSO (100 μl) were added and the mixture was mixed for 1 min. The cellular membranes were ruptured by thermal shock. This operation consisted in 5 successive immersions in liquid nitrogen (−173°C) and in water (37°C), alternately. The proteins were precipitated after addition of acetonitrile (2 ml) and centrifugation at 11 000 *g* for 30 min. The organic phase was concentrated by evaporation under a gentle stream of nitrogen at 30°C. The residue was reconstituted in acetonitrile (200 μl) by placing the tubes into an ultrasonic water-bath for 10 min and transferred to an autosampler microvial. The tumour tissue concentration of *m*THPC (ng/g protein/g tissue) was calculated from a calibration curve covering the anticipated range 10–1000 ng/ml.

2.8. Clinical study

This method has been applied to measure plasma and tumour tissue *m*THPC levels for a patient with squamous cell carcinoma who agreed to participate in this clinical study after having been informed of it aims and nature. This patient received *m*THPC at a dose of 0.15 mg/kg by intravenous administration. A blood sample and an endoscopic biopsy specimen were obtained four days after drug injection and just before photodynamic treatment.

3. Results and discussion

3.1. Electrochemical detection

The optimal potential between the two electrodes for the detection of *m*THPC and *p*THPC was determined by performing voltammograms. Fig. 2 shows the voltammograms for *m*THPC, *p*THPC both at 3.5 μg/ml and the background current from mobile phase constituents. The optimal potential for the detection of *m*THPC and *p*THPC was found to be +0.80 V.

3.2. Detection limit, linearity and precision

At a signal-to-noise ratio of 3, the minimal detectable concentration after the extraction procedure was 5 ng/ml by injecting 30 μl of 200 μl of reconstituted sample extract. *m*THPC could be determined with an acceptable precision (≤7%) (Table

Table 1
Within-day variability of *m*THPC plasma assay ($n=3$)

*m*THPC (ng/ml)	Concentration measured (ng/ml)	Coefficient of variation (%)	R.S.D. (%)
5	5	9	7
20	22	5	7
40	43	2	5
100	104	2	5
200	207	1	3
1000	1020	1	4
2000	2026	1	3

1) at a concentration of 5 ng/ml (Fig. 3). In order to investigate the linearity of the extraction procedure, blank plasma samples were spiked with *m*THPC (5 to 2000 ng/ml).

The calibration curve showed good linearity as expressed by the following equation:

$$y = 59.8(\pm 12.7)x + 17.8(\pm 7.1), \quad r \geq 0.993 \ (n = 3)$$

3.3. Reproducibility

The precision and accuracy of the assay (within-day analysis) for *m*THPC were evaluated over plasma concentrations from 5 to 2000 ng/ml. The results are shown in Table 1. The relative standard deviations (R.S.D.) based on triplicate determinations were less than 8% for *m*THPC at all concentrations (Table 1).

The between-day R.S.D. were calculated by performing analyses of plasma samples containing *m*THPC at seven concentrations on three different days. These results are summarized in Table 2. The between-day R.S.D. ranged between 3 and 18%. These results indicate a relatively good precision of the assay.

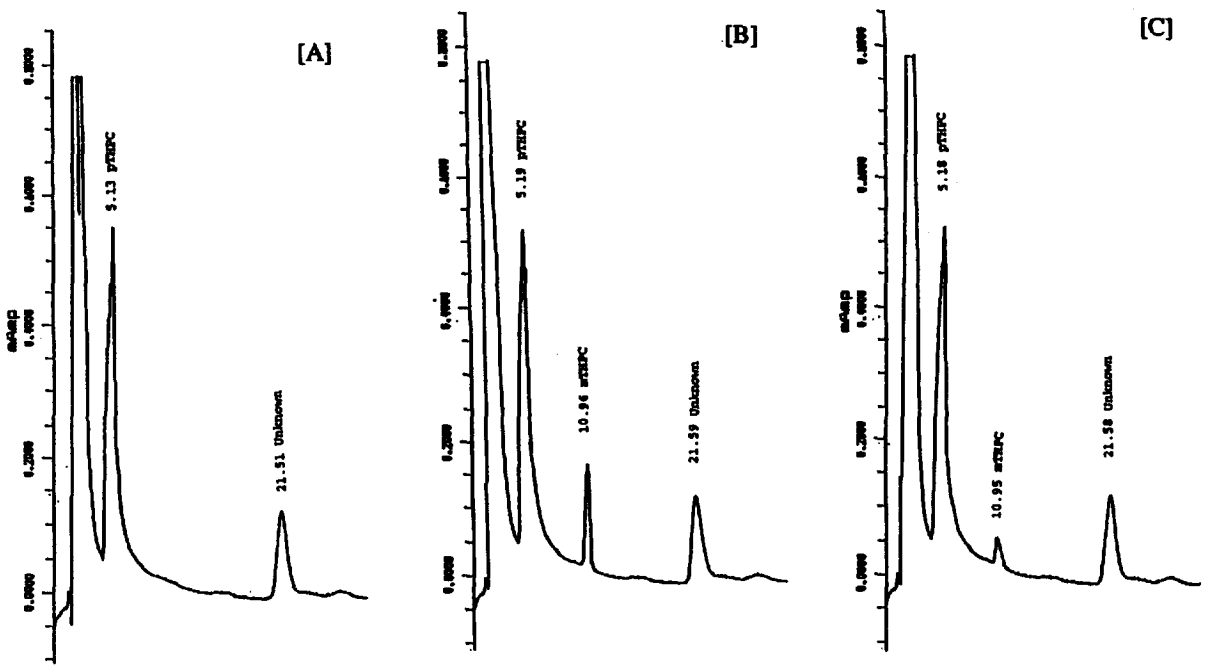

Fig. 3. Representative chromatogram of plasma control extract, supplemented with 100 ng *p*THPC as internal standard (A), plasma control extract (500 μl) supplemented with 100 ng *p*THPC and 200 ng/ml *m*THPC (B), plasma sample spiked with 100 ng *p*THPC as internal standard obtained from a cancer patient treated with 0.15 mg/kg *m*THPC, 4 days after photosensitizer administration and just before photodynamic treatment (C). Concentration of *m*THPC was 32 ng/ml. The mobile phase was fixed as 0.01 *M* KH_2PO_4 buffer (pH 2.5)–acetonitrile (55:45, v/v). For chromatographic conditions and extraction procedure, see Section 2.3 and Section 2.5.

Table 2
Between-day variability of *m*THPC plasma assay ($n=3$)

*m*THPC (ng/ml)	Concentration measured (ng/ml)	Coefficient of variation (%)	R.S.D. (%)
5	6	8	16
20	25	8	17
40	47	9	18
100	109	9	15
200	216	4	13
1000	1027	1	4
2000	2061	1	7

3.4. Extraction procedure and recoveries

Several techniques were tested to extract *m*THPC efficiently from plasma and tissue. Solid-phase extraction was examined using C_8 and C_{18} columns. Alternatively, liquid–liquid extraction procedures including solvent extraction and using a preparative column containing a specially modified form of diatomaceous earth were studied. In fact, by using these different approaches, the recoveries remained below 35%. Methanol combined with ammonia solution and DMSO (4, 0.2, 0.1, v/v/v) and (2, 0.1, 0.1, v/v/v) for plasma (0.5 ml) and tumour tissue (1 ml) respectively, yielded the purest extracts, free from interfering impurities (Figs. 3 and 4). Although *m*THPC is highly soluble in methanol, ethanol and acetonitrile, these solvents were unsuitable for the precipitation of *m*THPC directly in plasma and tumour tissue since at physiological pH the photosensitizer was found to be coprecipitated with the proteins. It has been shown that by adding DMSO, hydrophobic tetrapyrroles could be effectively extracted from proteins [7].

Recoveries were calculated as ratio of the areas under the curve for *m*THPC after extraction from plasma or tissue and the area under the curve obtained after injection of a standard solution at the same concentration. The recovery depends on the concentration range 5 to 2000 ng/ml or 10 to 1000 ng/ml as shown in Table 3 Table 4. The mean values of recovery were 90% (82–106%) and 89% (83–95%) for plasma and tumour samples, respectively (Tables 3 and 4).

The first step of tumour tissue extraction was to wash it in distilled water to remove blood prior to homogenization and using the present extraction procedure no interfering peak was observed at the retention time of *m*THPC (Fig. 4). The ability to separate correctly hemin from *m*THPC is crucial as all tissues contain blood [4,5,9].

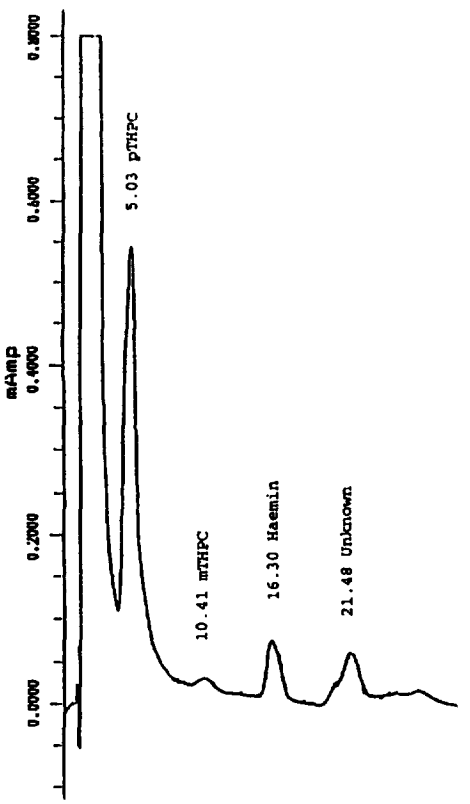

Fig. 4. Representative chromatogram of mouse tumour supplemented with 100 ng *p*THPC as internal standard and containing 79 ng/ml (70 ng/g protein/g tissue) of *m*THPC. The mobile phase was fixed as 0.01 *M* KH_2PO_4 buffer (pH 2.5)–acetonitrile (55:45, v/v). For chromatographic conditions and extraction procedure, see Section 2.3 Section 2.7.

Table 3
Mean recovery of *m*THPC in plasma at seven different concentrations ($n=6$)

*m*THPC (ng/ml)	Recovery ±C.V. (%)
5	84±8
20	82±7
40	84±5
100	84±5
200	89±6
1000	103±4
2000	106±6

Table 4
Mean recovery of *m*THPC in tumor tissue at five different concentrations ($n=2$)

*m*THPC (ng/ml)	Recovery ±C.V. (%)
10	83±5
50	85±8
100	95±6
500	87±4
1000	92±4

In mouse tumour tissue after intraperitoneal injection of *m*THPC (0.30 mg/kg), the highest levels were detected 72 h after drug administration (Table 5).

3.5. Sample analysis

The composition of the mobile phase was fixed as 0.01 *M* KH_2PO4 buffer, pH 2.5–acetonitrile (55:45, v/v). The retention times for *p*THPC, *m*THPC and hemin were 5.0±0.3, 10.6±0.5 and 16.8±1.2 min, respectively (Figs. 3 and 4).

pH variations exerted a major effect on the retention time. At pH 2.5, *m*THPC is present in an undissociated form. Since in acidic solution, the carboxylic chains of *m*THPC remain fully protonated, the addition of orthophosphoric acid to the mobile phase led to improved separation and reproducibility.

Fig. 3B shows the chromatogram of *m*THPC and *p*THPC extracted from plasma incubated 1 h at 37°C with 200 ng of *m*THPC. Peaks appearing from 1.5 to 3.1 min, were also found in control (Fig. 3A) and patient (Fig. 3C) plasmas, and are due to unidentified oxidising materials. In all cases, when the extraction procedure was applied an unknown peak appeared with a retention time of 21.5 (±1.2) min (Figs. 3 and 4) but this peak never interfered with the *m*THPC peak, as encountered with the extraction procedure previously reported [4,5].

Table 5
Tumor *m*THPC concentrations as a function of time

Time (h)	*n*	Mean (ng/g protein/g tissue)	S.E.M (%)
4	3	129	16
8	4	138	25
48	4	172	43
72	4	216	41
96	3	360	188
120	4	162	73

3.6. Plasma and tissue determination

This method has been applied successfully to measure plasma (32 ng/ml) and tumour tissue (28 ng/g protein/g tissue) *m*THPC levels following intravenous administration in a patient (0.15 mg/kg). This procedure which involves extraction has been also validated for mouse tumour tissue after intraperitoneal administration at the dose of 0.3 mg/kg 4, 8, 48, 72, 96 and 120 h after drug administration (Table 5, Fig. 4).

4. Conclusion

*meso*THPC has a lipophilicity which is supposedly in the optimal region with regard to cellular and tumoral uptake [8]. The high visible absorption of *m*THPC ($\epsilon=146\,000$ l/mol/cm, at 415 nm in methanol) resulted in UV detection being more sensitive than fluorescence [9]. For Whelpton et al., the use of dual detection gave additional qualitative assurance of peak purity [9]. But this double approach is a complex process and more time consuming than the electrochemical detection method. The present method appears suitable for determination of *m*THPC in plasma and also in tissue since it provides the sensitivity (10 ng/ml) required to quantify this photosensitizer in small (60 mg) endoscopic biopsy specimens. Owing to the rapid plasmatic extraction procedure by a highly efficient precipitation method and because of the sensitivity (5 ng/ml), the selectivity and the rapidity of the procedure, this HPLC assay is also suitable for routine analysis in pharmacokinetic investigations.

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