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Research paper

Determination of hypericin in human plasma by high-performance liquid chromatography after intravesical administration in patients with transitional cell carcinoma of the bladder

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Abstract

In the present study, the systemic absorption of hypericin was investigated after intravesical instillation of the compound in nine patients with superficial transitional cell carcinoma (TCC) bladder tumors. Hypericin (8 μM) was instilled in the bladder for 2–3 h before photodynamic diagnosis of bladder tumors. Blood was then collected from a peripheral vein 1 h after termination of the instillation. Solid phase extraction with ammonium acetate buffer and methanol was used to extract hypericin from the plasma. A reversed-phase high performance liquid chromatographic method with fluorescence detection was used to identify and quantify hypericin in the extracts from plasma samples. Analysis of standard plasma samples, which were spiked with known amounts of hypericin, indicated that the pH of the buffer was a determining factor in the extraction yield. The results obtained using ammonium buffer (pH 3.5) and methanol showed the mean extraction recovery of hypericin to be 64% (RSD = 12%, $n = 6$). The limits of detection and quantification were 6 and 20 nM, respectively. Extraction and analysis of the plasma of patients after intravesical administration showed hypericin concentrations below the detection limit (< 6 nM). In addition, photodynamic treatment of in vitro cultured HeLa cells incubated with 1–100 nM hypericin concentrations showed that lower concentrations (1–20 nM) of hypericin do not induce significant photocytotoxic effects. Taken together, these results imply that photosensitization or other systemic side effects in patients are not to be expected after photodynamic diagnosis of TCC bladder tumors with hypericin.

Keywords: Hypericin; Bladder carcinoma; Plasma; Photodynamic therapy; HPLC

1. Introduction

Bladder cancer is the sixth most common malignant disease world-wide [1]. It is a heterogeneous group of tumors, which arise primarily from the urothelium. On initial presentation, approximately 75% of all bladder cancers are limited to the mucosa or submucosa [2]. For these 'superficial' tumors recurrence rates are high (50–80%) after initial treatment, and a small percentage of patients (15–25%) show also carcinoma in situ (CIS) or flat non-invasive high-grade transition cell carcinoma (TCC). The latter superficial lesions have a very high malignant potential and are inexorably progressive and invasive [3]. Hence, recognition of early stage urothelial cancer or CIS is essential in order to offer the patients the most appropriate treatment and the highest cure rate. However, detection of CIS is difficult or impossible with cystoscopy.

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Fluorescence photodetection of flat TCC tumors is becoming of increasing importance. Already 30 years ago, urologists evaluated the use of the fluorescence properties of photosensitizers to improve diagnostic accuracy for bladder tumors [4]. Methods of bladder tumor detection using synthetic porphyrins did not give good results because the fluorescence yield of porphyrins in the tissue is slight [5]. Also since intravesical instillation of synthetic porphyrins does not give reliable results, these compounds are normally given systemically. However, the systemic use of porphyrins is normally associated with prolonged skin photosensitivity [6].

The use of an intravesical instillation is considered an attractive route of photosensitizer administration as it could give a better urothelial selectivity as well as providing an opportunity to administer a high dose of photosensitizer to the bladder tumor with little or no systemic side effects [7]. Intravesical instillation of methylene blue was abandoned because of the high rate (70–85%) of false negative results [8]. Another compound, which is being used for photodynamic diagnosis of bladder cancer is protoporphyrin IX (PpIX), which is formed intracellularly after intravesical instillation of 5-aminolevulinic acid (ALA) or ALA-derivatives. However, as our group and others have demonstrated, ALA-induced PpIX shows limited specificity with high number of false positive results [9–11]. With the use of more lipophilic hexyl ester, a two-fold increase of PpIX fluorescence can be observed, but without significant enhancement of the specificity [12]. In addition, fluorescence induced by ALA is rapidly bleached out and exposure to blue light must be limited to short illumination periods to inspect the whole bladder.

Hypericin is a polycyclic aromatic compound belonging to the chemical class of phenanthroperylenequinones. We previously investigated the possibility of using hypericin as a diagnostic tool for the fluorescence detection of flat bladder carcinoma [13,14]. In these clinical studies, it was demonstrated that following intravesical application of the compound in humans and blue light irradiation, hypericin fluorescence is dramatically enhanced and selectively localized in transitional papillary carcinoma and CIS. From the data obtained it was calculated that the specificity in detecting CIS (and dysplasia) was 98.5% and the sensitivity was 93%. Besides, all papillary lesions present showed bright red fluorescence. Moreover, hypericin induced fluorescence is stable and the bladder can be fully inspected during the procedure. Hypericin is also slowly cleared from bladder carcinoma lesions and the fluorescence can still be visualized 16 h after the instillation [13].

More recently, Pytel and Schmeller have described a technique for fluorescence cytology [15]. After 1–2 h instillation with a 8 μM solution of hypericin, bladder washings were obtained in eight patients with bladder cancer. After centrifugation, fluorescence microscopy revealed selective fluorescence from tumor cells, without fluorescence in benign urothelial cells. Taken together, these figures prove that hypericin is an outstanding tool for the fluorescence detection of urothelial carcinoma.

The level of hypericin in human plasma following intravesical administration has never been investigated. In a previous in vivo rat tumor model study, we used a tetrahydrofuran/dimethyl sulfoxide extraction method followed by a fluorimetric quantitation to determine whether intravesical hypericin instillation leads to systemic absorption in rats [16]. At the hypericin concentrations (8–30 μM) and instillation times (1–4 h) used, there was no measurable hypericin concentration in the plasma (<1 nM).

We carried out the present study in order to determine whether intravesical hypericin instillation in human bladders leads to systemic absorption of the photosensitizer. The information obtained from this study is essential for the clinical set-up of photodynamic diagnosis using hypericin. The presence or absence of hypericin in the plasma following intravesical instillation should determine whether or not patients must protect themselves from a direct exposure to sunlight, so as to avoid skin photosensitivity. In this work, we report a solid phase extraction and a HPLC method, which utilizes C18 extraction cartridges and fluorescence detection for the analysis of hypericin in plasma after intravesical instillation. In addition, the photocytotoxic effect of lower concentrations of hypericin (1–100 nM) was investigated using in vitro cultured HeLa cancer cells.

2. Materials and methods

2.1. Preparation of hypericin for bladder instillation

Hypericin was synthetised as reported previously [17]. The stock solution of hypericin was made in ethanol and kept at −20 °C in the dark. Hypericin is insoluble in water and plasma proteins were used as an effective hypericin carrier for intravesical bladder instillation. The solution for bladder instillation was prepared as follows: 16 mM hypericin in ethanol was diluted 1000-fold in a 1% plasma protein solution in buffered saline and sterilized by membrane filtration. Aliquots of 20 ml were kept frozen, and immediately before use 20 ml saline was added to obtain a 40 ml instillation solution containing 8 μM hypericin.

2.2. Instillation of hypericin in the bladder and sample collection

Nine male patients with superficial TCC bladder tumors were included in the study. The study was carried out under the approval of the ethics committee of the Academic Hospital Leuven (UZ Leuven). The clinical protocol used complied with the recommendation. All patients were provided with a written informed consent prior to the entry. Each patient was instilled with 40 ml of a 8 μM

hypericin instillation solution for 2–3 h. Photodynamic diagnosis of bladder epithelial lesions was carried out immediately after termination of the instillation. The procedure used a xenon arc lamp with a band-pass filter (380–450 nm) and a long-pass filter (>520 nm) in the observation lens (D-Light, Storz, Tuttlingen, Germany) as reported previously [13,14]. One hour after terminating the instillation, blood was withdrawn from a peripheral vein. The blood samples were collected into 10-ml ethylene diamine tetraacetic acid (EDTA) tubes. The tubes containing the samples were spun (1500 g, 15 min) and the plasma kept in the dark at −20 °C before analysis. Similarly, plasma samples (standard plasma) were collected from patients that were not instilled with hypericin.

2.3. Reagents

Potassium dihydrogen orthophosphate and LiChrolut RP-18 (100 mg) cartridges were purchased from Merck (Darmstadt, Germany). Ammonium acetate and tetrahydrofuran were from Acros Organics (Geel, Belgium). Methanol was purchased from BHD (Poole, England). Water was purified in the laboratory by distillation of demineralised water.

A stock standard solution of hypericin in ethanol (40.8 μM) was prepared and stored at −20 °C. Immediately before use, this stock solution was further diluted into working stock solutions of 408 nM. The ammonium acetate buffer consisted of a 25% (m/v) ammonium acetate solution, adjusted to the desired pH using diluted hydrochloric acid, before final dilution.

2.4. Solid phase extraction of hypericin from plasma samples

For hypericin extraction from plasma samples, the LiChrolut RP-18 cartridges were conditioned consecutively with 1.0 ml of methanol and 1.0 ml of 25% (m/v) ammonium acetate buffer. In extracting hypericin using ammonium acetate buffer, we found that extraction of hypericin at pH above 5 resulted in the loss of almost the whole analyte. For this reason, the pH of the ammonium acetate buffer was varied over a range of pH 5.0 to pH 2.5 in order to find an optimal pH, which resulted in less analyte losses. Plasma spiked with hypericin (20 nM) was prepared, and an aliquot (250 μl) was applied to the conditioned extraction cartridge. The cartridge was washed with 1.0 ml of the ammonium acetate buffer. The analytes were then eluted with 1.0 ml of methanol and the eluate was dried under reduced pressure at 40 °C. The residue was reconstituted in 1.2 ml of methanol followed by filtration. The filtrate (900 μl) was evaporated to dryness and the residue was reconstituted in 300 μl of the mobile phase (see further). An aliquot (200 μl) of this solution was injected for analysis by HPLC (see further). The limit of detection (LOD), i.e. peak area equal to three times baseline noise, and limit of quantitation (LOQ), i.e. peak area equal to ten times baseline noise, were determined by analyzing hypericin standard samples at various concentrations. The results showed that ammonium acetate buffer at pH 3.5 was optimal.

To extract hypericin from patient's plasma, an aliquot of the plasma sample (250 μl) was brought on the extraction cartridge and the cartridge was washed with 1.0 ml of the ammonium acetate buffer (pH 3.5). Hypericin was then eluted with 1.0 ml of methanol and the eluate was treated as described above.

2.5. Liquid chromatography instrumentation and chromatographic conditions

The HPLC apparatus consisted of a L-6200 Intelligent Pump (Merck Hitachi, Darmstadt, Germany), an Autosampler Model 655A-40 (Merck Hitachi), a F-1050 Fluorescence Spectrophotometer (Merck Hitachi), with the excitation and emission wavelengths set at 315 and 590 nm, respectively and a Hewlett-Packard integrator Model HP 3396 Series III (Avondale, PA, USA). The column was kept in a water bath at 60 °C. A Supelcosil LC-ABC (C18 base deactivated), 5 μm, 250×4.6 mm ID, (Supelco, Bellefonte, PA, USA) column was used. The flow rate was set at 1.0 ml/min.

The mobile phase was composed of methanol:tetrahydrofuran:buffer (495:330:175, v/v). The buffer consisted of 0.01 M potassium dihydrogen phosphate adjusted to pH 4.0 with 0.1 M phosphoric acid. The organic solvents were first mixed and the buffer added. The mixture was degassed by sparging helium.

2.6. Cell culture

HeLa cells (human cervical carcinoma cell line) were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were grown at 37 °C in humidified 5% CO_2 and 95% air atmosphere in Minimum Essential Medium (MEM) with Earle's Salt containing 2 mM L-glutamine, 1% antibiotic/antimycotic solution, 1% non-essential amino acids, 1% anti-PPLO (pleuropneumonia-like organisms) agent and 10% fetal calf serum (FCS). The medium, cell culture ingredients and phosphate-buffer saline (PBS) were obtained from Gibco BRL (Paisley, Scotland). All cell manipulations related to hypericin were performed under subdued light conditions (<1 μW/cm^2).

2.7. Photocytotoxicity assay

Cells were seeded onto 96-well tissue microtitre culture plates (Costa, Cambridge, MA, USA) at 5×10^3 cells per well and incubated for 24 h at 37 °C. The medium was replaced under subdued light conditions (<1 μW/cm^2) with fresh medium containing 10 or 100% FCS (controls), or fresh medium containing 10 or 100% FCS and different

concentrations (1–100 nM) of hypericin. Subsequently, the cells were incubated under dark conditions at 37 °C for 24 h. The medium and hypericin-containing medium were replaced with PBS and the cells were immediately exposed to light. For irradiation, light emitted by a Rhodamine 6G dye laser (375B, Spectra-Physics, Mountain View, CA, USA) pumped by a 4 W Argon-laser (Spectra-Physics, Mountain View, CA, USA) was coupled into a fiberoptic microlens (Medlight, Ecublens, Switzerland) to obtain an ultra uniform intensity distribution.

The laser was tuned at 595 nm and the laser beam was directed to the 96-well tissue microtitre culture plate containing the cells. The light doses of 1, 5, 10 and 20 J/cm^2) were delivered at a fluence rate of 5.5 mW/cm^2, as measured by an IL 1400A photometer (International light, Newburyport, MA, USA). Cell proliferation was determined by the use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO, USA) reduction assay. For this purpose, MTT was dissolved in the medium and 200 μl (1 mg/ml) of this solution was added to each well containing the cells. The plates were incubated at 37 °C for 4 h. Afterwards, MTT solution was removed and the resulting formazan crystals were dissolved in 200 μl of dimethyl sulfoxide. The plates were read on a microtitre plate reader (SLT, Salzburg, Austria) at 550 nm, and the light-induced cytotoxicity of different concentrations of hypericin with respect to the controls was determined after curve fitting using nonlinear regression using GraphPad Prism 4.00 (GraphPad Software, San Diego, CA, USA). All experiments were performed at least three times.

3. Results and discussion

Diffusion or absorption of endogenous and exogeneous substances from the bladder are prevented by the barrier function of the bladder epithelium. Because the cell membrane on the intravesical site is thick, asymmetrical and contains glycoglucosamines adherent to the cells, the urinary bladder is completely tight [18]. However, in case of TCC of the bladder, the normal urothelium is transformed and its protective function might be lost, leading to systemic absorption following intravesical instillation of some compounds.

Emerging evidence clearly indicates the need for early diagnosis of CIS of the bladder. Intravesical instillation of fluorophores, with limited systemic absorption and thus systemic side effects, is the method of choice when considering photodynamic diagnosis of bladder cancer. We therefore undertook the present study to determine whether there is systemic absorption following intravesical instillation of hypericin for photodynamic diagnosis of TCC human bladder tumors.

Before analyzing hypericin plasma samples after intravesical instillation in humans, we first optimized the solid phase extraction method using plasma spiked with hypericin. Since preliminary experiments showed a loss of almost the whole analyte when hypericin was extracted at pH above 5, the pH of the buffer was therefore varied from 5.0 to 2.5 in order to find the optimal pH resulting in a better recovery. The results showed that pH 3.5 was optimal. This allows the better recovery of hypericin without protein precipitation. The 25% (m/v) ammonium acetate buffer was chosen since a strong buffer was required to avoid competition with the plasma buffer.

The repeatability of the extraction method was assessed using hypericin-spiked plasma at the concentration of 20 nM. The relative standard deviation (RSD) was 10% ($n=6$). The linearity of both extraction procedure and the detector response was verified. This was done by assaying hypericin-free plasma spiked with hypericin at concentrations ranging from 20 nM to 100 nM. A calibration curve was calculated for hypericin using its concentration and the peak area over the standard range. The equation for the calibration curve using linear regression analysis was $y = 53021\times + 80985$ ($r^2=0.9868$, number of concentration points = 5, number of analyses at each concentration point = 3).

The extraction recovery was calculated from the ratio between the amount of hypericin recovered from standard plasma containing 20 nM and an equivalent hypericin solution, which was not extracted but treated identically to the plasma standard. The mean extraction recovery was 64% (RSD = 12%, $n=6$), which is similar to that reported by others [19]. The limit of detection was 6 nM, and the limit of quantification was 20 nM. This quantification limit is similar to that reported by Chi and Franklin [20], but better than that of Liebes et al. [19]. On the other hand, Kerb et al. [21] reported a quantification limit of 0.4 nM.

In extracting and analyzing hypericin from the patient's plasma samples, we first used the plasma samples from three patients who were instilled with hypericin. The results showed no peaks above the detection limit. It was therefore decided to enrich the solution to be injected. For this purpose, several extractions were performed for each patient's plasma sample (for all nine patients) and all the eluates were collected in the same glass tube. The whole eluate was treated as for a single extraction. Table 1 gives for each sample, the number of extractions, and the content expressed as nM of hypericin in plasma. Fig. 1 shows some typical chromatograms obtained from some patient's plasma samples, hypericin-spiked plasma samples and hypericin solution in ethanol.

After improving the sensitivity of the method by multiplying the number of extractions, it is observed that concentrations of up to 3 nM are present in some samples. Based on the assumed phototoxic hypericin threshold (>200 nM) in the plasma [21,22], these results imply that photosensitization or other systemic side effects are not to be expected after photodynamic diagnosis of TCC bladder tumors with hypericin. These results are consistent

Table 1
Extraction and analysis of patient's plasma samples

Sample number	Number of extractions	Contents of hypericin (nM)
1	2	<LOD
2	5	1.86
3	3	<LOD
4	3	<LOD
5	4	<LOD
6	4	1.62
7	3	2.03
8	6	1.78
9	3	<LOD

The table presents the contents of hypericin (expressed as nM) in plasma of patients. The patient's plasma sample numbers and the number of extractions for each sample are also given. <LOD, means that the content of hypericin is below the limit of detection.

with the clinical observation following intravesical administration of hypericin. In these studies, no local or systemic side effects have been reported in all of the patients who have undergone photodynamic diagnosis with hypericin [13,14].

Next we determined the photocytotoxic effect of hypericin in in vitro cultured HeLa cells. The aim of this experiment was to find out whether low concentrations of hypericin could induce significant cytotoxic effects in in vitro cultured HeLa cells after photo-activation. In this study, we incubated hypericin in the medium containing either 10% or 100% FCS. Hypericin is known to strongly bind to plasma proteins, and this binding is known to slow the release of the compound into the cells, thereby reducing its photo-activity [23]. The extent of hypericin-induced skin photosensitization will therefore not only depend on the extracellular concentration of hypericin, but also on its binding to the plasma proteins and subsequent release into the cells/tissues.

Fig. 2 presents the results of the photocytotoxicity effect of hypericin on HeLa cells. The results show that, even at the highest light dose used, lower hypericin concentrations (1–20 nM) did not induce significant photocytotoxicity on the cells. The intensity (<200 μW/cm^2) of the polychromatic light measured indoor under normal working light conditions is less activating than that of the monochromatic light (5.5 mW/cm^2) used in this study, an indication that low concentrations of hypericin (1–20 nM) are less likely to induce skin photosensitivity in patients [21,22]. At higher light doses (10–20 J/cm^2), pronounced cytotoxicity of hypericin was observed at the hypericin concentrations of 50 and 100 nM, especially in case hypericin was incubated in medium containing 10% FCS. As mentioned, the less pronounced cytotoxicity of hypericin in case hypericin was incubated in 100% FCS is due to the strong binding of the compound to the plasma proteins, leading to a slow release of hypericin into the cells. This results in relatively lower intracellular hypericin concentrations, and hence less photo-activity on the cells.

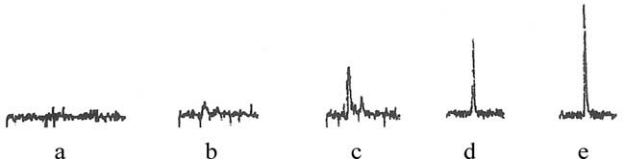

Fig. 1. Chromatograms obtained with: (a) a blank solution, (b) patient's sample number 1, (c) enriched (three extractions) patient's sample number 1, (d) hypericin standard plasma sample (20 nM), (e) hypericin solution in ethanol (20 nM).

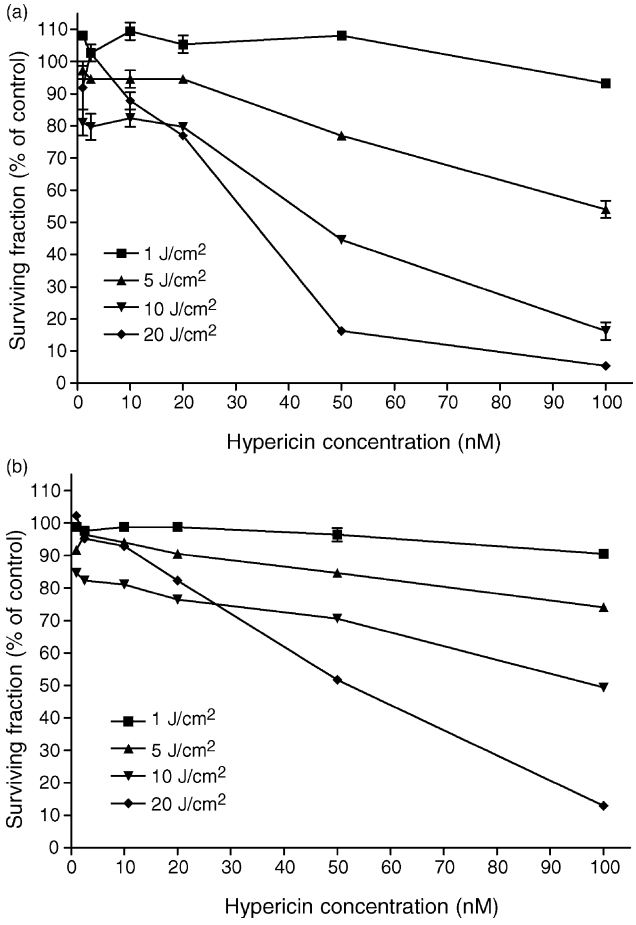

Fig. 2. Photocytotoxicity of hypericin in HeLa cells. Cells were incubated for 24 h with hypericin (1–100 nM) in the medium containing 10% foetal calf serum (FCS) (A) or 100% FCS (B). Hypericin treated cells were irradiated with 595 nm laser light at different light doses (1–20 J/cm^2) delivered at a fluence rate of 5.5 mW/cm^2. The cytotoxic effect was then evaluated using an antiproliferative assay, as described in materials and methods. Mean ± standard deviation (n = 3).

In our previous study, microscopic analysis of hypericin fluorescence in the sections of human bladder biopsies showed bright fluorescence in the TCC tumors, and no fluorescence was apparent in the underlying submucosa and muscle layers [14]. Hypericin is not metabolized by mammalian cells [24], and no hypericin metabolites have been detected after oral or intravenous administration in humans [21,25]. It is therefore unlikely that a conversion of

hypericin to other compounds takes place in the human body following intravesical administration of the compound.

Taken together, the results show that following intravesical administration there is negligible concentration of hypericin (<6 nM) in the plasma. These results demonstrate that high concentrations of hypericin can be effectively maintained in the urinary bladder for photodynamic diagnosis of TCC tumors without significant exposure to the systemic circulation.

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