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HPLC Method for Quantification of Halofuginone in Human Ureter: *Ex-Vivo* Application

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Abstract

A new high-performance liquid chromatography method was developed to study the diffusion of halofuginone in the wall of the ureter. The human ureter extracts were prepared by trypsin digestion of the tissues followed by liquid–liquid extraction using the isopropanol after precipitating the proteins. The method used a reversed-phase C18 column with a mobile phase delivered to the analytical column according to a gradient program starting at a composition of ammonium acetate (pH 4.7; 10 mM)-acetonitrile-triethylamine (70:30:0.2, v/v/v) and linear changes to 90% of acetonitrile at 11 min. Liquid-liquid extraction proved to be selective for the HFG and provided a high recovery rate of 97.7%. The HPLC method was successfully validated by applying the novel validation protocol using the accuracy profile based on a new concept, that of the total error. The protocol V4, with five levels of concentration and 105 trials, was selected according to the algorithm designed by the SFSTP 2003 committee. Acceptance limits were set up at ± 20%, while the risk was settled at 5%. The method was found accurate over a concentration range of 0.2–10 µg/ml. The limit of detection for HFG was 0.06061 µg/ml. In order to demonstrate the applicability of this method in *ex vivo* application, the quantification of HFG in the wall was applied to study its distribution from a gel (0.03% w/w of HFG) in the human ureter.

Keywords: Halofuginone; HPLC-UV; Wall ureter

Introduction

Halofuginone (HFG) is a coccidiostat used in veterinary medicine (Figure 1). It is a halogenated derivative of febrifugine, a natural quinazolinone alkaloid [1]. Furthermore, it inhibits the expression of the collagen type 1 gene and is becoming one of the most intersting novel antifibrotic [2-6].

The efficacy of halofuginone in preventing the occurrence and recurrence of urethra narrowing has been proven in animal studies using HFG orally or locally [7,8]. Indeed, HFG is a promising compound in the treatment of the human urethra structures when used as an intraurethral gel to attain a local effect.

Before starting the clinical phase 1 trials in humans, *in vitro* studies, using an experimental model simulating the different layers of the human urethra, are needed to determine the qualitative and quantitative composition of the gel. Indeed, the gel formula used is one that would allow rapid diffusion of HFG in the wall without passage into the bloodstream. The human ureter has the same composition as the urethra and may be obtained following renal transplantation. Thus, it is used in *ex vivo* experiments to simulate the different layers of the urethra.

In this context, the quantitative determination of HFG in the ureter wall is essential to study its distribution from different gels in the human ureter *ex vivo*.

Several methods are available for determining HFG in feeds and tissues [9-16]. In fact, in broiler production, HFG is used during almost all the breeding periods except for pre-slaughter withdrawal. There is a considerable risk of HFG residues in edible tissues. The concern for consumers' safety was the reason for the establishment of maximum residue limits for most of the coccidiostats in tissues [17,18]. Therefore, the monitoring of residues of coccidiostats is performed in all European countries [17,18]. As far as we know, this is the first study to date aiming at determining HFG while using a new method of extraction from the wall of a human ureter, developed and validated using high-performance liquid chromatography (HPLC). In order to demonstrate the applicability of this method in *ex vivo* application, the quantification of HFG in the wall was applied to study its distribution from a gel (0.03% w/w of HFG) in the human ureter in *ex vivo*

Experimental Part

Materials

HFG hydrobromide standard was obtained from Discovery Fine Chemicals, Unit 4A, Old Forge Roadster, Ferndown Industrial Estate, Wimborn, Dorset, United Kingdom. The internal standard (IS) imipramine hydrochloride was purchased from Sigma Ultra (England). HPLC grade acetonitrile (ACN) and propan-2-ol was purchased from Lab Scan. Ammonium acetate, sodium carbonate, and trypsin were purchased from Sigma Aldrich. Acetic acid was purchased from Merck

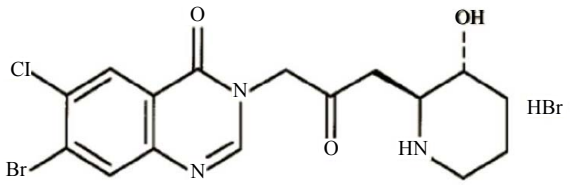

Figure 1: Structure of halofuginone

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and triethylamine from Fluka. Ammonium sulfate was obtained from Riedel-de Haên. Purified water was obtained by the distillation apparatus Millipore (Arium 611DI/611UV) in our laboratory. Acetate buffer (10 mM, pH=4.3) was prepared by dissolving 19.27 g of ammonium acetate and 25 ml of acetic acid in distilled water, and by diluting them to 1 L of water. Sodium carbonate solution (10% w/v) was prepared by dissolving 100 g of sodium carbonate in distilled water and diluting it to 1 L. The pH was adjusted to 8 - 8.5 with acetic acid. All other chemicals used were of an analytical grade.

Liquid chromatography instrumentations

Chromatographic analysis was performed using Agilent 1200 HPLC equipped with G1311A quaternary pump, G1322A degasser , G1315D diode array detector, thermostated column compartment, manual injectors (syringe volume =5 µl). Separation was performed at 30°C using Lichrospher® C18 analytical column with 5 µm particle size, 4 mm internal diameter, and 250 mm in length, and using Lichrospher ® 100 RP18 guard column (4 x 4 mm) with 5 µm particle size.

HPLC conditions

HFG and IS were eluted in gradient mode. The mobile phase consists of 10 mM ammonium acetate buffer (pH adjusted to 4.3 with acetic acid), triethylamine, and acetonitrile. It is delivered to the analytical column according to a gradient program, starting at a composition of ammonium acetate (pH 4.7; 10 mM)-ACN-triethylamine (70:30:0.2, v/v/v) and linear changes to ammonium acetate (pH 4.7; 10 mM)-ACN-triethylamine (10:90:0.2, v/v/v) at 11 min. The flow rate was 1 ml/min. A 5µl injection volume was used for each analytical run. The detector was set at 243 nm.

Preparation of standards

Stock solutions of HFG and IS were prepared at a concentration of 0.5 and 1 mg/ml, respectively, in ammonium acetate buffer (pH 4.3; 25 mM). These were stored at 4°C. The stock standard solution was stable for 3 months [19].

A series of standard HFG solutions were prepared by the appropriate dilution with mobile phase to obtain concentrations across a range of 0.2-10 µg/ml in human ureter extracts.

A working internal standard solution was freshly prepared every day from stocks at 0.1 mg/ml in distilled water.

Sample preparation

Fresh, surgically excised samples of human ureter were obtained directly after kidney transplant surgery. All the specimens were transferred to our laboratory within 1 h after being placed in a transport fluid (Eurocollins®) to provide 48 hours conservation. Ethical approval for the use of the ureter was provided by the Research Ethics Committee of the Hospital. After trimming away excess connective and adipose tissue, all specimens were immersed in physiologic water and distilled water, respectively, for 5 seconds. Human ureters were taken up into a Whatman filter paper to dry. Samples were cut into circular discs by means of a pair of scissors and a pair of tweezers (exposed areas 50 mm^2).

Extraction

The extraction of HFG from the human ureter was described for the first time in our study. Put each circular disc of human ureters into a 2-ml Eppendorf tube. One milliliter of sodium carbonate solution (10% w/v) was added to each sample. Manual grinding was carried out using scissors. We added 100 µl of 0.1 mg/ml IS and HFG to the homogenate. Enzymatic hydrolysis was performed by 1 mg of trypsin for digesting the proteins. The homogenate was incubated for 3 h in a bath at a temperature of 37°C. The mixture was transferred into 15-ml tubes and 600 mg of ammonium sulfate was added to precipitate the proteins. It was mixed for 1 minute on a vortex. Four milliliters of propan-2-ol were added. The mixture was homogenized for 20 minutes by agitation. Then, samples were centrifuged for 15 min at 4000 rpm. An aliquot of 2 ml of supernatant was removed and evaporated to dryness at 55°C under reduced pressure, using a rotary evaporator. The residue was dissolved in 100 µl of the mobile phase. Five microliters were injected into the HPLC system.

To determine the extraction yield, samples containing HFG and IS drugs with internal standard were injected into the HPLC system, with and without extraction. The direct injection samples contained an equivalent amount of drugs as spiked in the extracted samples. Extraction efficiency is given as: *extraction efficiency % = (peak area of extracted sample/peak area of un-extracted sample)*100*.

Validation of analytical method

The validation was carried out in accordance with the performance criteria described in SFSTP 2003 using human ureter tissue [20]. This validation is based on the use of the accuracy profile based on a new concept, that of the total error [20,21]. This approach has been used in a wide range of methods such as liquid chromatography (LC-UV, LC–MS), spectrophotometry, and ELISA [22]. The accuracy profile of the analytical procedure is based on the expectation tolerance interval and the concept of total error (bias + standard deviation). It allows not only bringing together the objectives of the procedure with those of the validation but also visually grasping the capacity of the procedure to fulfill its objectives and to control the risk associated with its use in routine [20,21,23-25].

There are five experimental validation protocols [26]. Protocol V4 was selected according to the algorithm designed by the committee SFSTP 2003 [20,26]. The following criteria were considered: a prior knowledge of the assay procedure of HFG by HPLC; the matrix effect is possible and unknown; and calibration is not done at a single level of concentration.

The V4 protocol includes:

- Calibration standards at five concentration levels (0,2, 1, 2.5, 5 and 10 µg/ml) with and without matrix with two independent replicates for each standard.

- Standards validation, five concentration levels (0,2, 1, 2.5, 5 and 10 µg/ml) with the matrix with three independent replicates for each standard.

All these standards were prepared in 3 days with a total of 105 trials.

To test the selectivity/specificity of the method, representative blank samples (six human ureters from different patients) were analyzed and checked for interferences (peaks) at the retention times of the HFG and IS. Peak identification was performed by spectral information provided by a diode array detector.

During the validation step, acceptance limits were enlarged in accordance with a risk decrease. The first ones were set up at ± 20%, a value adopted by the American Association of Pharmaceutical Scientists guidelines when the validation results are expressed in terms of total error, while the risk was settled at 5% [27].

Results and Discussion

Development and optimization of liquid-liquid extraction of HFG

The wall of a human ureter is a complex biological matrix. No previous study has described the extraction of HFG from this matrix. A new method of HFG extraction was established after performing different screening runs based on the previously reported liquid-liquid extraction method for HFG extraction [9,14,28]. After a homogenization step and enzymatic hydrolysis performed by trypsin, a chemical deproteinization with ammonium sulfate was used. Indeed, the salt plays a dual role; not only does it allow a differential precipitation of the proteins but it also permits separation of the aqueous phase and organic phase by increasing the ionic strength of the solution. In fact, the extraction solvent selected during optimization is isopropanol, a solvent which is miscible with water. However, the presence of ammonium sulfate ensures the trapping of water molecules to a separate organic phase.

Ethyl acetate is the most commonly used solvent in the literature [9,14,28]. We tested different extraction solvents ethyl acetate, dichloromethane and isopropanol . However, isopropanol gave the best extraction efficiency of HFG. Indeed, it is a slightly more polar solvent than ethyl acetate with an additional protic effect. Isopropanol is a hydrogen bond donor, thus it can form hydrogen bonds with two oxygen acceptors of HFG. This may explain why isopropanol was the solvent of choice to extract the HFG. However, the disadvantage of a polar solvent lies in it's poor selectivity toward polar compounds. But, the specificity of the method has been proven and there is no interference with endogenous peaks at 243 nm. Increasing the volume of extraction and using of a slow mechanical stirring by inversion increases the extraction yield of HFG and IS. The extraction was carried out using a carbonate alkaline solution. At this pH, HFG and IS have an overall neutral charge and are extractable by organic solvents. The extraction yield is 97.7% and 90.0% for HFG and IS, respectively. This method is simple and has a high extraction yield.

Optimization of the chromatographic method

The assay of HFG by HPLC was commonly used [9,11-15]. The absorption maxima of HFG are at 243, 285, 315, and 325 nm (Figure 2). In the literature, most authors have used a wavelength of 243, which allows a maximum absorption of the molecule and is in agreement with the absorbance of the IS, which has an absorption maximum at 250 nm [9,14,16].

HFG and IS have two cyclic structures with a basic nitrogen. These are moderately polar molecules that have the same chromatographic behavior (Figure 3). We then opted for reverse-phase chromatography for this molecule. We have chosen, as a stationary phase, a column based on octadecylsilane bonded silica C18 (25 cm × 4 mm, 5 µm). The choice of the composition of the mobile phase was directed by the literature. Indeed, many authors use a mixture of acetate buffer and acetonitrile [9,11-14,16,29]. The tests were carried out on solutions of 20µg/ml HFG in a solution of ammonium acetate 0.25 M. Preliminary tests were performed by varying several parameters: the elution system of the mobile phase, the proportion of organic solvent, the concentration of the buffer, and the pH of mobile phase.

By varying the proportions of the organic solvent, we concluded that the HFG is better eluted with the aqueous phase. Indeed, with a high proportion of acetonitrile, HFG peaks are wider and the retention times are slightly higher, whereas IS is less polar than HFG and presents less restraint with high proportions of acetonitrile. In order to have an acceptable analysis time, we experimented with a gradient elution, which allowed us to have a retention time for 4.9 min and 9.2 min of HFG and IS, respectively. Indeed, starting with a high proportion of acetate buffer, the HFG, more polar than the internal standard, is eluted first; and increasing the proportion of acetonitrile decreases the retention time of IS.

The two molecules analyzed are basic compounds. Streaks were observed in preliminary trials. Indeed, they are attributed to interactions with residual silanol remains. Two factors have enabled us to refine the peaks: increasing the concentration of the acetate buffer and adding triethylamine, which is a modifier whose role is to saturate the remaining sites. A pH of 4.3 allows a sharing phenomenon between the ionized form and the ideal molecular shape for optimal retention of the two molecules.

The best chromatogram was obtained with a mobile phase composition starting at a composition of ammonium acetate (pH 4.7; 10 mM)-ACN-triethylamine (70:30:0.2, v/v/v) and linear changes to ammonium acetate (pH 4.7; 10 mM)-ACN-triethylamine (10:90:0.2, v/v/v) at 11 min. In these conditions, HFG and IS have retention times of 4.9 and 9.2 min, respectively (Figure 4).

Validation of analytical method

Selectivity: A diode array test of purity was applied to verify the purity of HFG and IS. Referring to the chromatogram of blank extracts, no interference was observed from these extracts at the retention times of the peak corresponding to HFG and IS.

Response function: The response function of an analytical method is, within the range selected, the existing relationship between the response (signal) and the amount (quantity) of the analyte in the sample system [26]. The validation results for the response function

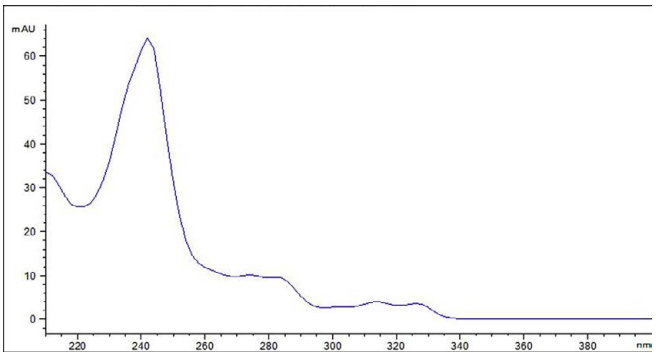

Figure 2: Absorption spectrum of halofuginone concentration of 1 mg / L in 0.25M acetate buffer

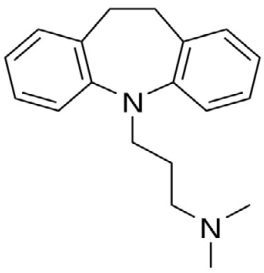

Figure 3: Structure of imipramine

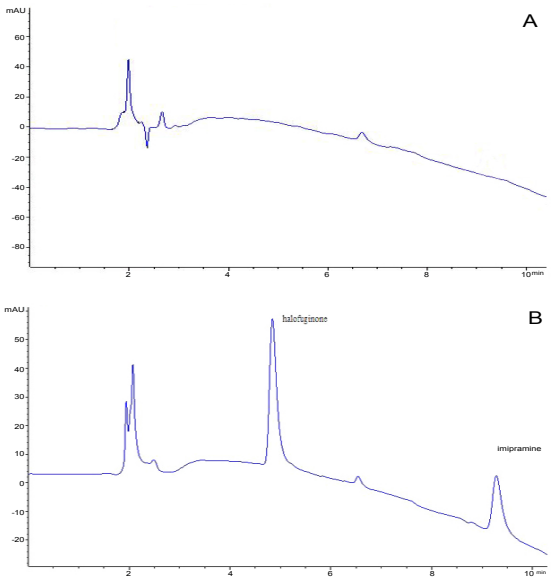

Figure 4: Representative HPLC chromatogram obtained from the analysis of halofuginone and imipramin as internal standard in human ureter extract.(A) blank human ureter extract; (B) standard solution of halofuginone (5 µg/ml) and imipramine (10 µg/ml) spiked in human ureter extract.

(t=0 min ammonium acetate (pH 4.7;10 mM)-ACN-triethylamine (70:30:0.2, v/v/v) ; t=10 min ammonium acetate (pH 4.7; 10 mM)-ACN-triethylamine (10:90:0.2, v/v/v))

in the present study are presented below in Table 1. A weighted (1/X) quadratic regression (with matrix) model was used for the determination of HFG in the human ureter.

Trueness and precision: Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one [26,30]. Precision is the closeness of agreement among measurements from multiple sampling of a homogenous sample under recommended conditions. Trueness and precision are not considered here as decisional parameters but their determination allows assessing the quality of the analytical method as they correspond respectively to random errors and systematic ones [20]. As can be seen in Table 1, the overall relative bias does not exceed 6.4% and the relative standard deviation for repeatability and intermediate precision are below 6.8%.

Accuracy profile: Accuracy takes into account the total error, which is the sum of systematic and random errors related to the test result [26]. The upper and lower β expectation tolerance limits expressed in µg are presented in Table 1 as a function of the introduced amounts. As can be seen from these results, the proposed method was accurate, because the different tolerance limits did not exceed the acceptance limits of total error for all amount levels tested, including the lowest one. The tolerance interval was within the 20% acceptance limit at all amounts. This validation suggests that the method offers sufficient guarantees, providing results ± 20% of the true value in at least 95% of cases (Figure 5).

Linearity: The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the quantities of the analyte in the sample [26].Therefore, a linear model was fitted in a calculated amount of the validation standards for all series as a function of the introduced amounts. The regression equation is presented in Table 1. A linear regression model (Figure 6) is fitted on the back-calculated amounts as a function of the introduced amounts in order to obtain the following equation:

$$Y = 0.08998 + 0.9973\ X$$

where Y = back-calculated amounts (µg) and X = introduced amount (µg). The coefficient of determination (r^2) is equal to 0.9979. The residual sum of squares (RSS) is equal to 1.154.

In order to prove method linearity, the absolute β expectation tolerance interval was applied. The linearity of the HFG HPLC method was also demonstrated since the β-expectation tolerance limits were included in the absolute acceptance limits for the whole concentration range investigated as shown in Figure 6.

Detection and quantification limits: The limit of detection (LOD) is the smallest quantity of the targeted substance that can be detected, but cannot be accurately quantified in the sample. The LOD was estimated using the mean intercept of the calibration model and the residual variance of the regression [20,22,25]. By applying this computation method, the LOD was found to be equal to 0.06061 µg/ml.

The lower limit of quantification (LOQ) is the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy (22). *LOQ is given as: LOQ = LOD * 3. With this method, the lower LOQ was equal to 0.2 µg/ml."*

Ex-vivo model application

The purpose of this application was to evaluate a gel formulation of HFG, an inhibitor of collagen synthesis, for the treatment and the prevention of occurrence and recurrence of urethra narrowing. The effectiveness of the therapy, following the intraurethral injection, is based on the availability of a pharmacologically active element, able to reach its biological target, and penetrate the wall. HFG should penetrate into the deep structures and should remain there at an effective concentration. The kinetics of diffusion in this case is important as the patient can only refrain from urinating, and in turn eliminating the gel, for two hours maximum. HPLC assay was applied to kinetic study to ensure adequate release kinetics which guarantees the therapeutic effect.

The male urethra is composed of several layers: epithelium transitional, chorion, muscular and adventitia. These four layers are also found in the human ureter, which has a very similar composition. The male urethra is surrounded by the anterior part of the corpus spongiosum, a vascular structure. To simulate in *ex vivo* the male urethra, wall absorption has been studied on samples of fresh human ureter mounted on modified Franz cells (exposed areas 50 mm^2). The fresh, surgically excised samples of human ureter used for the tests were obtained following kidney transplant surgery kept in a fluid conservation (Eurocollins®).

Four cells were performed for each ureter to quantify HFG in the wall after 30, 60, 90, and 120 minutes and were immediately tested. The ureter was cut and clamped between the receptor and donor compartments. The receptor compartment was filled with 2.5 ml of diffusion medium (phosphate buffer pH 7.4) through sampling port taking care to remove all the air bubbles. The contents were stirred by small magnetic bead to keep them well mixed.

Three hundred milligrams of the gel were filled in the upper compartment (on the internal surface of ureter). The cells, with stirring, were stabilized in a water bath at 37°C and closed with Parafilm® to avoid formula evaporation. The experiment was repeated five times

Response function (p=3; n=2)		Weighted (1/X) Quadratic Regression (within matrix)		
		Calibration range (m=5): 0.2-10 µg/ml		
		series 1	series 2	series 3
	Slope	0.4921	0.4729	0.4972
	Intercept	-0.05278	-0.04351	-0.05301
	r^2	0.9939	0.9974	0.9958
Trueness (p=3; n=2)				
		Absolute bias (µg/ml)	Relative bias (%)	Recovery (%)
	0.1 µg/ml	0.004295	2.148	102.1
	1 µg/ml	0.05569	5.569	105.6
	2.5 µg/ml	0.07220	2.888	102.9
	5 µg/ml	0.3170	6.339	106.3
	10 µg/ml	-0.04940	-0.4940	99.51
Precision (p=3; n=2)				
		Repeatability (RSD%)	Intermediate precision (RSD%)	
	0.1 µg/ml	6.822	6.822	
	1 µg/ml	3.179	3.421	
	2.5 µg/ml	1.892	1.892	
	5 µg/ml	0.8241	1.447	
	10 µg/ml	1.865	2.281	
Accuracy (p=3; n=2)				
		Beta-expectation tolerance limits (µg/ml)	Relative Beta-expectation tolerance limits (%)	
	0.1 µg/ml	[0.1709 , 0.2377]	[-14.54 , 18.84]	
	1 µg/ml	[0.9690 , 1.142]	[-3.101 , 14.24]	
	2.5 µg/ml	[2.456 , 2.688]	[-1.742 , 7.517]	
	5 µg/ml	[5.066 , 5.568]	[1.325 , 11.35]	
	10 µg/ml	[9.325 , 10.58]	[-6.751 , 5.763]	
Linearity (p=3; n=2)				
	Range (µg/ml)	0.2-10		
	Slope	0.9973		
	Intercept	0.08998		
	r^2	0.9979		
LOD (µg/ml)		0.06061		
LOQ µg/ml)		0.2000		

P: number of series of analysis; m: number of amount levels; n: number of replicates; RSD: Relative Standard Deviation.

Table 1: Validation results

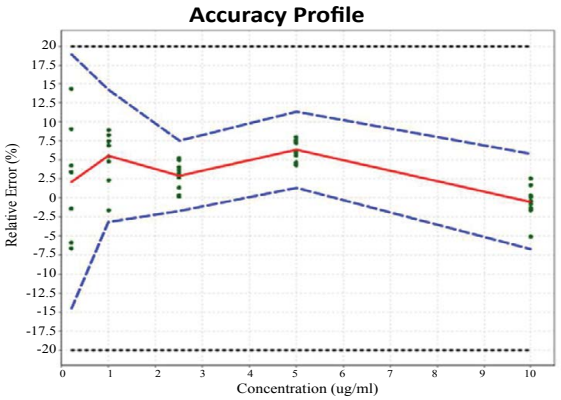

Figure 5: Accuray profile Accuracy profile obtained by considering Weighted (1/X) Quadratic Regression (with matrix) The plain line is the relative bias, the dashed lines are the β-expectation tolerance limits and the dotted curves represent the acceptance limits. The dots represent the relative back-calculated concentrations and are plotted with respect to their targeted concentration.

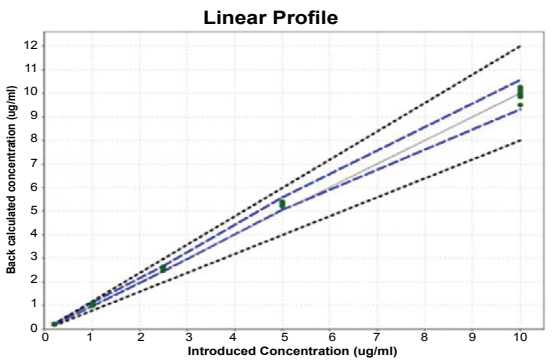

Figure 6: Linear profile of the analytical method for the determination of HF in human ureter extracts using a linear regression model as calibration curve. The dashed limits on this graph correspond to the accuracy profile, i.e. the expectation tolerance limits expressed in absolute values. The dotted curves represent the acceptance limit at ± 20% expressed in the amount unit. The continuous line is the identify line y=x.

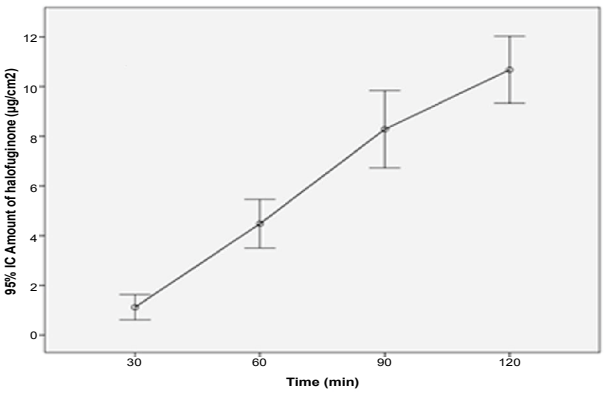

Figure 7: Permeation profile of halofuginone (through wall ureter from a gel at a concentration of 0.03% w/w of halofuginone). Applied dose: 300mg of gel. The cumulative amount of halofuginone (µg) that penetrated wall (per cm2 of wall). Each data point represents the averaged value of samples from wall replicates (n=5). The vertical error bars represent the 95% confidence intervals.

with five different ureters.

Wall diffusion was determined by measuring the amount of drug residing in the ureter layer. After the application, wall samples were washed off with water and dried with absorbent paper. HFG content in the various samples was measured by HPLC. Gel formulation was prepared by dissolving HFG at concentration of 0.03% w/w in water with carboxymethylcellulose 0.06% w/vol.

The HFG content of the wall was expressed as mean (µg/cm^2) ± standard error of mean. Amount of HFG measured in the wall (µg/cm^2) was plotted as a function of time.

As demonstrated in Figure 7, the results of the curve shows that the amount diffused in the wall increases during the two hours after application of the gel to reach an amount of 10.6 µg/cm^2 at 120 min. Various parameters can influence the diffusion of a molecule in the wall. This method could, in more extended studies, examine different gel formulations to keep the one whose diffusion kinetics ensures optimal therapeutic effect during the two hours of application.

Conclusion

The determination of HFG is essential to study its diffusion from different gels in the human ureter *in vitro* experiments. In this work, we have developed and validated a method for the determination of HFG from the wall of a human ureter by HPLC. To the best of our knowledge, this method is the first assay for the quantitative determination of HFG in the human ureter. Liquid-liquid extraction proved to be selective for the halofuginone and provided a high recovery rate of 97.7%. A rapid and reliable RP-HPLC assay has been developed and validated in accordance with the performance criteria described in SFSTP 2003. The assay provides a linear response across a range of concentrations 0.2-10 µg/ml. The assay is fast, accurate, and precise for the quantification of HFG in human ureter tissue extracts. The method of determination of HFG developed in this study allowed us to check the diffusion of HFG from a gel 0.03% w/w in the wall of the ureter.

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