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Reversed-phase ion-pair analysis of 5-fluorouracil in pig bile, liver homogenate, plasma and urine after administration by isolated liver perfusion

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Abstract: 5-Fluorouracil is at present one of the most administered cytostatic drugs in cancer chemotherapy. However, due to its high toxicity, local administration of the drug, e.g. by isolated liver perfusion, may be advantageous.

A bioanalytical procedure, suitable for the routine analysis of 5-fluorouracil in pig bile, liver homogenates, plasma and urine is described using reversed-phase ion-pair chromatography with absorbance detection at 268 nm. Using a protein precipitation procedure with acetonitrile, a determination limit of 3500 ng g^{-1} was achieved for liver samples, and 5 ng ml^{-1} for plasma samples using a liquid–liquid extraction step.

Keywords: *Liquid chromatography of 5-fluorouracil; reversed-phase ion-pair analysis; absorbance detection; analysis in bile, liver homogenates, plasma and urine.*

Introduction

The anti-metabolite 5-fluorouracil (FUra) is one of the most important cytostatic drugs used in cancer therapy nowadays [1, 2]. The analyte is especially used in the treatment of liver metastasis originating from colon cancer. Moreover, FUra is used, alone or in combination with other cytostatic agents, against stomach, pancreas, breast and ovarian cancer [1]. The main disadvantage of FUra is its toxicity, related with cell death of tissues with a high proliferative activity such as bone-marrow, mucous membrane and gonadal tissue [3]. In the treatment of liver metastasis, therefore, local administration may prevent the development of systemic side effects. In this study, an isolated liver perfusion in a pig was investigated in order to examine the possibility of administering higher doses of the analyte without the development of systemic side effects. To evaluate these studies, a routine analysis applicable for the determination of FUra in liver homogenates and biological fluids was required.

In the literature, several gas chromatographic (GC) and liquid chromatographic (LC) procedures have been reported. Because of the lack of sensitivity and selectivity,

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especially when FUra is used in combination with other fluoropyrimidines, LC methods are to be preferred over the described GC systems [4].

LC analysis of FUra can be executed by normal-phase (NP) [5, 6], ion-exchange (IE) [7], reversed-phase (RP) [4, 8–13] and RP-ion pair (RP–IP) modes [2, 14, 15]. In bioanalysis, NP is not very promising because of the low solubility of the analyte in organic solvents and because of the composition of the matrix, but it can be applied in combination with a pre-chromatographic derivatization of FUra [6].

RP chromatography, without the addition of an ion-pairing agent, results in unacceptable small capacity ratios (k') or needs laborious and time-consuming sample pretreatment procedures [4, 8, 9]. RP–IP with cetyltrimethylammonium bromide, results in acceptable k' values, unlike systems with tetraalkylammonium halogens [14], but requires careful control of column temperature and equilibrium-time. In the RP–IP mode, octyl (C_8) [2, 14] or octadecyl (C_{18}) [14] chemically modified silica or polymeric PRP-1 stationary phase [15] can be applied.

Sample pretreatment can be performed using several procedures such as: protein precipitation [15], liquid–solid isolation with RP [6] or IE materials [4], liquid–liquid extraction [2, 5, 11, 12, 16], applying relatively polar extraction solvents (e.g. ethyl acetate), ultrafiltration [10] or with combinations of these techniques, e.g. liquid–solid isolation after protein precipitation [7, 8, 17], and liquid–solid isolation after liquid–liquid extraction [9].

Detection normally takes place with an absorbance detector, in the ultra-violet (UV) mode, however after pre-chromatographic derivatization with a fluorescence introducing reagent, a fluorimeter can be used as a detection system [6, 16, 18]. An alternative analytical method for the bioanalysis of FUra is the rather time-consuming isotachophoresis [17] or radioactivity detection after LC analysis [14].

This paper describes a method for the routine bioanalysis of FUra using an RP–IP system with a polymeric PRP-1 column and is suitable for the determination of the analyte in plasma, urine, liver homogenates, bile and perfusion liquid after administration of the analyte by means of an isolated liver perfusion to a pig. With the described combination of sample pretreatment, protein precipitation for liver samples and liquid–liquid extraction for plasma samples, determination limits of about 5 ng ml^{-1} are obtained, which is sufficient for pharmacokinetic studies.

Experimental

Materials

FUra was obtained from Hoffmann-La Roche (Basle, Switzerland) in ampoules containing 25 mg ml^{-1} or 50 mg ml^{-1} in 0.9% sodium chloride solution and stored in polypropylene vials at 253 K in the dark. The organic solvents and hydrochloric acid (35%) were purchased from Baker (Deventer, The Netherlands) and tris-(hydroxymethyl)-aminomethane (Tris) and cetyltrimethylammonium bromide (cetrimonium) came from Janssen Chimica (Beerse, Belgium). All solvents and reagents were of analytical reagent grade and were used as such. Ethyl acetate was freshly distilled before use and saturated with water and, throughout the study, deionized water (Milli-Q Purification System, Millipore, Bedford, MA, USA) was used.

Chromatography

The LC system consisted of a model 1044 high pressure Orlita pump (Giessen, FRG),

a model 7125 Rheodyne (Berkeley, CA, USA) injection valve (fixed 100-μl loop), and a Spectroflow model 757 variable wavelength detector (Kratos, Ramsey, NJ, USA), operating at 268 nm.

A stainless steel column (100 × 3.0 mm, i.d.) packed with 10-μm PRP-1 (Hamilton, Reno, NV, USA) material was used. The column was packed with the pressurized slurry technique [19]. The applied eluent was a mixture of 50 mM Tris and 5 mM cetyltrimethylammonium bromide, adjusted to pH 8.2 with concentrated hydrochloric acid. Chromatography was performed at ambient temperature and at flow rate of 1.0 ml min^{-1}.

Sample preparation

The liver samples of 250 mg each were taken every 15 min (0, 15, 30, 45, 60, 75 min) during the isolated liver perfusion, pulverized (2 × 6 s) in 2 ml of acetonitrile with a Polytron (Kinematica, Luzern, Switzerland) and immediately frozen in liquid nitrogen. Just before the actual analysis, the thawed samples were homogenized (vortex mixing), centrifuged (1000*g*) and an aliquot (25–50 μl) of the acetonitrile phase was evaporated to dryness under a stream of dry nitrogen. The residue was dissolved in 1 ml of the eluent and a 100 μl aliquot was injected into the LC system.

Heparinized blood samples were taken at intervals during and after the isolated liver perfusion (0, 30, 60, 75, 80, 85, 90, 120, 180, 240, 360 min), and centrifuged at 1000*g*. The resulting plasma samples were stored under the described conditions (253 K) and just before the actual analysis the defrosted samples were homogenized (vortex mixing) and centrifuged (1000*g*). Aliquots of 250 μl were vortex mixed, with 250 μl of a 50 mM Tris solution (pH 6.0), for 40 s and subsequently mixed with 5 ml of ethyl acetate. The extraction was carried out for 5 min, using a homemade rotating device at 60 rpm (20 g). The resulting ethyl acetate fraction was evaporated to dryness (N_2) and the residue was dissolved in 250 μl of the eluent, from which 100 μl was subjected to LC analysis.

The bile (0, 30, 60 min) and perfusion liquid (0, 5, 15, 30, 45 min) samples contained relatively high concentrations of the analyte and therefore, dilution of the samples was the only sample pretreatment required before LC analysis. The urine (0, 30, 60 min) was analysed to check whether any leakages had occurred during the perfusion.

All biological samples, containing FUra, were stored in polypropylene tubes in the dark at 253 K until the actual analysis took place.

Results and Discussion

Sample pretreatment and recoveries

Plasma samples. The plasma samples were extracted, according to the literature, with ethyl acetate [2, 12, 15]. Liquid–liquid extractions at pH 6 resulted in considerably cleaner blank chromatograms in comparison with extractions at acidic pH values [12]. Because the reproducibility and the accuracy of the procedure was not significantly increased by the addition of an internal standard, the internal standard was omitted during this study. No additional clean-up method was necessary because the selectivity of the system was sufficient to separate the analyte from the endogenous interfering compounds (Fig. 1).

Ultrafiltration is basically an elegant sample pretreatment procedure, but because it is rather expensive and because no concentration is achieved during this clean-up

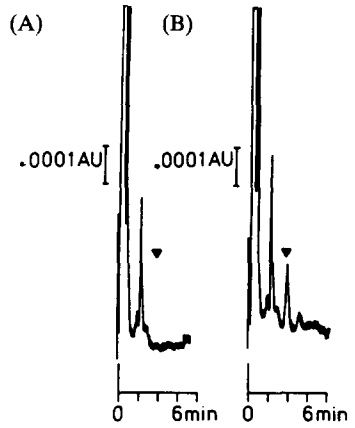

Figure 1
Chromatograms obtained after analysis of blank plasma sample (A) and after analysis of plasma sample spiked with 50 ng ml^{-1} of FUra (B).

procedure, this technique is mostly applied for calculating the degree of drug protein binding and not for sample clean-up in routine bioanalysis [10].

The mean recovery of the FUra in plasma samples was 50 ± 4% RSD, calculated after the analysis of four samples, in duplicate, containing 20, 200, 2000 and 20,000 ng ml^{-1}. The recovery was independent of the plasma concentration of the drug, within the examined concentration range. This relatively low recovery, in comparison with some data listed in the literature, is due to a different phase volume ratio. However, the concentrations in plasma, depending on the dose, are very high (200 ng to 20 μg ml^{-1}), so that a relatively low recovery does not limit the usefulness of the procedure.

The determination limit for 100 μl injections and a signal-to-noise ratio of 3, was 5 ng ml^{-1} for spiked plasma samples.

Urine samples. Because the urine samples were only used to check whether there was any leakage of FUra from the isolated liver, these samples were only diluted before analysis. When these diluted samples showed a small peak of FUra, then extraction and concentration of the samples was performed according to the described procedure for plasma samples. The selectivity and minimum detectable amounts for the analysis of FUra in urine samples are about the same as listed for the analysis in plasma samples, although relatively long system re-equilibration times are needed due to late eluting matrix peaks.

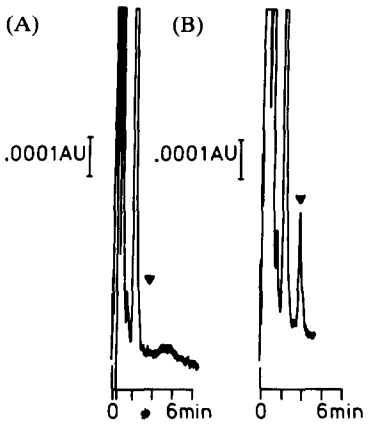

Figure 2
Chromatograms obtained after analysis of blank liver homogenate sample (A) and after analysis of liver homogenate sample spiked with 50 ng ml^{-1} of FUra using a 100-μl injection (B).

Bile and perfusion fluid samples. The bile and perfusion fluid samples contain a relatively high concentration (100 ng to 10 μg ml^{-1}) of the drug and a simple dilution step, 300 or 500 times according to the applied dose, was sufficient as a sample pretreatment procedure for an accurate quantitative determination. Analysis of the bile and the perfusion fluid was necessary to determine the distribution of the analyte over the body after an isolated liver perfusion.

Liver samples. For the analysis of liver homogenate samples, further pretreatment procedures are needed. First of all, the enzymatic activity must be stopped immediately, to avoid further metabolism of the drug. Therefore, the samples were pulverized in acetonitrile and frozen in liquid nitrogen immediately after sampling. This sample pretreatment procedure was reproducible and the recoveries were higher than 90%, when blank liver samples were spiked with 2.5 to 500 μg ml^{-1}. In order to increase the accuracy of the determination of FUra in liver homogenates the concentrations of the analyte were calculated with simultaneously analysed spiked liver homogenates samples. Enzyme activity can also be stopped by the addition of 50 mM borate buffer of pH 9.5. However, denaturation with acetonitrile has the advantage that it can also be used for other cytostatic drugs such as mitomycin C, which is sometimes given in combination with FUra. Liver samples containing FUra should be analysed as soon as possible, because of the limited stability of the drug in the extracts. For each experiment, a calibration curve was prepared using exactly the same procedure as the real samples and stored under the same conditions (Fig. 2).

The minimum detectable amount, signal-to-noise ratio 3, was 2.2 ng in liver homogenates, using UV absorbance detection at 268 nm.

Analytical variables

Linearity. Blank plasma samples ($n = 8$) were spiked with 200 ng to 20 μg ml^{-1}, blank liver ($n = 7$) samples were spiked with 2.5 to 500 μg ml^{-1}, and all samples were analysed in three separate runs. After the extraction of the drug from plasma samples the equation for the calibration curve was

$$y = 6.65\ (\pm 0.04)\ x - 2\ (\pm 4)\ (r = 0.9998),$$

and for the liver samples the equation for the calibration curve was

$$y = 0.24\ (\pm 0.03)\ x - 0.3\ (\pm 0.5)\ (r = 0.9996),$$

where y and x represents the peak height and the concentration (ng ml^{-1}) of FUra, respectively. The numbers in parentheses are the standard deviations, and r is the mean correlation coefficient. The calibration curves showed a linearity for at least 3 orders of magnitude. The differences in the slopes of the two calibration curves is mainly due to the fact that the liver samples were diluted before the analysis.

Precision. The mean slope of four calibration curves of extracted plasma samples was 6.78 with a standard deviation of 9.8%, and for extracted liver samples the mean slope was 0.256 with a standard deviation of 4.3%.

The within-day precision of the analytical method of the other samples and clean-up procedures were the same or even better than the extracted liver and plasma samples.

Chromatography

In the developed RP–IP LC system MOS-Hypersil [2] as well as PRP-1 [15] can be used as the sorbent. However, using silica based materials (e.g. MOS-Hypersil) the maximum applicable pH is about 8.0, but at this pH the k' value of FUra is very small, and an endogenous plasma component influences the quantitative results. Therefore, the PRP-1 material is preferred over silica based materials, because the pH of the mobile phase can be increased to pH 8.2 to achieve an acceptable k' value of the component and to eliminate the interfering component. The maximum pH of the mobile phase is 9.0 because of the limited stability of FUra. Furthermore, the equilibration time of the ion-pairing mobile phase on PRP-1, is three to five times that required for C_8 or C_{18} RP stationary phases.

The analytical columns could be cleaned with methanol or ethanol (50–100 ml), after which the column could be loaded again with the mobile phase (50 ml). The column could be used for over 1000 injections.

UV absorbance detection at 268 nm was chosen because it is the λ_{max} of FUra, and also resulted in the optimum signal-to-noise ratio.

Pharmacokinetic studies

In order to investigate the pharmacokinetics of FUra after isolated liver perfusion, an adult female pig of about 30 kg was chosen as a model to study this local administration route for human beings. Following the results obtained in this model, another study will be carried out in rats with an implanted tumour to evaluate the effectiveness of this administration route. In the last stage of this study, when FUra has been shown to inhibit the growth of tumour tissue, the experiments will be applied in human beings.

In Fig. 3 an example is given of an absorption curve of FUra in liver. The liver was perfused for 60 min, whereafter the liver was cleaned with a rinsing liquid for 15 min and subsequently the perfusion was stopped and the liver was reconnected with the blood circulation of the body. All the points of the concentration–time curve were analysed with the developed procedure for liver homogenate samples. The obtained plasma levels

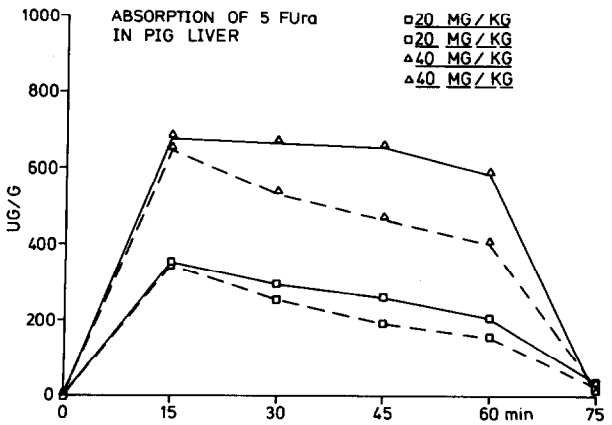

Figure 3
Liver homogenate concentration time curves obtained after the administration of 20 or 40 mg kg^{-1} of FUra to a pig.

with a dose of 20 mg kg^{-1} were, as expected, about half of the concentrations obtained by the administration of 40 mg kg^{-1} of the analyte.

The most important feature of this study is that the administration of FUra by means of an isolated liver perfusion resulted in much higher concentrations of the drug at the tumour site, and that the described bioanalytical procedure has adequate detectability, reproducibility, and precision for routine use.

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