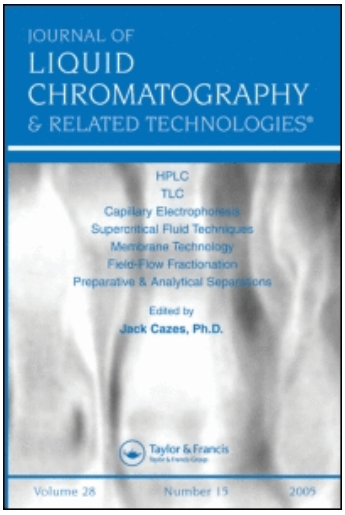

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Routine Analysis of Fluorouracil in Plasma by High-Performance Liquid Chromatography and Gas Chromatography. A Comparison and Implications for Pharmacokinetic Analysis

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Original Article

ROUTINE ANALYSIS OF FLUOROURACIL IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY. A COMPARISON AND IMPLICATIONS FOR PHARMACOKINETIC ANALYSIS

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ABSTRACT

A comparison between a high-performance liquid chromatographic (HPLC) and a gas chromatographic (GC) assay for routine determination of 5-fluorouracil (FUra) in plasma is described. Both assays are based upon a rapid and simple plasma pretreatment *without any derivatization step* and without application of gradient elution and temperature programming. More than 150 plasma samples obtained from breast cancer patients treated with FUra were compared. A good correlation as determined by perpendicular regression analysis was found with r = 0.9742. Consequences for pharmacokinetic analysis were also investigated. No significant differences between *mean* pharmacokinetic parameters based on the two assays were found but *intrapatient* variability for some pharmacokinetic parameters has been demonstrated.

INTRODUCTION

FUra[1] is still widely used as an antineoplastic agent, despite the development of a large number of new cytostatic drugs since FUra entered clinical chemotherapy. The drug has been studied extensively during the last decade with an emphasis on its pharmacokinetics and metabolism, intracellular concentrations and the intrinsic mode of action. To reveal more about the pharmacokinetics and metabolism of FUra, a reliable method of determination in body fluids and, if possible, in tissue is a requisite. From 1965 a number of papers dealing with the determination of FUra have been published (1-13). Most authors applied GC with derivatization, or HPLC. However, extensive pharmacological studies need time sparing and convenient assays, e.g. GC without derivatization (12) and HPLC (6, 13). To reveal advantages of HPLC and/or GC, a comparison between both techniques can be carried out, as has been described before (14). In this comparison a GC assay was used which included a time-consuming derivatization procedure (12). The present report describes a comparison between a GC and HPLC technique without using any derivatization at all (12, 14). Furthermore, the data obtained were applied for pharmacokinetic analysis of FUra *in vivo* behaviour.

[1] Abbreviations used are :
FUra : 5-fluorouracil; CUra : 5-chlorouracil; FUraH2 : 5,6 - dihydro-5-fluorouracil; GC : Gas Chromatography; HPLC : High Performance Liquid Chromatography; i.s. : internal standard; C.V. : Coefficient of Variation; AUC : Area Under the plasma Concentration time curve; $t_{1/2,z}$: elimination half-life; MRT : Mean Residence Time.

MATERIALS AND METHODS

Patients and sampling procedures

Blood samples were collected from 16 patients at specified times (0.02, 0.08, 0.25, 0.5, 1.0, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, 12.0 and 24.0 h) starting at the end of the injection. Before FUra administration was started, a blood sample was taken for use as a blank. All patients were treated with FUra for breast cancer (Dose : 500-600 mg/m2) in combination with cyclophosphamide and methotrexate. After the collection of the heparinized blood samples and centrifugation at 1000g for 10 min, the plasma was stored until analysis at -30 °C. All plasma samples were analyzed by both HPLC and GC. Use of glassware was avoided, since Fura is suspected of adsorption at glass surfaces (15, 16).

Chemicals

FUra and 5-fluorocytosine (i.s.), were kindly supplied by Hoffmann-La Roche, Mijdrecht, the Netherlands. CUra (i.s.) was purchased from Calbiochem, Los Angeles, Ca., USA). Solvents were of analytical grade (Merck, Darmstadt, FRG) and used without purification. Ethyl acetate was distilled twice before it was used in the GC assay.

Extraction

HPLC

To 1 ml of patient plasma 2.5 µg of 5-fluorocytosine (250 µg/ml) was added when FUra concentrations were in the range of 0 to

2 μg/ml, while 25 μg was added when FUra concentrations exceeded 2 μg/ml. Additionally, 50 μl 1.0 M sodium acetate buffer (pH4.8) together with 0.5 ml 1.4 M anhydrous sodium sulfate was added. The diluted plasma sample was extracted with 10 ml of n-propanol/diethylether (16/84, v/v). The solution was shaken vigorously for 90 s and centrifuged at 1000 g for 5 min. The organic layer (7 ml) was removed and transferred to another polythene tube. Then 1.0 ml of a 50 mM phosphate buffer (pH11) was added. After mixing and centrifugation as before, the organic layer was removed and discarded. The pH of the aqueous phase was adjusted to 7.0 by addition of 10 μl of a 1.0 M H_2SO_4 solution. Aliquots of 25 μl of the neutralized solution were injected onto the column of the liquid chromatographic system. Calibration curves were prepared by adding pure FUra in concentrations of 50, 100, 250, 500, 750, 1,000, 2,500, 5,000, 10,000, 25,000, 50,000 and 100,000 μg/l to pooled drugfree plasma. Concentrations of FUra were determined from a graph relating peak height ratios of FUra to 5-fluorocytosine and to the concentration of known standards.

GC

To 200 μl of plasma 10 μl of an internal standard solution (10 ng CUra/μl) was added. The CUra containing plasma (pH6-7) was extracted twice with 3 ml of ethyl acetate. The combined ethyl acetate layers were evaporated and the residue was redissolved in 200 μl ethyl acetate of which 20 μl was introduced on the stainless steel needle of the injection system. The lifetime of the column was elongated by the introduction of a purification step with hexane (12). Plasma samples analysed for the preparation of calibration curves were extracted by the same way.

Instrumentation

HPLC

The liquid chromatographic system consisted of a high pressure pump (Model 6000 A, Waters Associates, Milford, MA 01757, USA), an injector (Model U6K Universal injector, Waters), a 10 μm μBondapack/C18 column (300x 3.9 mm i.d.) and an ultraviolet absorbance detector operating at 254 nm (Model 440, Waters). Chromatograms were registered on a flat-bed recorder (BD8, Kipp & Zoon, Delft, the Netherlands). The mobile phase consisted of KH_2PO_4 buffer (50 mM, pH3). The flow rate was 1.3 ml/min at a pressure of 1000 psi.

GC

A gas chromatograph (Model 420, Packard Becker, Delft, The Netherlands) furnished with a nitrogen phosphorus selective detection system (Model 18-789 A, Hewlett Packard, Avondale, Pennsylvania, USA) was used for the GC procedure. Packed columns (0.45 m x 0.8 mm i.d.) were applied with gaschrom Q as support material, coated with 3% Versamid as absorbent. A solid phase injection system was used in order to prevent solvent interferences (modified pyrolysis system Becker Model 767) (17). Helium was used as carrier gas (8.5 ml/min) and as additional scavenger gas (up to 30 ml/min). The hydrogen flow-rate and air flow-rate amounted to 3.0 ml/min and 100 ml/min, respectively. The inlet and detector temperature were 300°C, the oven temperature 190°C.

Pharmacokinetic analysis and moment analysis

The $t_{1/2,z}$ of FUra was calculated by linear regression analysis of the terminal parts of the log plasma concentration versus

time curves (18), for all curves the concentration 30 min after drug administration was taken as the start of the terminal part. The Area Under the plasma Concentration - time Curve (AUC) was calculated by the trapezoidal rule with extrapolation to infinity. The mean residence time (MRT) was determined according to equation 1) (19). For calculation of the steady state volume of distribution (Vss) the MRT was used according to equation 2) (20), in which T represents the time of drug infusion which was close to zero.

$$MRT = \frac{AUMC_{0-\infty}}{AUC_{0-\infty}} \quad ; \quad AUMC = \int_0^{\infty} t.C_p.dt \qquad 1)$$

$$V_{ss} = \frac{dose \bullet AUMC}{(AUC)^2} - \frac{dose \bullet T}{2(AUC)} \qquad 2)$$

Statistical analysis

The relationship between HPLC and GC data were calculated using perpendicular least-squares regression since both variables are subject to error (21-23). A programme for a Hewlett Packard 85 according to Janssen & Helversteijn (24) was kindly donated by "Werkgroep redactie L.B.P.-Chemisch Weekblad/Magazine". The paired Student's t-test was used for the comparison of pharmacokinetic data, $p<0.05$ taken as the minimal level of significance.

RESULTS

HPLC

Figure 1 shows representative chromatograms for blank plasma and a plasma specimen which contained the compounds of interest.

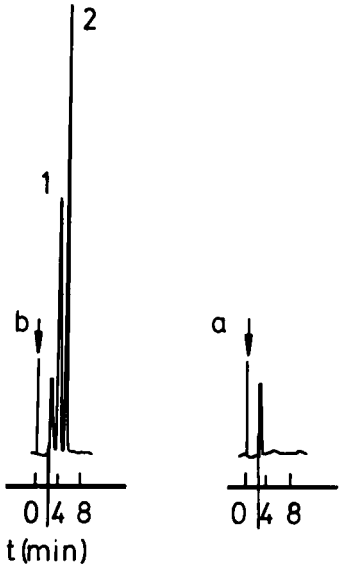

Fig. 1 A liquid chromatogram of blank plasma (a) and of plasma with FUra (b, peak 2) and the internal standard 5-Fluorocytosine (b, peak 1).

The retention times agreed well with the data of Christophidis et al., 3.8 and 5.0 min for 5-fluorocytosine and FUra, respectively.

Calibration curves, made up for each patient by plotting the ratio of added amount FUra and 5-fluorocytosine versus the added amount of FUra to plasma, in the ranges of 50 ng/ml to 1 µg/ml and of 1 µg/ml to 100 µg/ml showed good C.V. (C.V. = $S_{\frac{x}{x}}$. 100%) and a good linearity : r was 0.995 both for the low and high range curves with a C.V. of 8% at 50 ng/ml and 3% at 100 µg/ml. In our hands, the limit of determination appeared to be 25 ng/ml based on a signal to noise ratio of 3:1 and the recovery was 80 ± 6%.

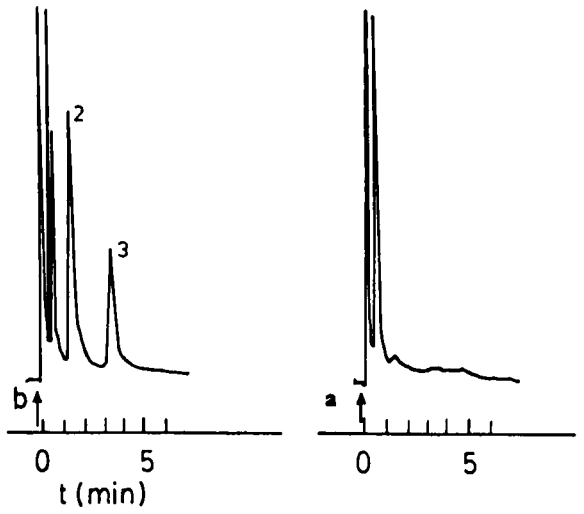

Fig. 2 A gas chromatogram of blank plasma (a) and of plasma with FUra (b, peak 2) and the internal standard Chlorouracil (b, peak 3).

GC

Figure 2 shows two chromatograms, representative for blank plasma and plasma enriched with FUra and CUra.

The retention times for FUra and CUra were 1.8 and 3.7 min respectively.

Again for each patient a calibration curve was made up. The calibration curves, ranging from 125 ng/ml to 5 µg/ml and 5 µg/ml to 100 µg/ml, achieved and calculated as described for the HPLC assay, showed comparable characteristics with respect to the linearity and C.V. : r was 0.997 and 0.993 for low and high range respectively, and a C.V. of 9% at the limit of determination and 3% at 100 µg/ml. The limit of determination (S/N = 3) was 125 ng/ml. The recovery for the extraction method was 65 $\pm$ 8%.

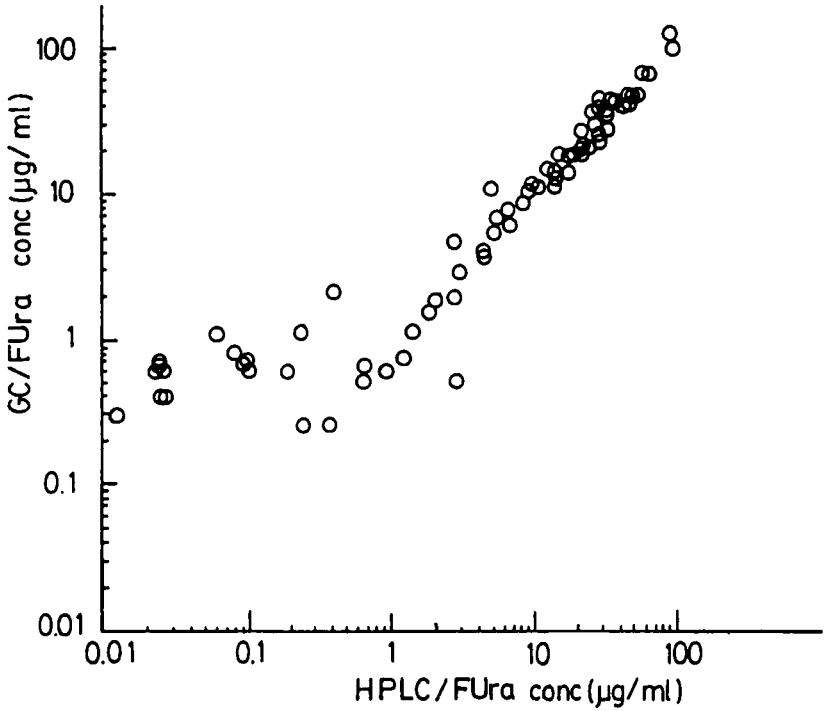

Fig. 3 Comparison between HPLC and GC determination of FUra in plasma samples. The relationship is described as $Y_i = 1005 \cdot X_i - 0.5146$; SD (a) = 0.0269 and SD (b) = 0.7961, n = 77.

The comparison

The correlation between plasma concentrations determined by HPLC was close with those determined by GC with r = 0.9742. The relationship is described as Yi = 1005Xi - 0.5146 with SD (a) = 0.0269 and SD (b) = 0.7961 and n = 77. The concentration of FUra in 12 plasma samples was 0.1 µg/ml as determined by HPLC while the concentration of FUra in the samples as determined by the GC assay varied between 0.1 and 1 µg/ml (Fig. 3). Pharmacokinetic data of FUra for 12 patients are presented in Table 1.

TABLE I

Pharmacokinetic data of FUra based on GC and HPLC data of 12 Breast Cancer Patients treated with the antimetabolite (D : 500-600 mg/m^2)

Patient number	GC				HPLC			
	$t_{½,z}$ [1] (min)	CL (l/h)	MRT (min)	V_{ss} (l)	$t_{½,z}$ (min)	CL (l/h)	MRT (min)	V_{ss} (l)
1	18	38.3	26	8	12	45.0	17	6
2	14	88.4	20	15	9	111.5	13	12
3	12	84.3	16	12	14	76.0	20	13
4	13	40.8	19	6	9	51.2	12	5
5	18	74.6	28	19	10	34.0	15	5
6	14	45.8	19	10	12	43.9	17	8
7	17	64.9	27	17	17	49.6	27	13
8	18	33.6	27	9	18	29.8	28	8
9	14	58.6	19	11	9	63.2	12	7
10	14	57.3	19	12	12	54.7	19	7
11	10	77.0	15	12	16	72.2	15	11
12	8	100.0	10	10	8	87.0	11	10
$\bar{x}$	14.2	63.6	20.4	11.8	12.2	59.8	17.2	8.8
SD	3.2	21.5	5.6	3.7	3.4	23.5	5.6	3.0

[1] $t_{½,z}$ = elimination half-life; CL: clearance; MRT: mean residence time; V_{ss} : steady-state volume of distribution.

For some pharmacokinetic parameters considerable differences between data of one patient based on HPLC and GC can be demonstrated , e.g. $t_{1/2,\alpha}$ (HPLC) and $t_{1/2,\alpha}$ (GC) of patient 5. However, no significant differences were found between mean pharmacokinetic parameters based on HPLC data and those based on GC data.

DISCUSSION

Treatment with FUra continues to be amongst the main topics in cancer pharmacotherapy. Therefore reports still appear dealing with monitoring of the antimetabolite (25-29).

The study reported here compares the capacity of GC and HPLC in routine, selective and sensitive analysis of FUra in plasma without using derivatization. Data of GC and HPLC have been compared by perpendicular regression analysis of the raw data together with pharmacokinetic evaluation. Limits of determination reported for the GC and HPLC assay revealed that the HPLC assay is five fold more sensitive (12, 14, 16).

Our study pointed out that interfering peaks originating from plasma matrix compounds can hinder both the simple GC and HPLC assays described here. For instance, the HPLC showed some hindrance of patient plasma compounds with respect to 5-fluorocytosine which was reported earlier by Christophidis et al. (14). Furthermore, when FUra was assayed by HPLC in plasma derived from rats and other mammals, endogenous compounds, e.g. uracil (29), present in plasma can interfere with FUra.

The GC assay however was hindered by some interferences at FUra concentrations below 100 ng/ml occuring in patient plasma. A

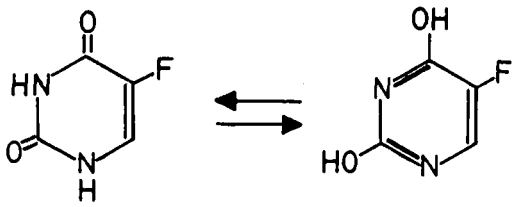

Fig. 4 FUra tautomerism.

metabolite of FUra, i.e. FUraH2, was suspected to be responsible for the interference. Addition of FUraH2 to plasma samples excluded this possibility; co-elution of both fluoropyrimidines using Versamid packed columns did not occur.

Duration of analysis of the HPLC assay was somewhat longer due to the two step extraction procedure and to a slight difference in retention times in favour of the GC assay.

An interesting point is the behaviour of FUra at elevated temperatures with respect to its tautomerism as depicted in figure 4.

It can not be excluded that the equilibrium is changed at higher temperatures during elution on the GC column, which might cause the slight tailing observed with FUra and CUra.

The difference between the two assays seems only marginal taking the whole concentration range (0.1 - 100 ug/ml) into account. However, differences between individual pharmacokinetic parameters can be large as depicted in Table I : $t_{1/2,\alpha}$ of patient 5 was 1.8 longer when data of GC analysis were used in comparison to $t_{1/2,\alpha}$ as determined by HPLC data. This is not reflected in mean pharmacokinetic data; no significant differences between

mean pharmacokinetic parameters as determined by HPLC and those by GC could be demonstrated (Table I). When individual pharmacokinetic parameters however have to be correlated with pharmacodynamic ones (30) large differences between correlation patterns can be observed (31). This is of importance since assays as described here are used to generate pharmacokinetic data which might be used as predictive tool in clinical settings. Alternative assays for those described here have been published recently (31-38) : a GC assay for simultaneous determination of FUra and its quantitatively most important metabolite FUraH2 using capillary columns (27, 28); a GC/MS assay allowing determination of FUra concentrations in plasma down to 0.39 ng/ml (38) and HPLC assays for simultanous determination of FUra and metabolites of the anabolic pathway (25, 26, 31-33, 35). However, these assays generally require expensive equipment and experienced personnel.

A comparison generally results in a preference for one of the methods tested. Here the preference is determined by the biological matrix in which FUra has to be determined. In our opinion the HPLC method is the method of choice in case of clinical studies. When FUra has to be determined in plasma originating from laboratory animals, the GC method proved to be superior with respect to selectivity (37).

In conclusion it has been shown in this study that a good correlation exists between the GC data and HPLC data concerning routine determination of FUra in plasma of cancer patients. No significant differences were found between derived *mean* pharmacokinetic parameters but it appeared that this is not a guarantee for absence of large *intra*patient variation.

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