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A GAS CHROMATOGRAPHIC ASSAY FOR THE DETERMINATION OF 5,6-DIHYDROFLUOROURACIL AND 5-FLUOROURACIL IN HUMAN PLASMA

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SUMMARY

A gas chromatographic assay for the determination of 5-fluorouracil (5-FU) and 5,6-dihydrofluorouracil (FDHU) is described. The selectivity and sensitivity of the method allows the determination of both 5-FU and FDHU in 200 μl of plasma. Diphenylsuccinimide and chlorouracil were used as external and internal standard, respectively. The assay including the extraction shows a good linearity in the range 0—5000 ng/ml plasma for 5-FU as well as for FDHU. 5-FU and FDHU plasma concentrations of a number of patients with breast cancer treated with 5-FU were determined in order to demonstrate the usefulness of the method.

INTRODUCTION

Although 5-fluorouracil (5-FU) has been used in clinical oncology for more than two decades, the literature [1, 2] offers only a few data about one of its quantitatively most important metabolic products, 5,6-dihydrofluorouracil

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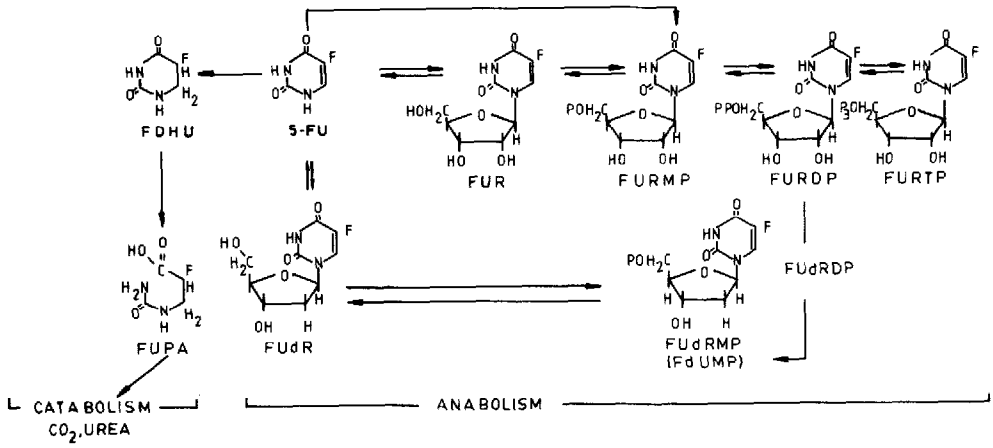

Fig. 1. Metabolic pathways of the 5-FU metabolism.

(FDHU). Most effort has been put into the determination of products of the anabolic pathway of 5-FU metabolism [3—6], since these products show cytostatic activity.

Determination of FDHU might be of great importance in 5-FU studies. From Fig. 1 it can be deduced that saturation in the catabolic pathway may lead to non-linearity in 5-FU pharmacokinetics, as suggested in the literature [1, 2].

An overflow towards the anabolic pathway might result in relatively higher antineoplastic activity. Besides this, a practical point demands attention. Plasma levels of FDHU can be monitored more easily than intracellular concentrations of metabolites from the anabolic pathway like 5-fluorouridine (FUR), 5-fluoridine-5′-monophosphate (FURMP), 5-fluoridine-5′-diphosphate (FURDP), 5-fluorodeoxyuridine-5′-diphosphate (FUdRDP), 5-fluorodeoxyuridine-5′-monophosphate (FdUMP) and 5-fluorodeoxyuridine (FUDR) (Fig. 1).

Since pharmacokinetic studies require the determination of the time course of several disposition phenomena like drug absorption, distribution, elimination and metabolism, the frequent measuring of drug and metabolite concentrations in plasma is the most obvious way to obtain an insight into these phenomena.

The low concentrations to be measured in pharmacological studies require selective and sensitive analytical techniques. At present high-performance liquid chromatography (HPLC) and gas chromatography (GC) are the methods of choice for routine measurements. FDHU shows neither fluorometric nor electrochemical activity and owing to its low molar absorptivity, GC with nitrogen-selective detection is preferred to HPLC. In spite of the selective detection an extraction procedure is required.

EXPERIMENTAL

Apparatus

A gas chromatograph (Model 420, Packard Becker, Delft, The Netherlands) equipped with a nitrogen—phosphorus detection system (Model 18-789 A.

Hewlett-Packard, Avondale, PA, U.S.A.) and a flat bed recorder (BD 7, Kipp & Zn., Delft, The Netherlands) were used for the experiments. SCOT OV-275 capillary columns with a length of 7 m were prepared according to ref. 7. A ball-valve solid-sample injector as described in the literature [8] was used. The inlet and detector temperatures were set at 245°C and 300°C, respectively, while FDHU was chromatographed at an oven temperature of 195°C and 5-FU at 215°C. Helium was used for both carrier gas (12 ml/min) and make-up gas (30 ml/min).

Reagents

All reagents were of analytical grade (J.T. Baker Chemicals, Deventer, The Netherlands). Ethyl acetate was distilled twice; chloroform was washed twice with distilled water; methanol was used without any purification. 5-FU and FDHU were kindly supplied by Hoffmann-La Roche (Mijdrecht, The Netherlands). Diphenylsuccinimide (DPS) was synthesized by Chemische Industrie Katwijk (Katwijk, The Netherlands) and purified by recrystallization from tetrahydrofuran (THF). Chlorouracil (CU) was purchased from Calbiochem (Los Angeles, CA, U.S.A.).

The stock solutions of 5-FU (100 mg/l methanol), FDHU (10 mg/l ethyl acetate), DPS (250 mg/l ethyl acetate) and CU (100 mg/l methanol) were stored at 4°C.

Extraction

Polythene tubes were chosen to avoid loss of compound (5-FU and FDHU) by adsorption onto the surface [9, 10]. In order to remove interfering compounds, 0.2 ml of plasma with 100 ng of CU as internal standard was extracted twice with 3 ml of chloroform. Subsequently the plasma fraction was extracted twice with 3 ml of ethylacetate under vigorous shaking on a whirlmixer for 20 sec. In order to obtain a good phase separation, the mixture was centrifuged at 1000 *g* for 5 min. The ethyl acetate fractions were collected in a tube containing the external standard (DPS, 250 ng), to correct for injection volume variations, and dried under a gentle nitrogen stream at ambient temperature. The residue was dissolved in 100 μl of ethyl acetate and aliquots of 10 μl were brought onto the needle of the solid-sample injection system.

Patients and drug administration

Five hospitalized female patients, aged 57—72 years, treated for breast cancer with 5-FU (500 mg/m^2 intravenously), were sampled via an indwelling intravenous catheter in the arm opposite to that of 5-FU application. Blood samples, collected in heparinized polythene tubes, were taken at 0, 0.04, 0.08, 0.25, 0.5, 1.0, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, 12.0 and 24 h after drug administration. The samples were centrifuged at 1000 *g* within 1 h after sampling; the plasma was decanted and stored at −15°C until analysis.

RESULTS AND DISCUSSION

Chromatography of FDHU

The GC conditions were optimized with respect to temperature and

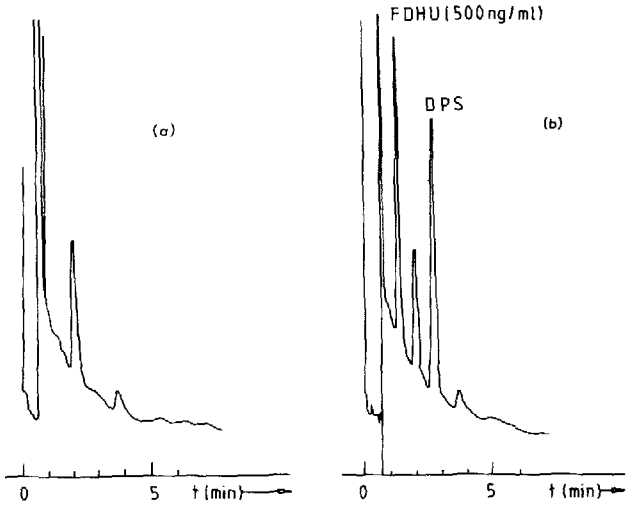

Fig. 2. (a) Chromatogram of an extract of blank plasma. (b) Chromatogram of an extract of plasma spiked with FDHU and DPS.

flow-rate. At 195°C an adequate separation of FDHU and DPS (external standard) was combined with a reasonable time of analysis. Under these conditions the peak shape is good (asymmetry factor, measured at 0.1 of peak height, smaller than 1.2), allowing a quantification based on peak height.

The detection limit based on a signal-to-noise ratio of 3:1 was determined to be 500 pg for FDHU. Fig. 2a and b shows the chromatograms of an extract of blank plasma and plasma spiked with FDHU, respectively. From the figures it can be seen that concentrations down to 100 ng/ml plasma can be determined easily.

Chromatography of 5-FU

If only 5-FU has to be determined, the column temperature can be increased to 215°C. At this temperature the retention times of 5-FU and CU amount to about 3 and 6 min, respectively, allowing a high throughput of samples. In spite of an adequate separation of FDHU, DPS, 5-FU and CU under these conditions, it is not possible to determine all components in one single run because of interfering peaks of the plasma matrix. Although 5-FU and FDHU can be determined in one single run by applying a temperature program, we prefer two isocratic runs for routine measurements.

Linearity and precision of the method

Quantification of FDHU. The linear dynamic range of the method was investigated by injecting different amounts of FDHU, directly as well as after extraction from spiked plasma. From Fig. 3, showing the relation between relative peak area of FDHU/DPS and FDHU concentrations, the recovery of the extraction can be calculated as the ratio of the slopes of the two calibration curves after direct injection and after extraction, respectively; this was about 60%. The coefficient of variation for the assay ranges from 6.6% (n = 3) at a level of 100 ng/ml to 1.6% at 5 μg/ml (n = 3).

Quantification of 5-FU. The calibration curve for 5-FU (Fig. 4) was con-

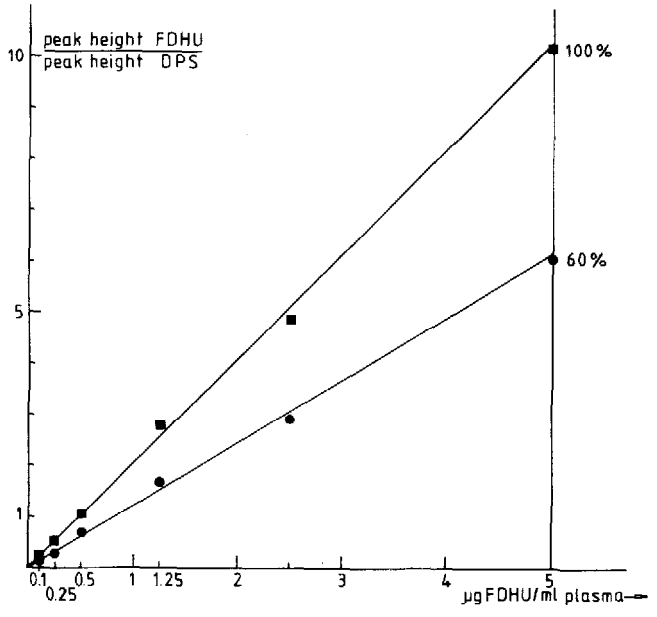

Fig. 3. Calibration curve for FDHU obtained after extraction from plasma (lower curve). The upper curve corresponds to 100% (direct injection).

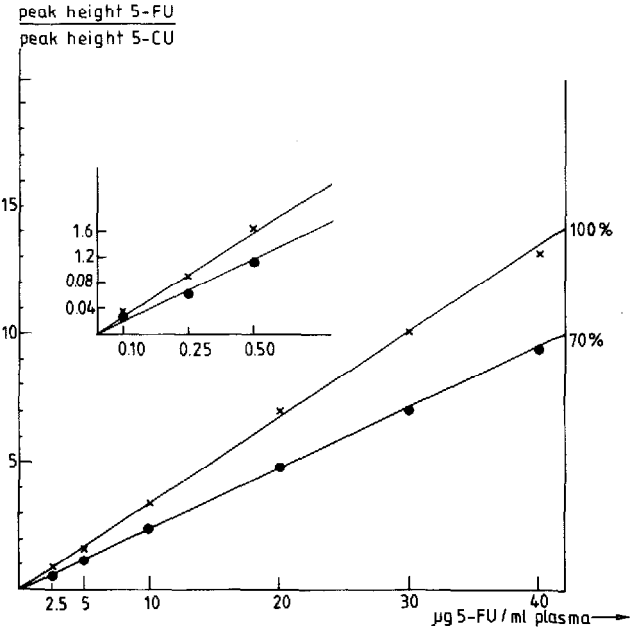

Fig. 4. Calibration curve for 5-FU obtained after extraction from plasma (lower curve). The upper curve corresponds to 100% (direct injection).

structed as described for FDHU. The recovery of 5-FU extracted from plasma amounted to about 70%, the absolute limit of detection being about 1 ng, resulting in measurable concentrations down to 150 ng 5-FU per ml plasma. The coefficient of variation of the assay ranges from 11% at a level of 100 ng/ml to 1.5% at 40 µg/ml.

In the case of the intravenous bolus injection of 5-FU it can be generally stated that samples taken just after administration have to be diluted to determine the 5-FU. These diluted samples cannot be used for determining FDHU. Plasma samples taken 15 min after 5-FU infusion can be used for the determination of both FDHU and 5-FU in one extract.

The described assay for 5-FU in plasma samples was compared with a method using a packed column, described in the literature [11]. There appeared to be a good correlation at levels higher than 2 μg/ml (correlation coefficient 0.988, slope 0.927, intercept −0.550, n = 46). However, at levels below 2 μg/ml the correlation was poor (correlation coefficient 0.768, slope 0.861, intercept −0.527, n = 12), which could be explained by an irreversible adsorption of 5-FU to the support material of the packed column. For this reason we prefer capillary columns. Owing to the wide range of physicochemical properties, relatively short capillaries can be used. Comparison of the developed method with an HPLC assay [12] shows a good correlation (correlation coefficient 0.994, slope 1.082, intercept −0.9962, n = 25).

Examples of FDHU plasma concentration—time curves of four patients treated with 5-FU are given in Fig. 5. Remarkably, a fifth patient had no detectable FDHU in the plasma at all. As can be seen, FDHU plasma profiles cannot be readily fitted by using common pharmacokinetic models.

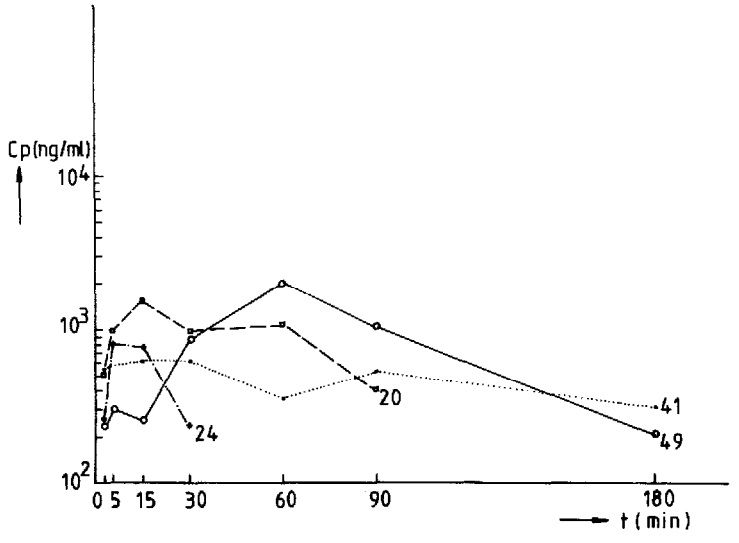

Fig. 5. Pharmacokinetic profiles of four patients treated with 5-FU (dose: 500 mg/m² intravenously).

CONCLUSION

The GC assay developed for the determination of 5-FU and FDHU in plasma can be used routinely for monitoring both compounds in plasma samples from patients treated with 5-FU.

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