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HIGHLY SENSITIVE DETERMINATION OF 5-FLUOROURACIL IN HUMAN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY AND NEGATIVE ION CHEMICAL IONIZATION MASS SPECTROMETRY

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SUMMARY

A sensitive method using gas chromatography and electron-capture negative ion mass spectrometry for the determination of 5-fluorouracil in plasma is described. 5-Chlorouracil was used as internal standard. Sample clean-up consisted of extraction of the 5-halogenated uracil derivatives with 2-propanol—diethyl ether (22:78, v/v) at pH 6, followed by a back-extraction into aqueous buffer at pH 10.5. Pentafluorobenzyl derivatives of 5-fluoro- and 5-chlorouracil were prepared by extractive alkylation with pentafluorobenzyl bromide with dichloromethane as solvent and tetrabutylammonium as counter-ion. The limit of sensitivity employing this technique was 50 fg on-column. Quantitation in human plasma was possible down to $3 \cdot 10^{-9}$ *M* (0.39 ng/ml). Mass fragmentographic analysis of 5-fluorouracil in plasma of patients after a bolus injection of 500 mg/m^2 is presented. Plasma concentrations could be determined for at least 8 h after administration.

INTRODUCTION

5-Fluorouracil (5-FU) is widely used for the treatment of patients with cancer [1, 2]. Extensive pharmacokinetic studies have been hampered by a short initial half-life (10—15 min) of 5-FU in humans. Within 3 h after a bolus

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injection of 5-FU, the plasma concentration falls below the reported detection limit of most methods. 5-FU in plasma has been determined by various techniques, including gas chromatography (GC), high-performance liquid chromatography (HPLC) and electron impact (EI) and chemical ionization (CI) mass spectrometry combined with gas chromatography (GC—MS). Typical limits of detection were reported for HPLC and GC, $5 \cdot 10^{-6}$ *M* down to $5 \cdot 10^{-8}$ *M* [3—7], and for GC—MS, $7.5 \cdot 10^{-8}$ *M* and $1.5 \cdot 10^{-8}$ *M* [8, 9]. A GC—MS method was reported with a detection limit of $8 \cdot 10^{-9}$ mol [10]; however, for this method unstable silyl derivatives were used [7]. These techniques are often of limited value for detailed pharmacokinetic studies in humans, because of either poor specificity or inadequate sensitivity.

Electron-capture negative ion (ECNI)-MS is known to be one to three orders of magnitude more sensitive than EI-MS for suitable compounds. ECNI-MS requires compounds with a high electron affinity and a large cross-section [11] for sensitive analysis. Although 5-FU contains a halogen atom, the native electron affinity is low. The dipentafluorobenzyl (di-PFB) derivative of 5-FU fulfils this demand to a great extent. PFB bromide has been reported to be a suitable reagent for making PFB derivatives in an extractive alkylation procedure [12]. These derivatives show extremely good electron-capture properties [11].

This paper describes a method for the assay of 5-FU in plasma by extractive pentafluorobenzylation and GC coupled with ECNI-MS, as demonstrated by plasma determinations of 5-FU during 8 h after a bolus injection of 500 mg/m^2 in cancer patients.

EXPERIMENTAL

Instrumentation

All GC—MS analysis were carried out on a Kratos MS-80 GC—MS system, equipped with an EI/CI source with negative ion detection capability. Negative ion chemical ionization was performed with ammonia as reagent gas. The source pressure was adjusted and optimized to obtain maximum sensitivity. Ionization was initiated with 40-V electrons with an emission current of 1.5 mA. The source temperature was 250°C.

The GC column was interfaced directly to the ion source by connection to a deactivated fused-silica capillary (40 cm × 0.1 mm I.D.). The connection was made with a single ferrule zero-dead-volume union (Chrompack, The Netherlands). The interfacial region between the chromatograph and the spectrometer was maintained at 250°C. Chromatographic separation was performed on a fused-silica CPSIL19 chemically bonded capillary column (25 m × 0.22 I.D., film thickness 0.12 μm) (Chrompack, The Netherlands). The GC oven temperature was 290°C. Samples were introduced using a solid injector [13]. Before a series of analyses was started, this injector was extensively cleaned by flushing with hydrogen fluoride and was successively washed with water and acetone. The injector was deactivated by flushing with 10% dimethylchlorosilane in dichloroethane and washed with acetone.

Reagents and chemicals

5-FU, 5-chlorouracil (5-ClU) and 5-bromouracil (5-BrU) were purchased

from Sigma (St. Louis, MO, U.S.A.) and pentafluorobenzyl bromide from Pierce (Rockford, IL, U.S.A.). Tetrabutylammonium hydrogen sulphate (TBA) was from Fluka (Buchs, Switzerland). All organic solvents and other chemicals were of analytical grade.

Laboratory equipment

All analyses were carried out using disposable polypropylene tubes and pipet-tips, to prevent adsorption and cross-contamination of the 5-halogenated uracil derivatives during the sample processing.

Sample clean-up

Plasma levels with an expected 5-FU concentration higher than 10^{-6} *M* were diluted with pooled plasma, free of 5-FU.

To 0.9 ml of plasma, 30 μl of a stock solution of a suitable 5-ClU concentration were added, to obtain a final 5-ClU concentration of 10^{-7} *M*. Then 0.1 ml of a 2 *M* Tris—HCl buffer (pH 6.0) was added and the plasma was extracted twice with 4 ml of 2-propanol—diethyl ether (22:78, v/v) with vigorous shaking for 5 min, using a whirlmixer. After centrifugation at 2000 *g* for 10 min, the extracts were combined and 5-FU and 5-ClU were back-extracted into 0.5 ml of 0.2 *M* phosphate buffer (pH 10.5) by vigorous shaking for 5 min. The organic layer was discarded and the aqueous layer was used for extractive pentafluorobenzylation.

Preparation of PFB derivatives

An aqueous solution (0.5 ml) containing 0.5 *M* TBA and 0.2 *M* phosphate buffer, adjusted to pH 10.5 with 3 *M* sodium hydroxide, was added to 0.5 ml of aqueous solution containing the 5-halogenated uracils. Dichloromethane (5 ml) and PFB bromide (10 μl) were added, and the tube was stoppered and vigorously shaken at room temperature for 60 min. After centrifugation at 2000 *g* for 10 min the aqueous layer was discarded and the organic layer was washed with 1 ml of 0.1 *M* hydrochloric acid and 1 ml of 0.1 *M* phosphate buffer (pH 8). Then 4 ml of the dichloromethane layer were transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 50°C. The residue was dissolved in 0.5 ml of hexane—chloroform (3:1, v/v).

Determination of the recovery of 5-FU and 5-ClU during sample clean-up

5-FU and 5-ClU were added to plasma so that their concentrations were 10^{-7} *M* for 5-ClU and 10^{-9}—10^{-6} *M* for 5-FU. These samples were extracted and back-extracted as described. Standard solutions were also prepared with concentrations of 5-FU and 5-ClU in the same way as the spiked plasma samples. To the plasma extracts and standards, 30 μl of a suitable stock solution of 5-BrU, which was used as internal standard for recovery calculations, were added, to obtain a final 5-BrU concentration of $5 \cdot 10^{-7}$ *M*. The extracts and standards were pentafluorobenzylated using the extractive alkylation procedure, and analysed by GC- ECNI-MS.

The recoveries were calculated from the relative peak area ratios as obtained from the plasma samples, and the ratios obtained from the standards.

Time dependence of the extractive pentafluorobenzylation of 5-FU, 5-ClU and 5-BrU

Eight aqueous solutions of 0.5 ml each, containing 10^{-5} *M* of 5-FU, 5-ClU and 5-BrU, were derivatized to PFB derivatives as described, with the following exceptions: the dichloromethane contained $5 \cdot 10^{-7}$ *M* *n*-hexacosane as internal standard and the reaction was terminated after 5, 10, 20, 30, 40, 60, 90 and 120 min. These samples were analysed with GC–MS operated in the EI mode, and the total ion current was recorded. The yield of the reaction was calculated from the peak area ratio of the di-PFB derivative formed and the internal standard.

Patients

Patients with advanced colorectal carcinoma received 5-FU (500 mg/m^2) intravenously in the arm. Blood samples were taken at various times up to 8 h after injection, from the patient's other arm. Blood was collected in heparinized tubes. The samples were spun down within 1 h and stored at −20°C until analysis was performed.

RESULTS AND DISCUSSION

Extractive derivatization

The time dependence of the yield of extractive pentafluorobenzylation of 5-halogenated uracils with PFB bromide at room temperature is shown in Fig. 1. A constant amount of the PFB derivatives was reached after 1 h. The di-PFB derivatives of 5-FU, 5-ClU and 5-BrU were found to be stable in solution for at least a month when stored at room temperature.

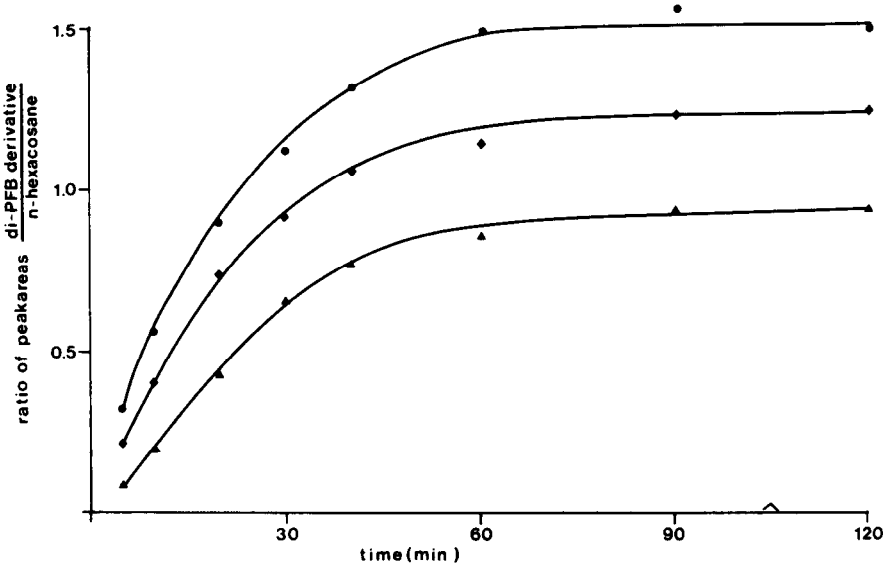

Fig. 1. Yield of the reaction between 5-FU (●), 5-ClU (♦) and 5-BrU (▲) with pentafluorobenzyl bromide under extractive alkylation conditions. Yield is expressed as the ratio of the peak areas of the di-PFB derivative (10^{-5} *M*) formed and *n*-hexacosane ($5 \cdot 10^{-7}$ *M*).

Gas chromatography and mass spectrometry

The three di-PFB derivatives exhibited good GC properties. The separation of the compounds on a fused-silica CPSIL19 capillary column is shown in Fig. 2.

The MS properties have been examined under EI and positive and negative ion chemical ionization (PCI and NCI) conditions. The identity of the derivatives was confirmed with EI. The EI spectra demonstrated molecular and fragment ions consistent with the addition of two PFB groups. Both EI and PCI spectra showed quite extensive fragmentation, dominated by the ion at *m/z* 181 ($CH_2C_6F_5$). The NCI spectra, however, showed only one major fragment $[M—C_7H_2F_5]^-$ and an ion of very low abundance at *m/z* −291 for all three analogues. The NCI mass spectrum of 5-FU di-PFB is given in Fig. 3. The ionization efficiency of NCI was found to be 15 to 20 times higher than using EI. Both the higher ionization efficiency of NCI and the high relative intensity (80% of total ion current) of the diagnostic ion $[M—C_7H_2F_5]^-$ resulted in an approximately 300 times higher sensitivity compared with EI or CI.

Quantitative determinations were performed by monitoring the ions at *m/z* −309, *m/z* −325 and *m/z* −369 for the di-PFB derivatives of 5-FU, 5-ClU and 5-BrU, respectively. The linearity of the detection by the GC–MS system

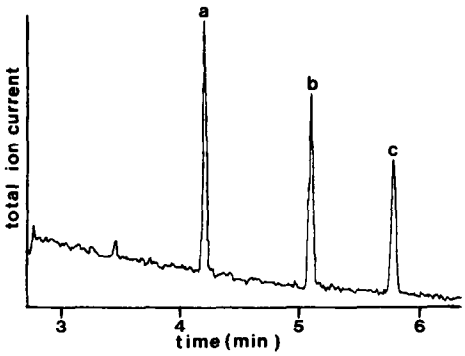

Fig. 2. Total negative ion current chromatogram of the PFB derivatives of 5-FU (a), 5-ClU (b) and 5-BrU (c). Separation was achieved on a CPSIL19 fused-silica capillary column, 290° C isothermal.

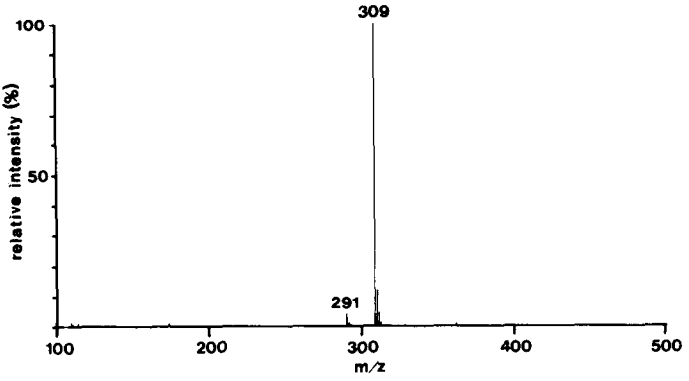

Fig. 3. Negative ion mass spectrum of the di-PFB derivative of 5-FU.

was tested by injections of amounts of 5-FU di-PFB in the range from 0.5 pg to 5 ng, and a constant amount of 50 pg of 5-ClU di-PFB as internal standard. A linear response curve was obtained with a correlation coefficient of 0.9991. The detection limit of 5-FU was 50 fg (0.38 fmol) on column, with a signal-to-noise ratio of 5.

For good analytical practice, it was found to be important to analyse the samples in order of increasing concentration. We found that traces of the compounds could adhere persistently to the injector. Therefore each series of analysis should be preceded by extensive cleaning of the injector (see Experimental) to diminish sample carry-over.

Plasma determinations

Extractive alkylation could not be performed directly from plasma, owing to protein precipitations. Therefore, the plasma was first extracted. The recovery was 70% for 5-FU and 59% for 5-ClU, as measured in the aqueous layer after back-extraction.

Calibration curves were obtained, using spiked plasma samples which were carried through the whole procedure. Linear curves were obtained in the concentration range from $3 \cdot 10^{-9}$ *M* to 10^{-6} *M* for 5-FU (0.39—130 ng/ml) in plasma with correlation coefficients greater than 0.998. For 5-FU concentrations of 10^{-9} *M*, $3 \cdot 10^{-9}$ *M* and 10^{-8} *M*, standard deviations were obtained of 22%, 5.8% and 5.4%, respectively ($n = 7$), indicating a limit of quantitation in plasma of $3 \cdot 10^{-9}$ *M*.

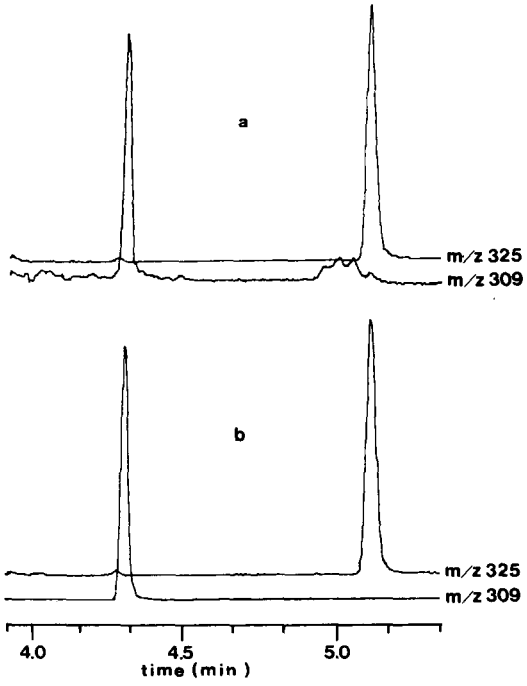

Fig. 4. Determination of 5-FU in plasma of two patients. The recordings of 5-FU at *m/z* —309 and 5-ClU at *m/z* —325 were each normalized to full-scale deflection. The calculated concentrations of 5-FU were $5.5 \cdot 10^{-9}$ *M* (a) and $4.0 \cdot 10^{-7}$ *M* (b). The concentration of 5-ClU added to the sample was 10^{-7} *M* in both cases.

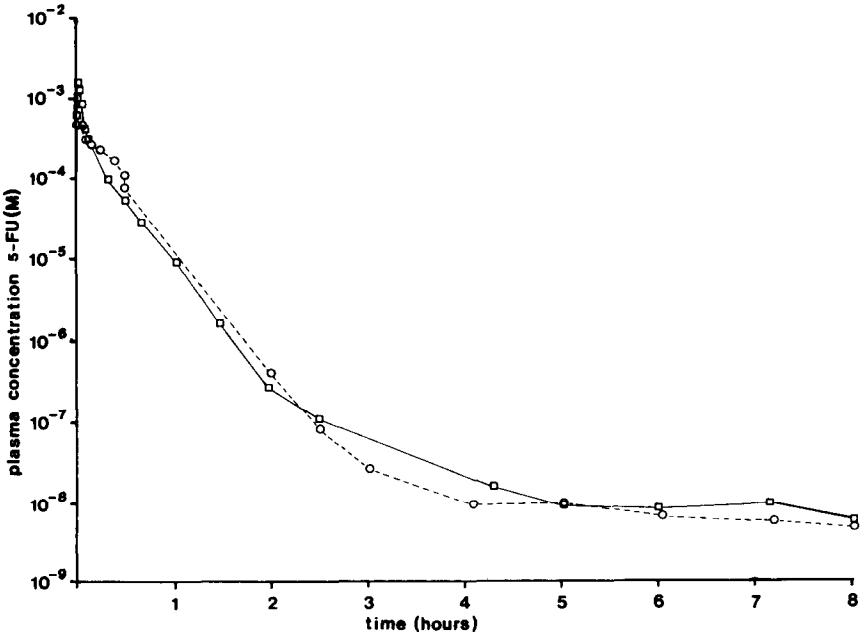

Fig. 5. Plasma concentration versus time curve of 5-FU in two patients who had received a bolus injection of 500 mg/m² intravenously.

With this method, no impurities were encountered in the elution region of the compounds of interest or at longer retention times. This allowed the continuous analysis of samples at 7-min intervals. Two selected ion monitoring recordings of plasma samples are presented in Fig. 4.

Application to patients

Fig. 5 represents the plasma concentrations in two patients. At 8 h the concentration was still higher than the limit of quantitation. The highest concentrations (above 10^{-5} *M*) have been determined by HPLC (slightly modified from ref. 14). The sharp initial fall of plasma concentrations is followed by a more horizontal phase in the plasma versus time curve. The terminal half-life may contain the information on the elimination of nucleotides derived from 5-FU [15].

CONCLUSION

The detection limit of the method described allows measurement of plasma concentrations in patients for over five decades. The limit of quantitation in plasma is not determined by the sensitivity of the mass spectrometer but rather by traces of 5-FU and 5-ClU from the chromatographic system. Further cleaning of the system may lower the limit by one order of magnitude. More complete knowledge of the pharmacokinetics of 5-FU than that obtained during the first 3 h after a bolus injection may be helpful in finding correlations with clinical observations. The pharmacokinetics of 5-FU were reported to be non-linear [16]. Therefore a different uptake or metabolism pattern may be

expected to occur at different plasma concentrations. If knowledge of the metabolism is combined with more complete pharmacokinetic data than those obtained during the first 3 h after a bolus injection, then it may prove possible to devise modifications to the therapy.

An extended examination of 5-FU concentrations in plasma of patients and its evaluation with regard to observed toxicity and antitumor effect is in progress.

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