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Note

Sensitive gas—liquid chromatographic assay of underivatized 5-fluorouracil in plasma

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Several gas-chromatographic methods have been reported for the analysis of 5-fluorouracil (5-FU) in biological fluids [1—4]. These methods have in common that 5-FU is determined as a methyl or silyl [1, 2, 4] derivative. In our opinion gas—liquid chromatographic (GLC) analysis of underivatized 5-FU is possible, which might lead to a more direct analysis of the drug. In accordance with this view, already put into practice in the analysis of underivatized barbiturates [5, 6], we have developed a suitable GLC method for underivatized 5-FU.

MATERIALS AND METHODS

Apparatus

A Becker Model 420 gas chromatograph equipped with a Hewlett-Packard dual nitrogen—phosphorus flame ionization detector Model 18789 A was used. The radiochemical measurements were performed on a Packard Model 2450 Tri-carb® liquid scintillation spectrometer.

Solvents, standards and reagents

The solvents used were of analytical grade and obtained from Baker (Deventer, The Netherlands). Dimethyldichlorosilane was from Merck (Darmstadt, G.F.R.). Gas-Chrom Q, Versamid 900 and Carbowax 20M were obtained from Chrompack (Middelburg, The Netherlands). RBS-25 was from Hicol (Rotterdam, The Netherlands). 5-Fluorouracil was kindly supplied by Hoffmann-La Roche (Mijdrecht, The Netherlands). 5-Chlorouracil (5-CU) was purchased from Calbiochem (Los Angeles, Calif., U.S.A.) and 5-fluoro-[6-^{3}H]uracil (2.1 μCi/mmol) from the Radiochemical Centre in Amersham (Great Britain).

Instafluor was from Packard (Downers Grove, Ill., U.S.A.) and Triton X-100 from Koch-Light Labs. (Colnbrook, Great Britain).

Packing of the column

To 500 mg of Gas-Chrom Q (140—160 μm, carefully sieved and dried at 120°), 30 ml of a 10% (v/v) solution of dimethyldichlorosilane in toluene were added, and the mixture was refluxed for 1 h. The resilanized Gas-Chrom Q was filtered off, washed with toluene and refluxed in methanol. After filtration and washing with acetone the powder was dried at 120°. A solution of Versamid 900 (15 mg) in 15 ml chloroform—methanol (85:15) was added to the hot Gas-Chrom Q, and the solvent was removed using a Rotavap. A glass column (0.45 m × 0.8 mm I.D.) was cleaned with warm RBS 25 solution, water and acetone, and dried at 120°. The column was then silanized with dimethyldichlorosilane, cleaned by three methanol washings and dried at 120°. A 1% (w/v) solution of Carbowax 20M in methanol was sucked through the hot column. The air-dried column was packed with 3% Versamid 900 on Gas-Chrom Q.

Recovery experiments

For the radiochemical experiments a solution of 5-FU in methanol (4 ml, 100 ng/μl) containing 5-fluoro-[6-^{3}H]uracil (about 22 × 10^5 dpm per ml) was prepared. Several 20-μl samples of this solution were stored in the scintillation liquid in a counting vial as a reference. Aliquots of 10—60 μl were then pipetted into vials of glass, silanized glass and plastic, or Eppendorf tubes. After evaporation of the solvent the residue was dissolved in 200 μl methanol, the vial was heated at 50° and shaken by means of a Vortex mixer. Subsequently 20 μl of the methanolic solution were added to the scintillation liquid (1 ml water and 9 ml Instafluor Triton X-100, 2:1). A sample of 20 μl of pure methanol added to the scintillation liquid served as a background reference. The gas-chromatographic recovery experiments were carried out by the same procedure, omitting the addition of radioactively labelled 5-FU. Standard solutions of 5—FU (10—600 ng/μl) and 5-CU (200 ng/μl) in methanol were used; after evaporation 100 μl methanol were added to the residue prior to detection. The recoveries were calculated by comparison of the absolute peak heights obtained with those detected with the corresponding reference solutions.

Extraction method

To 0.2 ml of plasma in a plastic tube were added 100 or 200 ng of the internal standard, 5-CU. After two extractions with 3 ml of ethyl acetate the combined ethyl acetate layers were evaporated and the residue could be used in the GLC procedure. However, the lifetime of the column can be extended by further purification. Hence the ethyl acetate residue was dissolved in 1 ml of hexane, 0.5 ml of water was added and the mixture was shaken by means of a Vortex and centrifuged. The hexane layer was separated and the water layer again extracted with 1 ml of hexane. After evaporation of the remaining water layer the residue was suitable for further manipulation in the GLC procedure. For the construction of a calibration curve (5—200 ng) known amounts of

5-FU in the range 50—2000 ng were added to 0.2 ml of blank plasma, after which the extraction procedure described above was followed.

GLC procedure

The residue obtained by the extraction method was dissolved in 100 μl water. The injections were carried out with a solid-phase injection system (modified pyrolysis system Becker Model 767 [5]). The reference solution and sample solution (10 μl) were applied to the tip (0.5 mm O.D.) of a stainless-steel rod (2 mm O.D.) with a microsyringe. After evaporation of the solute, the rod was conducted through the sluice system and maintained in the upper part of the column for 10 sec. The retention times for 5-FU and 5-CU were approximately 1.4 and 3.5 min, respectively. The operating conditions were: carrier gas (helium) flow-rate 8.5 ml/min, additional scavenger gas (helium) up to 30 ml/min; hydrogen flow-rate 3.0 ml/min; air flow-rate 100 ml/min; inlet and detector temperature 300°; oven temperature 190°.

RESULTS AND DISCUSSION

Initial observations whereby 5-FU was found to adhere to glass prompted us to investigate this phenomenon in more detail. A number of recovery experiments were performed using vials of different material. Solutions of 5-FU were evaporated in glass and plastic vials. After redissolving the residue, both radiochemical and gas-chromatographic measurements indicated that considerable loss of 5-FU had occurred in the glass vials, whereas with the plastic vials almost quantitative recoveries were obtained. Some results of experiments on the adhesive properties of several materials are summarized in Fig. 1. Awareness of the adherence of 5-FU to non-deactivated glass is important in developing extraction procedures for this compound. Although we have no indication that 5-FU is adsorbed by glass from a solution, we have avoided the use of non-deactivated glass equipment during the analysis of 5-FU.

GLC of the polar compound 5-FU requires special conditions. In the analytical system the number of adsorption sites should be minimized. Hence all glass equipment used, including the glass column, was silanized with dimethyldichlorosilane in toluene. The glass surfaces were then relatively inert to 5-FU (see Fig. 1). The support material, Gas-Chrom Q, was carefully sieved, resilanized and loaded with the selected stationary phase Versamid 900 (3%, w/w). The use of a narrow-bore short column, also deactivated with Carbowax 20M and packed to only 30—50%, contributed to the elimination of active sites and permitted a rapid analysis. As an internal standard 5-CU was found to be highly suitable because of the structural similarity to 5-FU. The adhesive behaviour of 5-CU closely resembled that of 5-FU. In order to prevent solvent interference a solid-phase injection system was used, and to obtain a high degree of sensitivity nitrogen—phosphorus detection was used. In this way excellent results for the determination of underivatized 5-FU were obtained. In Fig. 2 three chromatograms illustrate the GLC method for 5-FU.

For the assay of 5-FU in the plasma of patients a series of samples of blank plasma, to which a known amount of 5-FU was added, has been analysed simultaneously according to the extraction and GLC procedure described. In

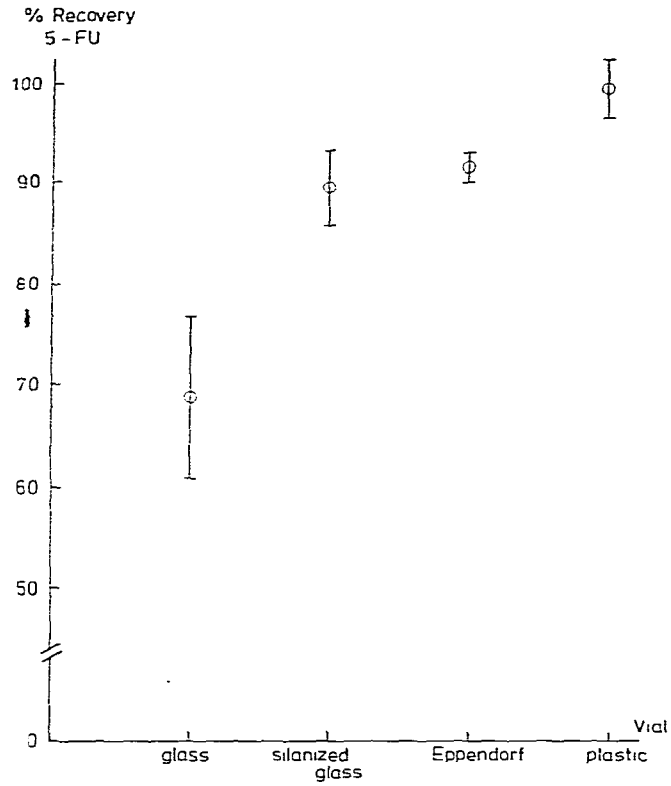

Fig. 1. Recovery of 5-fluorouracil from glass, silanized glass, Eppendorf tubes and plastic vials.

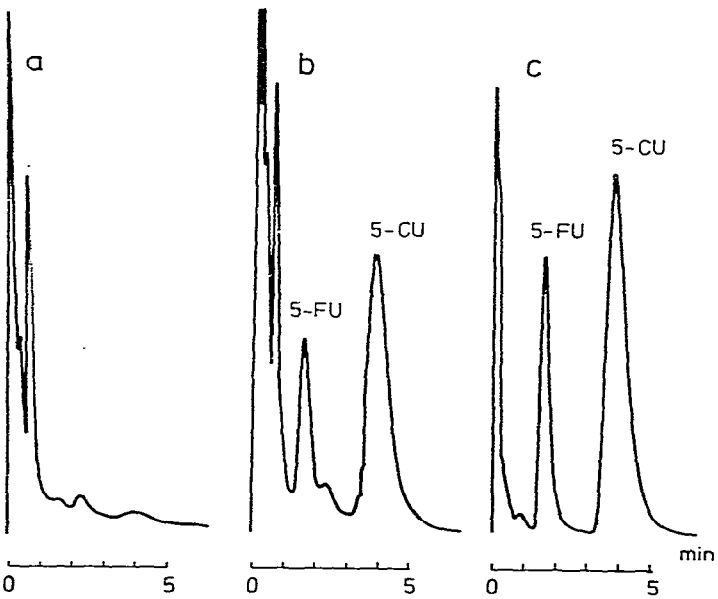

Fig. 2. GLC diagrams of: (a) blank plasma; (b) 50 ng of 5-FU and 200 ng of 5-CU from a plasma sample; (c) 50 ng of 5-FU and 200 ng of 5-CU in a standard solution in water.

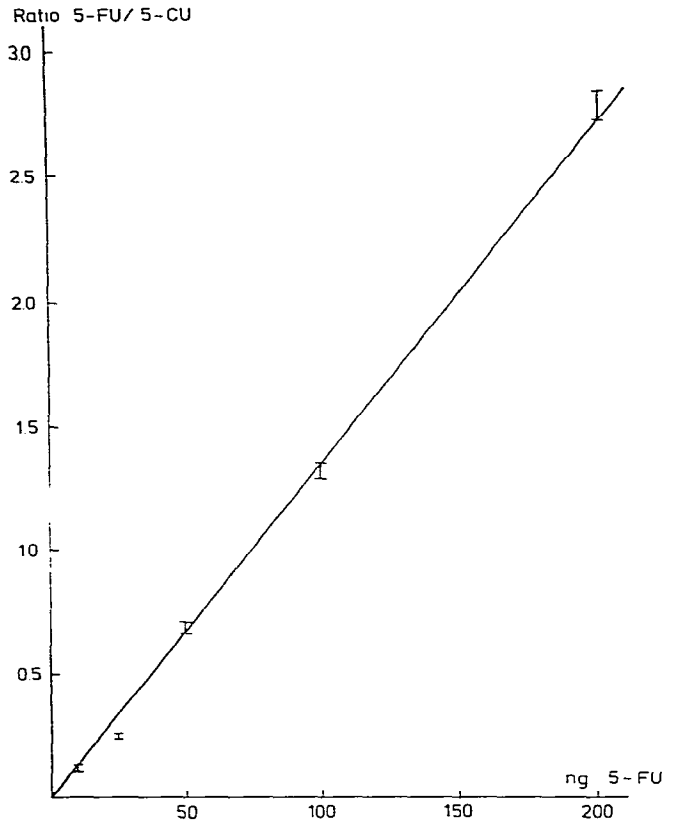

Fig. 3. Ratios of the peak heights of 5-FU and 5-CU measured according to the extraction method and the GLC procedure *vs.* the added amount of 5-FU.

Fig. 3 an example of a standard extraction line is presented. The recoveries for the extraction method were found to be in the range 50—80%.

The lower detection limit of 5-FU in patients' plasma according to the described extraction and GLC procedure is 50 ng/ml, whereas the limit per injection on the column is usually 1 ng per injection.

To demonstrate the practicability of the method, an example of a routine determination of 5-FU in plasma is shown in Fig. 4, which presents the 5-FU decay curve after intravenous and oral administration.

In conclusion, it can be stated that a suitable GLC analysis for underivatized 5-FU is now available, offering a more direct approach to the determination of 5-FU in only 0.2 ml plasma. The observed adherence of 5-FU to glass demands attention, as a considerable loss of 5-FU in extraction and GLC procedures may occur.

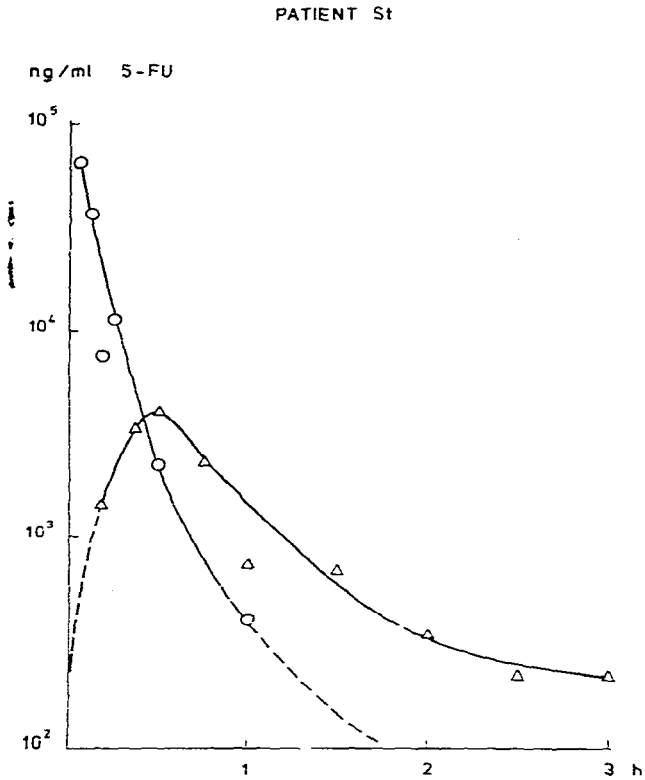

Fig. 4. Concentration of 5-FU in the plasma of a patient administered 500 mg of 5-FU orally (△) and by an intravenous (○) bolus injection.

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NOTE ADDED IN PROOF

As the described method was used routinely, it appeared that the estimation of the lower detection limit was too optimistic and is 5 ng per injection, or 250 ng/ml.

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