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ANALYSIS OF 5-FLUOROURACIL IN PLASMA BY PRECOLUMN DERIVATIZATION WITH 4-BROMOMETHYL-7-METHOXYCOUMARIN, FOLLOWED BY MULTI-DIMENSIONAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An assay for 5-fluorouracil (5-FU) has been developed that utilizes a double extraction with ethyl acetate, followed by precolumn derivatization with 4-bromomethyl-7-methoxycoumarin. The reaction mixture was quenched with 5% acetic acid, extracted with hexane, and analyzed by multi-dimensional high-performance liquid chromatography. Derivatized 5-FU was injected into a cyanopropyl column and a heart cut containing the analyte was then switched to an octadecyl column and quantitated by fluorescence detection. The assay had a limit of detection of 0.5 ng 5-FU/ml plasma and was linear to 20 μg/ml. It was shown to be free of interferences from the other anticancer agents commonly used in combination with 5-FU. This assay should have the sensitivity needed to measure the low levels that occur after low-dose, continuous infusion of 5-FU.

INTRODUCTION

5-Fluorouracil (5-FU, Fig. 1, I) is an antimetabolite which has been used in cancer chemotherapy for many years[1]. Although a large number of quantitative methods for 5-FU have been reported, including microbial assays[2], high-performance liquid chromatography (HPLC)[3–14] and gas chromatography with mass spectroscopic detection (GC–MS)[15–18], only GC–MS has enough sensitivity for determining the pharmacokinetic parameters for 5-FU following low-dose, continuous infusions. After the termination of a continuous intravenous infusion of 5-FU, the plasma concentration of 5-FU quickly decreases below the limit of detection of most assays (*ca.* 5–10 ng/ml), because 5-FU has an elimination half-life of 6–12 min[14,15]. Derivatization of 5-FU with 4-bromomethyl-7-methoxycoumarin (BrMmc, Fig. 1, II) allows the use of HPLC with the increased sensitivity of fluorescence detection[11–13]. Finn and Sadee[18] have discovered the existence of a second, much longer, β-

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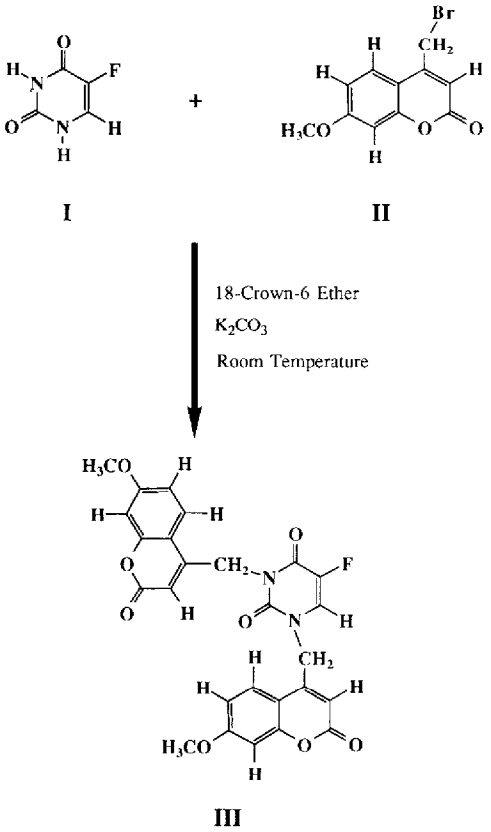

Fig. 1. Structure of 5-fluorouracil (I), 4-bromomethyl-7-methoxycoumarin (II), and the resulting 5-FU-$(Mmc)_2$ derivative (III).

elimination phase for 5-FU by GC–MS; however, their method of analysis was unable to quantify this parameter reliably. BrMmc was first synthesized by Secrist *et al.*[19] and used for the derivatization of thiouracil. Later[20], BrMmc was utilized for the derivatization of fatty acids. Iwamoto *et al.*[10,11] first reported the derivatization of 5-FU with BrMmc in acetone–acetonitrile with powdered potassium carbonate and 18-crown-6 ether as catalysts. Subsequently, it was found that the 18-crown-6 ether could be eliminated[12,13] by conducting the reaction in dimethyl sulfoxide (DMSO). This reaction proceeds quickly and quantitatively at room temperature. However, the full potential of this highly fluorescent derivative was not realized due to plasma interferences. Previously, the purification and identification of the 5-FU–BrMmc derivatization product [5-FU-$(Mmc)_2$, Fig. 1, III] was reported[21], along with preliminary evidence that multi-dimensional chromatography can provide the low detection limits needed to characterize the pharmacokinetics of 5-FU more thoroughly.

Using DMSO as the derivatization solvent gave satisfactory yields for 10 ng or more of 5-FU. However, attempts to derivatize 1 ng 5-FU or less with this reaction

system did not yield any detectable product. Returning to the original scheme of Iwamoto *et al.*[10,11], who used a 18-crown-6 ether catalyst and acetone–acetonitrile, it was possible to derivatize 0.2 ng 5-FU reproducibly. This reaction scheme, coupled with the multi-dimensional chromatography previously described[21], yielded an assay with a limit of detection of 0.5 ng/ml plasma and linearity up to 20 μg/ml on only 0.5 ml of sample.

EXPERIMENTAL

Chemicals

5-Fluorouracil (5-FU), prednisolone, prednisone, methotrexate, uracil, thymine and cytosine were purchased from Sigma (St. Louis, MO, U.S.A.). 4-Bromomethyl-7-methoxycoumarin (BrMmc), 18-crown-6 ether, and cyclophosphamide were obtained from Aldrich (Milwaukee, WI, U.S.A.). Injectable 5-FU (50 mg/ml, SoloPak Labs., Franklin Park, IL, U.S.A.), mitomycin C (0.5 mg/ml, Bristol Labs., Syracuse, NY, U.S.A.) and vincristine (1 mg/ml, DuPont Critical Care, Waukegan, IL, U.S.A.) were obtained from the University of Kansas Medical Center pharmacy. Adriamycin was obtained from Dr. S. Lindenbaum (University of Kansas) and 5,6-dihydro-5-fluorouracil was a gift from Dr. K. Chan (University of Southern California). Sodium acetate, glacial acetic acid, HPLC-grade solvents and potassium carbonate were purchased from Fisher Scientific (St. Louis, MO, U.S.A.). The potassium carbonate was powdered with a mortar and pestle and dried in a vacuum oven (30°C) overnight before use. All water was deionized and purified with a Milli-Q water system (Millipore, Bedford, MA, U.S.A.) before use. All glassware used for the extraction and derivatization was silanized with trimethylchlorosilane in chloroform.

Extraction of plasma

Initially, the extraction efficiencies of various solvents were compared by extracting 0.5 μg 5-FU from 0.5 ml phosphate buffered saline. Either 5 or 9 ml volumes of ethyl acetate, methyl-*tert.*-butyl ether (MTB), diethyl ether, chloroform and dichloromethane were used. After addition of solvent, the tubes were mixed for 2 min and centrifuged (1000 *g*) for 5 min. The solvent layer was removed, dried under nitrogen, and reconstituted in 50 m*M* KH_2PO_4 (pH 3.0). The recovery of 5-FU was assessed by HPLC with UV detection[7].

In the final assay procedure, plasma samples containing 5-FU (0.5 ml) were vortex-mixed with 100 μl 0.5 *M* KH_2PO_4 (pH 3.5) in screw-capped culture tubes (150 × 16 mm). Ethyl acetate (9.0 ml), previously saturated with water, was added, and the samples were mixed for 2 min. Following centrifugation (1000 *g*, 10 min) to achieve separation of the layers, the ethyl acetate was removed and the remaining aqueous layer was re-extracted with another 9 ml of ethyl acetate (water-saturated). The extracts were combined in silanized glass, conical centrifuge tubes and dried under nitrogen.

The extraction efficiency was determined by adding 250 ng 5-FU to 0.5 ml plasma and to the ethyl acetate extract from 0.5 ml blank plasma. The plasma samples were extracted as described above and derivatized with BrMmc, as described below. Following multi-dimensional HPLC, the recovery of 5-FU from plasma was expressed as the ratio of the peak heights from the two samples.

Derivatization of 5-FU with BrMmc

5-FU was derivatized with BrMmc, using a modification of the procedure previously reported by Iwamoto *et al.*[10,11]. 5-FU or dried plasma extracts were solubilized with 100 μl of ethyl acetate, containing 1 mg 18-crown-6 ether. This was followed by the addition of 5 mg potassium carbonate and 0.25 mg BrMmc in 400 μl acetone–acetonitrile (1:2, v/v). The samples were vortex-mixed after the addition of each compound. After 2 h at 75°C or 40 h at room temperature (22 ± 2°C) the reaction was quenched with 625 μl of 5% acetic acid in water and the mixture was extracted twice with 1-ml portions of hexane. The hexane extracts were discarded and the remaining aqueous layers were then ready for analysis by HPLC.

To determine the derivatization yield, 5-FU was derivatized in the presence of plasma extract and compared with previously isolated and purified 5-FU-$(Mmc)_2$[21]. 5-FU (250 ng) was added to a plasma extract, dried, derivatized as described above, and quantitated by multi-dimensional HPLC. The peak heights of these samples were divided by the peak heights from the molar equivalent of 5-FU-$(Mmc)_2$ to obtain the efficiency of derivatization.

Stability of 5-FU-$(Mmc)_2$

The stability of 5-FU-$(Mmc)_2$ in acetone–acetonitrile and buffer mixtures was investigated. A sample of 5-FU-$(Mmc)_2$ (50 ng) in 1 ml acetone-acetonitrile was mixed with 1.25 ml 10 m*M* acetic acid (pH 3.4), 10 m*M* sodium acetate (pH 5.0), or 50 m*M* potassium carbonate (pH 11.0) and kept in the dark at room temperature (22 ± 2°C). At various times, an aliquot of each mixture was removed and analyzed by multi-dimensional HPLC.

HPLC

The chromatographic system (Fig. 2) consisted of two Model 110 A pumps (Altex Scientific, Berkeley, CA, U.S.A.), a WISP 712 autosampler (Waters Assoc., Milford, MA, U.S.A.), an SLIC 1400 controller (Sys-Tec, New Brighton, MN, U.S.A.), a 6-port air-actuated switching valve (Rheodyne, Cotati, CA, U.S.A.), and a Model 535 fluorescence detector and C-R5A integrator (Shimadzu, Kyoto, Japan). The columns used were 150 × 4.6 mm I.D., packed in this laboratory[22] with 5-μm silica particles, bonded with cyanopropyl (CPS-Hypersil, Shandon Southern Products, London, U.K.) or octadecyl (ODS-Hypersil) side chains.

The CPS and ODS columns were eluted at 1.5 ml/min with methanol–10 m*M* sodium acetate, pH 4.75 (4:6, v/v) and methanol–10 m*M* sodium acetate, pH 4.75 (1:1, v/v), respectively. Both columns were jacketed and thermostated at 35°C with a circulating water bath (Haake, Saddlebrook, NJ, U.S.A.) to minimize fluctuations in retention due to periodic changes in the ambient temperature.

Samples (100 μl) were injected onto the CPS column with the WISP autosampler and a 2.5-min heart cut containing the derivative peak was switched onto the ODS column. The time of the cut was determined by injecting a standard solution of 5-FU-$(Mmc)_2$ onto the CPS column and monitoring the eluent with the UV detector (325 nm). The switching valve was actuated by the SLIC controller and immediately after the cut, the integrator was started. After further separation on the ODS column, the derivative was detected with the fluorescence detector (λ_{ex} = 325 nm, λ_{em} = 395 nm) and quantitated by peak height measurement.

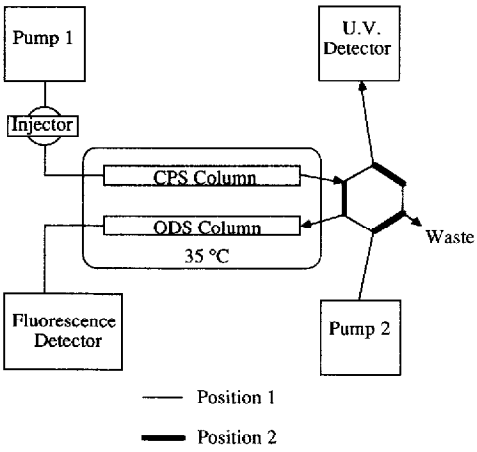

Fig. 2. Schematic drawing of the column switching HPLC system. The valve is in position 1 for normal operation. During the heart cut the valve is in position 2 and the eluent flows from the CPS column into the ODS column.

Standard curve

Linearity of the assay was established by generating standard curves of 5-FU from plasma over two concentration ranges. The low range was 0, 0.5, 1, 5, 10, 50, 100 and 200 ng 5-FU/ml, while the high range was 0, 0.5, 1, 5, 10 and 20 μg 5-FU/ml. Each solution was analyzed in triplicate with a reaction time of 40 h at room temperature. The 5, 10 and 20 μg/ml concentration levels were diluted 1:10 before injection to keep the peaks within the linear range of the fluorescence detector. For analysis of samples, a single standard curve that covered the expected concentrations of the samples was generated.

Rabbit pharmacokinetics

A preliminary pharmacokinetic profile of 5-FU in a rabbit was obtained. 5-FU (50 mg/kg) was administered to a New Zealand rabbit (4.3 kg) by intravenous bolus in the ear vein over 2 min. At 1, 4, 6.5, 10, 14.5, 18.5, 25, 28 and 43 min, and 1, 1.5, 2, 3 and 21.5 h after injection, blood samples (1.8 ml) were collected from the ear artery with a syringe, containing 0.2 ml of 0.1 *M* potassium oxalate. Plasma was obtained by centrifugation (1000 *g*, 15 min) and stored at −20°C until analyzed. The data were fit to a two-compartment model with bolus administration and first order elimination, using PCNONLIN (Statistical Consultants, Edgewood, KY, U.S.A.).

RESULTS AND DISCUSSION

Several assays for 5-FU have been reported[2–19], however, most of these have severe limitations. The most sensitive of these, GC-MS[15–18], requires expensive equipment and a high degree of technical expertise. The use of HPLC is less expensive and much simpler, however, it lacks the required sensitivity to determine the terminal β-elimination phase of 5-FU in plasma[18]. An assay that has the high sensitivity of

GC–MS and the ease of HPLC was required to characterize 5-FU pharmacokinetics further. The various stages of this assay were developed in a rational and systematic fashion. The optimization of the extraction and derivatization of 5-FU was required before multi-dimensional HPLC could be used to its fullest potential. The linearity, accuracy and reproducibility of the assay were established, once the assay development had been completed. Finally, the use of this assay for pharmacokinetic analysis was demonstrated.

Choice of extraction solvent

Extraction of 5-FU from phosphate buffered saline was investigated with a variety of solvents. The results are summarized in Table I and show that ethyl acetate and dichloromethane gave comparable extraction efficiencies. However, ethyl acetate was selected over dichloromethane, because it is less hazardous and yielded a cleaner organic layer after extraction (no precipitated plasma proteins in the upper layer after centrifugation). Further investigation revealed that acidification of the sample with 0.5 *M* KH_2PO_4 (pH 3.5)[10], followed by a double extraction of 0.5-ml samples with 9 ml ethyl acetate (water-saturated) gave the best results.

Because the derivatization reaction was dependent on the sample matrix, the determination of the extraction efficiency was performed while keeping the derivatization conditions constant. To achieve this, 5-FU was added to an extract from 0.5 ml blank plasma. The peak height of the derivative formed from 250 ng 5-FU, extracted from 0.5 ml plasma, was 21 619 $\pm$ 1869 μV ($\bar{x} \pm$ S.D.) and 23 275 $\pm$ 1170 μV ($\bar{x} \pm$ S.D.) from 250 ng 5-FU added to blank plasma extract. The recovery of 5-FU from 0.5 ml plasma with two 9-ml portions of ethyl acetate was calculated to be 92.9 $\pm$ 8.0% ($\bar{x} \pm$ S.D.). Since the standard curves were found to be linear, it is reasonable to assume that the recovery of 5-FU remains constant and is independent of concentration.

Derivatization kinetics

Initially, the effects of temperature and time on the derivatization reaction were determined with solutions of 5-FU in acetone–acetonitrile. 5-FU (100 ng) was derivatized at 75°C or at room temperature (22 $\pm$ 2°C), and the reaction was quenched at various times. It can be seen from Fig. 3a that the reaction reached a plateau after 15

TABLE I

RECOVERY OF 1 μg 5-FU/ml PHOSPHATE BUFFERED SALINE WITH VARIOUS EXTRACTION SOLVENTS

Solvent	*Volume (ml)*	*% Recovery*
Ethyl acetate	5	54
	9	77
MTB	5	23
	9	36
Diethyl ether	9	25
Chloroform	9	3
Dichloromethane	9	78

min at 75°C and 45 min at room temperature. However, when the derivatization of 5-FU from a plasma sample was carried out for 0.5 h at 75°C or 1 h at room temperature, the peaks were only 10% of the expected height. This led to further investigation of the derivatization of 5-FU extracted from plasma.

Human plasma was spiked with 200 ng 5-FU/ml, and 0.5-ml aliquots were extracted twice with 9 ml ethyl acetate. Following the evaporation of the solvent, the extract was derivatized at 75°C or room temperature and quenched at different times. When 5-FU extracted from plasma was derivatized, the reaction did not reach a plateau until 2 h at 75°C or 20 h at room temperature (Fig. 3b). This decrease in reaction rate could be attributed to several factors. Two of the most likely factors are either inhibition of the derivatization reaction by extracted plasma components or a decrease in the dissolution rate of 5-FU from the plasma residue. The latter seems to be the most probable, since the final yield of the reaction is the same for both 5-FU and the 5-FU extracted from plasma. Also, the solubilization of the extraction residue with 100 μl ethyl acetate before derivatization for 1 h at room temperature resulted in a 2-fold increase in peak height (data not shown). The chromatographic profile from

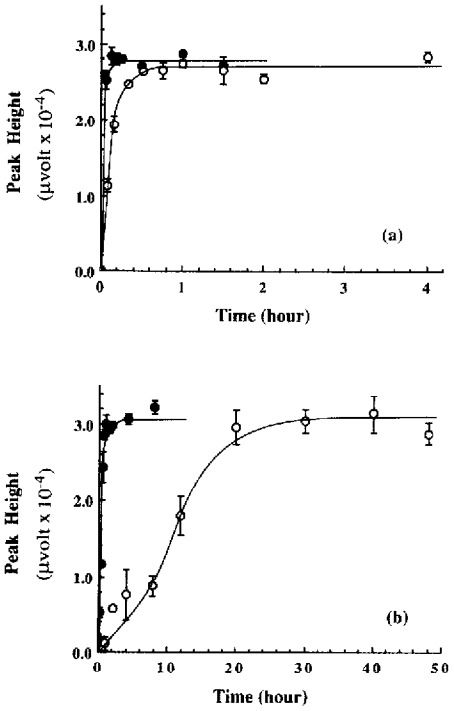

Fig. 3. Time profile for the derivatization of 5-FU with BrMmc. 5-FU in (a) neat solution (100 ng) or (b) extracted from plasma (200 ng/ml) was derivatized with 0.25 mg BrMmc in 500 μl acetone–acetonitrile (1:2, v/v), containing 0.1 mg 18-crown-6 and 5 mg potassium carbonate at (●) 75°C or (○) room temperature (22 ± 2°C). The reaction was quenched with 625 μl 5% acetic acid at various times and the product was analyzed by HPLC. The derivatization of 5-FU in neat solution is complete in 15 min at 75°C or 45 min at room temperature. The plasma extract extends the derivatization time to 2 h at 75°C or over 20 h at room temperature.

reversed-phase HPLC was significantly cleaner with derivatization at room temperature than at 75°C. Difficulty in maintaining the seal of some of the reaction tubes resulted in evaporation of the derivatization solvent from these tubes at 75°C. To alleviate these problems, the derivatization reactions were carried out in the dark at room temperature for 40 h. This time was chosen to insure that the reaction had gone to completion and because it was found to be the most convenient for scheduling the analysis of large numbers of samples. When the peak heights from 250 ng 5-FU, added to plasma extract, were compared with a molar equivalent of isolated 5-FU-$(Mmc)_2$, the extent of derivatization was calculated to be 92.5 ± 4.6% ($\bar{x}$ ± S.D.). Comparison of the peak heights from 5-FU added to plasma with those obtained with the 5-FU-$(Mmc)_2$ gives the absolute efficiency of the extraction and derivatization, which was calculated to be 85.9 ± 7.4% ($\bar{x}$ ± S.D.).

Stability of the derivative

To insure that there was no loss of the derivative when samples were stored in an autosampler, its stability was investigated at various pH values. The solutions in aqueous acetic acid or sodium acetate (pH < 5.0) showed no sign of degradation after 6 h (Fig. 4). However, in the potassium carbonate solution (pH 11.0) extensive degradation was seen with a half-life of the derivative estimated to be less than 50 min. Further investigation revealed that 625 μl of 5% acetic acid neutralized the potassium carbonate used as a catalyst in the reaction and kept the pH of the reaction mixture below 5.0. Repeated injection of a spiked plasma sample over 24 h showed no decrease in the resulting peak (data not shown), allowing the use of an autosampler.

Chromatography

The use of a single cyanopropyl (CPS) or octadecyl (ODS) HPLC column was investigated, but the late-eluted peaks and lack of baseline separation of 5-FU-$(Mmc)_2$ from other contaminants (Fig. 5a and b) led to the development of a multi-dimensional system (single-column HPLC would be adequate if one were working at the elevated

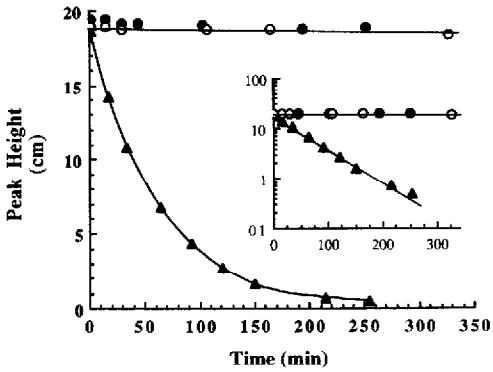

Fig. 4. Stability of 5-FU-$(Mmc)_2$ in the reaction mixture. A 5-FU-$(Mmc)_2$ solution (50 ng/ml) in acetone–acetonitrile (1:2, v/v) was diluted 1:1.25 with (○) 10 m*M* acetic acid (pH 3.4), (●) 10 m*M* sodium acetate (pH 5.0), or (▲) 50 m*M* potassium carbonate (pH 11.0) and analyzed by multi-dimensional HPLC. The 5-FU-$(Mmc)_2$ derivative is stable below pH 5.0. However, at pH 11.0, the half-life estimated from the semi-log plot (inset) is 50 min.

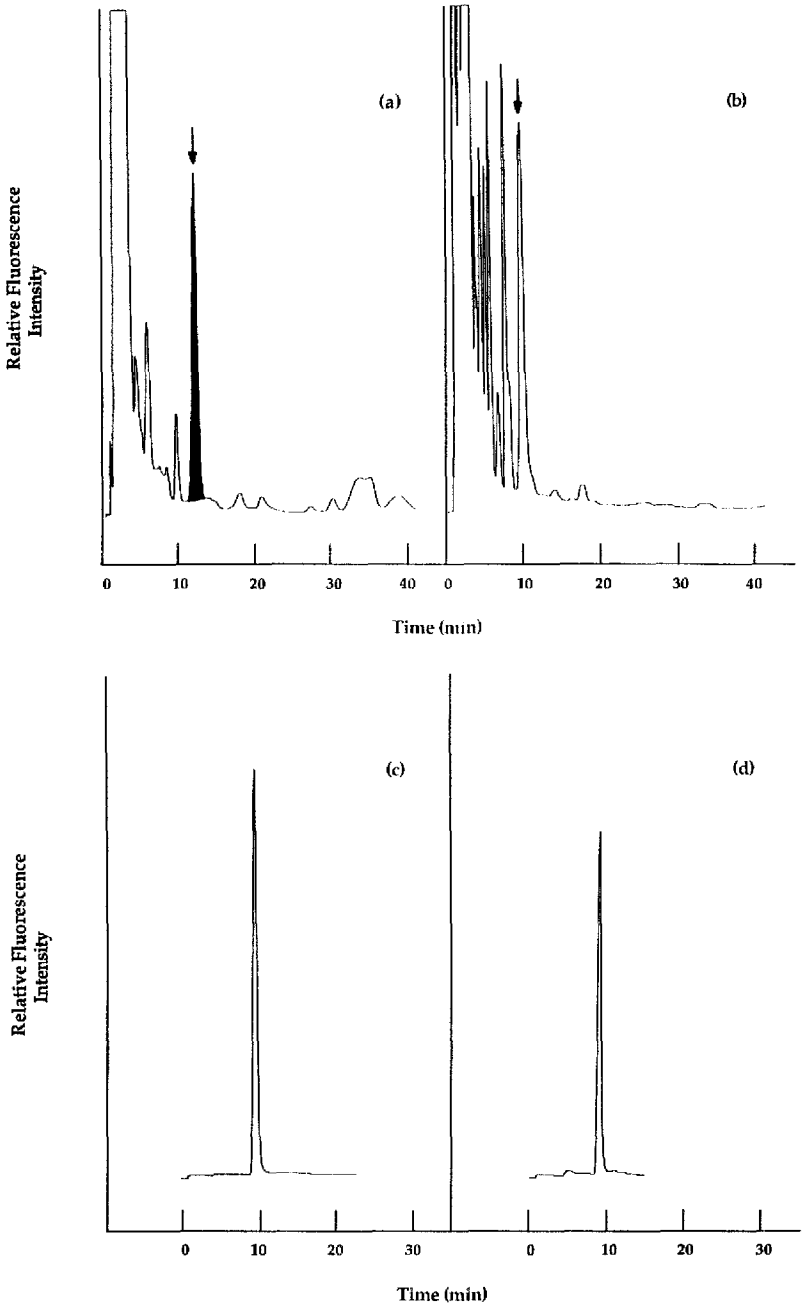

Fig. 5. Chromatograms from 5-FU-$(Mmc)_2$ or plasma samples. The arrows mark the analyte peak. The chromatograms from a patient sample containing 433 ng 5-FU/ml that was injected into (a) a CPS column eluted with methanol–sodium acetate (4:6, v/v) at 1.5 ml/min at 35°C, (b) an ODS column eluted with methanol–sodium acetate (1:1, v/v) at 1.5 ml/min at 35°C, or (c) the peak (filled in area) from the CPS column was switched to the ODS column. A chromatogram from a rabbit plasma sample (110 ng 5-FU/ml) after multi-dimensional HPLC is also shown in (d).

levels shown in Fig. 5a and b). Fig. 5c and d show the resulting chromatograms when multi-dimensional chromatography was used. The 5-FU-$(Mmc)_2$ peak was free of interfering peaks at concentrations of 110 and 433 ng/ml. The use of column switching with two different column packings improved the separation and shortened the analysis time. When the next sample is injected shortly after the heart cut is finished, the analysis time is about 15 min per sample. If either the CPS or ODS column were used alone, the late-eluted peaks extend the analysis time to more than 20 min.

5-FU is used in combination with many other anticancer agents. The most common of these drugs are adriamycin, methotrexate, vincristine, spirogermanium, prednisone, prednisolone, mitomycin C and cyclophosphamide. To insure that these drugs would not interfere with this assay, eight separate solutions, containing 110 ng 5-FU and 5 μg of one of these agents were derivatized. After quenching, the reaction mixtures were analyzed with the column switching system. There was no difference in the peak heights of the 5-FU derivative from the samples containing the other drugs when compared with 5-FU alone. Also, the chromatograms did not have any other peaks related to these other agents. Endogenous compounds with similar structures: 5,6-dihydro-5-fluorouracil (the major metabolite), uracil, thymine and cytosine were also tested. None of these caused any interference with the 5-FU-$(Mmc)_2$ peak. However, peaks believed to be the corresponding coumarin derivative for uracil and 5,6-dihydro-5-fluorouracil were found to be eluted before and after 5-FU-$(Mmc)_2$, respectively. Both of these other peaks were well separated from the analyte peak.

Assay validation

Representative standard curves for the high- and low-concentration ranges are shown in Fig. 6. The extraction and derivatization is linear from 0.5 ng to 20 μg 5-FU/ml plasma, although dilution of solutions containing more than 5 μg/ml is needed to remain in the linear range of the detector used in this study. Linear regression of peak heights *versus* concentration gave eqns. 1 and 2 for the high and low standard curves, respectively:

$$y = 8.7 \cdot 10^2 + 1.4 \cdot 10^2 x \qquad (r^2 = 0.999) \tag{1}$$

$$y = 1.3 \cdot 10^1 + 1.8 \cdot 10^2 x \qquad (r^2 = 0.999) \tag{2}$$

The units for the peak heights (y) are in μV and the concentrations (x) are in ng/ml. The slopes for the two curves are not significantly different (Student's t-test). The limit of detection was 0.5 ng 5-FU/ml (signal-to-noise ratio = 2), which is a great improvement over that reported previously with this reaction procedure[10–13].

The accuracy and precision of the assay (Table II) was determined, using the standard curve data. The error over the complete assay range was generally less than 6%. As expected, it was higher at the low concentration end of the two standard curves (*i.e.*, 0.5 and 1 ng/ml for the low curve and 500 ng/ml for the high curve). However, the accuracy could be improved by using a smaller concentration range than was used here. Although, it is preferable to use an internal standard to improve the reproducibility of the assay, a suitable compound with similar extraction, derivatization and chromatographic characteristics could not be found. Even without an internal standard, the relative standard deviation was acceptable with occasional scatter. At the 1000 ng 5-FU/ml level, two of the three determinations were within 10%

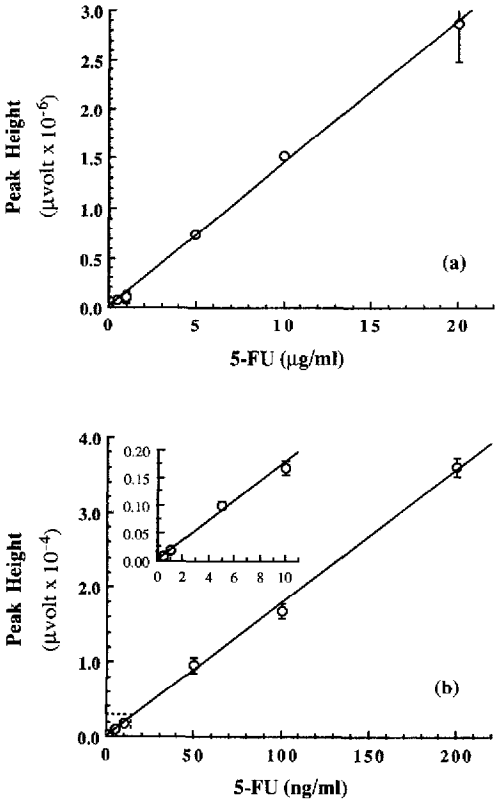

Fig. 6. Standard curves from spiked human plasma. Human plasma was spiked with 5-FU at varying concentrations and aliquots were analyzed, as described in the text. Points shown are the average of three determinations (±S.D.). (a) High-level standard curve of 0.5–20 μg 5-FU/ml. Samples containing more than 5 μg/ml were diluted 1:10 with acetone–acetonitrile–5% acetic acid (1:2:3, v/v/v) before analysis by HPLC. (b) Low-level standard curve of 0–200 ng 5-FU/ml. The points below 10 ng/ml are shown in the inset. The assay was linear to 20 μg 5-FU/ml plasma, with a limit of detection of 0.5 ng/ml.

of each other, however a third point caused the large standard deviation. It should be noted that even with the large standard deviation, the mean calculated concentration is in good agreement with the theoretical concentration. Also, subsequent experiments have shown (Table III) that the between-day reproducibility was acceptable. The between-day variability over the linear range of the assay was 10% for four concentrations over three days (Table III).

Rabbit pharmacokinetics

The utility of the assay was demonstrated by monitoring the plasma concentration of 5-FU in a rabbit after a bolus dose. One New Zealand rabbit (4.3 kg) was given 50 mg 5-FU/kg (225 mg) by intravenous bolus over 2 min. Blood samples (1.8 ml) were drawn at various times, and the plasma was analyzed after centrifugation to remove the erythrocytes. The data were fit to a two-compartment model. The experimental

TABLE II

ACCURACY AND PRECISION OF THE ASSAY OVER THE COMPLETE STANDARD CURVE RANGE

Concentration (ng/ml)			
Actual	*Calculated*	*%Error*	*%R.S.D.*[a]
0.5	0.6	20.0	20.0
1.0	1.2	20.0	6.0
5.0	5.8	16.0	8.0
10.0	9.5	−5.0	7.2
50.0	53.8	7.6	11.9
100	94.3	−5.7	5.5
200	201.3	0.7	3.6
500	594.8	19.0	15.4
1000	996.8	−0.3	34.7
5000	4801	−4.0	4.8
10000	10424	4.2	1.9
20000	19576	−2.1	13.3
	Mean	5.9	11.0

[a]Relative standard deviation.

data are shown (Fig. 7) along with the computer generated line. The semi-log plot (Fig. 7, inset) demonstrates that the elimination of 5-FU does not fit a two-compartment model, as others have recently reported[23]. The data suggest that the elimination profile may follow saturable Michealis–Menten kinetics. Non-linear pharmacokinetics of 5-FU has been demonstrated in humans[2,15,24], and further experiments are being conducted in this laboratory to define the pharmacokinetics of 5-FU in rabbits and humans better.

TABLE III

BETWEEN-DAY VARIATION OVER THE WORKING RANGE OF THE ASSAY

Concentration (ng/ml)						
Actual	*Calculated*			*Mean*		*%R.S.D.*
	Day 1	*Day 2*	*Day 3*			
1	1.6	1.6	1.0	1.4		21.4
10	11.2	11.4	9.0	10.5		12.4
100	91.6	101.5	101.8	98.3		5.9
1000	1087.0	1003.8	992.1	1027.6		5.0
					Mean	9.9

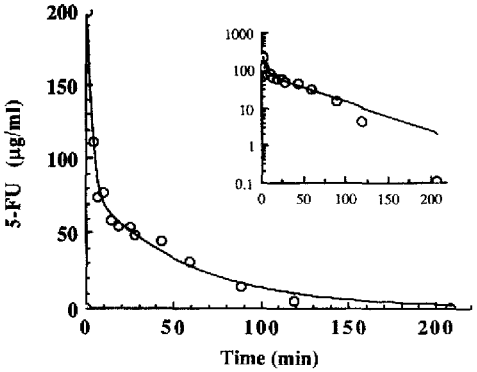

Fig. 7. Pharmacokinetic profile of 5-FU after a single intravenous dose in a rabbit. A rabbit was administered 50 mg 5-FU/kg (225 mg) by intravenous bolus over 2 min. Samples were drawn at various times and analyzed, as described in the text. The experimental data are shown along with the computer generated line for a two-compartment model. The semi-log plot (inset) shows that the data do not fit a two-compartment model and is more indicative of non-linear, saturable kinetics.

CONCLUSION

The resulting 5-FU assay described in this paper utilizes a double ethyl acetate extraction, followed by precolumn derivatization with BrMmc. Both, temperature and sample matrix were found to have profound effects on the derivatization reaction. The combined efficiency for the extraction and derivatization of 5-FU was more than 85%. The limit of detection was 0.5 ng/ml plasma, and the linear range of the assay extended over four orders of magnitude. This sensitivity and large range will be sufficient to further characterize the pharmacokinetics of 5-FU. Finn and Sadee[18] were able to measure a β-elimination phase of 5-FU on a few occasions. However, their assay could not do this on a routine basis. The present assay has the sensitivity to measure the low levels that occur during the terminal β-phase and the results of further pharmacokinetic studies being conducted in these laboratories will be published elsewhere. Since peaks for the uracil and 5,6-dihydro-5-fluorouracil derivatives were detected, adaptation of this method to uracil and 5,6-dihydro-5-fluorouracil determinations, in addition to 5-FU, should be straightforward.

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