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GAS–LIQUID CHROMATOGRAPHY OF SOME ALKYL DERIVATIVES OF 5-FLUOROURACIL

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SUMMARY

Various procedures for converting 5-fluorouracil into its methyl, butyl and hexyl derivatives are described. Structures were established as the N,N'-dialkyl derivatives using mass spectrometry or combined gas–liquid chromatography–mass spectrometry.

The reaction conditions, *i.e.*, the amount of derivatization reagents and reaction time, were optimized. Gas–liquid chromatographic characteristics of the derivatives were investigated on different stationary liquid phases, and 2% or 3% SP-2250, 5% XE-60 and 5% OV-1 were found to be superior. With 5-chlorouracil as the internal standard a linear response for the various derivatives was observed in the microgram range. The applicability of the different dialkyl derivatives in the measurement of 5-fluorouracil in biological materials is discussed.

INTRODUCTION

5-Fluorouracil (5-FU), an antimetabolite of uracil, plays an important role in the chemotherapy of certain forms of cancer[1–4]. A specific and sensitive assay for 5-FU in biological fluids is needed for the evaluation of its chemotherapeutic regimens.

In recent years, a few gas–liquid chromatographic (GLC) assays for 5-FU have been described using flame-ionization detection (FID)[5,6], electron-capture detection[7] and multiple-ion detection[8–10]. Most of these techniques are based on detection of the silyl derivative of the drug[5,8] and on the use of internal standards such as anthracene[6] or thymine[7,9]. Both substances appear to be inappropriate for determination of 5-FU in biological samples, as anthracene is structurally far removed from 5-FU and thymine may occur endogenously.

This paper describes some rapid, simple and virtually quantitative alkylation methods for 5-FU, yielding stable derivatives that are amenable to GLC and to mass fragmentographic analysis[10]. 5-Chlorouracil (5-ClU) is proposed as an internal standard for quantitative measurements.

EXPERIMENTAL

Materials

Sources of reference compounds and reagents are as follows: 5-FU, Sigma (St. Louis, Mo., U.S.A.); 5-ClU, Calbiochem (Lucerne, Switzerland); *n*-alkanes, Poly-Science Corp. (Evanston, Ill., U.S.A.); dried dimethyl sulphoxide (DMSO), Merck (Darmstadt, G.F.R.); N,N-dimethylacetamide (DMA), >99%, Aldrich-Europe (Beerse, Belgium); tetramethylammonium hydroxide (TMeOH), 20% methanolic solution, Aldrich (Milwaukee, Wisc., U.S.A.).

Tetrapentylammonium (TPeA$^+$) and tetrahexylammonium (THeA$^+$) counter ion solutions (about 0.15 and 0.01 M, respectively) were prepared as described previously[11].

The methylsulphinyl carbanion reagent, potassium *tert.*-butoxide in dried DMSO (6 g per 100 ml), was prepared according to De Leenheer and Gelijkens[12].

Tetrahexylammonium hydroxide (THeAOH) (0.2 M solution in methanol) was prepared as follows. A 9.6-g amount of tetrahexylammonium iodide (Eastman-Kodak, Rochester, N.Y., U.S.A.) was dissolved into 100 ml of methanol, 3.5 g of finely divided silver oxide were added and the solution was mixed for at least 1 h at room temperature to precipitate the iodide. The mixture was filtered and the filtrate, consisting of 0.2 M THeAOH, stored in well stoppered, amber-glass bottles. This solution is stable for at least 3 months when stored at room temperature.

All other chemicals were of analytical-reagent grade and were used without further purification.

Apparatus

GLC was performed on a Hewlett-Packard (Avondale, Pa., U.S.A.) Model 5750G or 5830A instrument equipped with dual flame-ionization detectors and a Hewlett-Packard 3370B integrator or a built-in recorder/integrator (HP 18850A GC terminal), respectively. Spiral silanized glass columns (1.8 or 2.3 m × 2 mm I.D.) were packed with the following stationary liquid phases (Supelco, Bellefonte, Pa., U.S.A.): 3 or 5% OV-1 (methyl silicone polymer), 2 or 3% SP-2250 (methyl phenyl silicone polymer), 3% OV-17 (methyl phenyl silicone polymer), 3% OV-25 (methyl phenyl silicone polymer), 2 or 3% QF-1 (fluoroalkyl silicone polymer), 3 or 5% XE-60 (nitrile, silicone gum), 3% Dexsil-300 (carborane–methyl silicone polymer) and 3 or 1% FFAP (reaction product of Carbowax 20M and *m*-nitroterephthalic acid). All liquid phases were coated on 80–100 or 100–120 mesh Gas-Chrom Q (Supelco) using the filter/fluidizing technique of Horning *et al.*[13] and Kruppa *et al.*[14]. The injector and detector (FID) block temperatures were maintained 10° higher than the oven temperature except for the flash-alkylation technique, where the injector heater was at 270°. Nitrogen was used as the carrier gas at a linear velocity of about 7 cm/sec. The hydrogen and air flow-rates were adjusted so as to give optimal sensitivity and good stability.

Mass spectrometric (MS) analysis was carried out on an LKB 9000S instrument (LKB, Bromma, Sweden). The electron energy was 70 eV, the ionization current 60 μA, the accelerating voltage 3.5 kV and the ion source temperature 270°. Crystallized products were introduced into the mass spectrometer using the direct inlet system. The analysis was performed at the evaporation temperature of the

compound. The hexyl derivatives of 5-FU and 5-ClU were prepared by flash alkylation and analysed in the GLC–MS mode. Therefore, derivatives were separated on a 3% Dexsil-300 column (Gas-Chrom Q, 100–120 mesh, 1.8 × 2 mm I.D.) using helium at a flow-rate of 30 ml/min as the carrier gas. The injector block and oven temperatures were 260° and 230°, respectively.

Analytical derivatization study

Extractive methylation

To 1.0 ml of dichloromethane eluate[11] containing 100 μg of 5-FU was added 1.0 ml of 0.14 M TPeA$^+$ solution. When THeA$^+$ was used as counter ion, the methylation conditions were as follows: to 1.0 ml of an aqueous solution of 5-FU (25 μg/ml), previously adjusted to pH 10.0 with 0.1 M sodium hydroxide, was added 1.0 ml of 0.005 M THeA$^+$ solution. In both instances methyl iodide and 2 ml of dichloromethane were added and the mixture was shaken for several hours. This procedure was studied by adding 5, 10, 25, 50, 75 and 100 μl of methyl iodide and by shaking the mixture for 0.25, 0.5, 0.75, 1, 2 and 4 h. The phases were separated and the organic layer was washed with an equal volume of a saturated solution of silver sulphate in 0.1 M sulphuric acid. The organic layer was dried over sodium sulphate and evaporated to dryness at 40° under a stream of nitrogen. When THeA$^+$ was used as counter ion, the residue obtained after evaporation was redissolved in 1 ml of water and the derivative extracted into 2 ml of diethyl ether. The ether phase was washed with two 1-ml portions of water, dried over sodium sulphate and evaporated to dryness at 30° under a stream of nitrogen. The residue obtained by the TPeA$^+$ and THeA$^+$ procedures was dissolved in 200 μl of dichloromethane to which 0.2 mg of n-C$_{24}$/ml was added. A 1–2-μl aliquot was submitted to GLC analysis.

Wet alkylations

Methylation. A volume of 50 μl of methylsulphinyl carbanion reagent was added to 0.5 ml of a 5-FU solution in dried DMSO (200 μg/ml). After a few minutes, 100 μl of methyl iodide were added and the reaction mixture was kept in the dark for a given time. The reaction was stopped by adding 5 ml of water, 100 μg of crystallized N,N'-dimethyl-5-ClU were added and the derivatives were extracted into 5 ml of chloroform. The organic layer was washed with three 5-ml portions of water, dried over sodium sulphate and evaporated to dryness under a stream of nitrogen at 50°. The residue was dissolved in 100 μl of chloroform and 1 μl was injected on to the top of the GLC column. The influence of the amount of methylsulphinyl carbanion was checked by carrying out the same experiment as described above using 3:4, 2:4, 3:8, 1:4 and 1:10 dilutions of the stock methylsulphinyl carbanion reagent in dried DMSO. The methylsulphinyl carbanion and the methyl iodide reaction times were varied from 30 sec to 5 min and from 0 (after adding methyl iodide and mixing, water was added) to 2 h, respectively.

Butylation. An amount of 50 μg of 5-FU was dissolved in a mixture of 50 μl of DMA and 15 μl of 2% TMeAOH in methanol, 20 μl of n-butyl iodide were added and the solution was thoroughly mixed. After a certain time, 1 ml of water was added and the derivative was extracted into 2 ml of diethyl ether. The organic layer was dried over sodium sulphate and evaporated to dryness at 30° under a stream of

nitrogen. The residue was dissolved in 50 μl of methanol and a 1-μl aliquot was submitted to GLC analysis.

Optimization experiments were carried out following the procedure described using 50 μg of 5-ClU and varying the reaction time between 1 and 30 min. An amount of 50 μg of N,N'-dibutyl-5-FU was added to the reaction mixture as an internal standard before the ether extraction step.

Flash hexylation

A volume of 0.2 ml of a methanolic solution of 5-FU (50 μg/ml) placed in a glass tube and the solvent removed by evaporation. The residue obtained was dissolved in 25 μl of 0.2 M THeAOH in methanol and an aliquot was introduced directly into the injection port of the gas chromatograph.

The reproducibility of this gas-phase reaction was investigated by repeated injections of 1-μl aliquots of a solution containing 4 μg of 5-FU and 5 μg of 5-ClU in 25 μl of 0.2 M THeAOH in methanol.

Preparative derivatization procedures

Extractive methylation

An amount of 5 mg of 5-FU was extracted as an ion pair using a cellulose column[11]. To the eluate were added 5 ml of 0.14 M TPeA$^+$ and 0.5 ml of methyl iodide. When using THeA$^+$ as counter ion, 5 mg of 5-FU was dissolved in 40 ml 0.005 M THeA$^+$ and shaken for 2 h with a mixture of 20 ml of dichloromethane and 0.5 ml of methyl iodide. The solutions were further purified as described above.

Wet alkylations

Methylation. The procedure was carried out as outlined above using 50 mg of 5-FU (or 5-ClU), 5 ml of dried DMSO, 4 ml of methylsulphinyl carbanion reagent and 2 ml of methyl iodide. The reaction was stopped by adding 10 ml of water and the derivative was extracted into 20 ml of chloroform. The organic layer was subsequently washed with equal volumes of 0.5 M sodium thiosulphate and water.

Butylation. An amount of 10 mg of 5-FU (or 5-ClU) was dissolved in a mixture of 200 μl of 20% TMeAOH in methanol, 0.8 ml of methanol and 4 ml of DMA. Subsequently 0.5 ml of *n*-butyl iodide was added. After keeping the mixture in the dark at room temperature for 2 h, 5 ml of water was added and the derivative was extracted into 20 ml of diethyl ether.

For all instances described, the organic layer was evaporated to dryness in a water-bath under a stream of nitrogen. The residue obtained was dissolved in a minimal volume of diethyl ether and light petroleum (b.p. 40–60°) was added until the solution became turbid. The product was crystallized by refrigeration overnight and the crystals were separated and dried under vacuum.

Linearization experiments

The different alkylation reactions were carried out in the range 0–100 (25, 50, 75, 100), 0–50 (10, 20, 30, 40, 50), 0–25 (5, 10, 15, 20, 25) and 0–10 (2, 4, 6, 8, 10) μg of 5-FU using optimal reaction conditions as discussed below. Depending on the FID response, an appropriate amount of crystallized corresponding dialkyl deriv-

ative of 5-ClU was added to the mixture after stopping the reaction. For the flash hexylation procedure 5-ClU was added directly to the 5-FU solutions. A linearity check was made by plotting peak-height or peak-area ratios of 5-FU to 5-ClU against the amount of 5-FU.
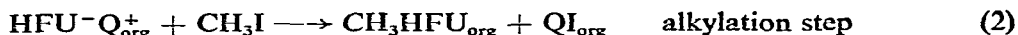
RESULTS AND DISCUSSION

Identification of derivatives

The results for the MS and GLC–MS analysis are given in Table I. Fig. 1 proposes a rationale for a generalized mass fragmentation pattern for the compounds studied. All of these data strongly indicate the formation of the corresponding N,N'-dialkyl derivatives.

Dibutyl and dihexyl derivatives exhibit an ion intensity pattern which differs significantly from that of the underivatized substances and the dimethyl derivatives. On lengthening the alkyl chain, abundant ions originate from fragmentation of the alkyl chain and are superimposed on the entire mass spectra.

Extractive methylation

The extractive methylation process can be described as a two-step reaction, in which the anionic form of 5-FU (HFU$^-$) is first extracted as an ion pair with a suitable quaternary ammonium ion (TPeA$^+$, THeA$^+$–Q$^+$) into an organic phase where methylation takes place.

$$HFU^- + Q^+ \rightleftharpoons HFU^-Q_{org}^+ \qquad \text{extraction step} \qquad (1)$$

$$HFU^-Q_{org}^+ + CH_3I \longrightarrow CH_3HFU_{org} + QI_{org} \qquad \text{alkylation step} \qquad (2)$$

The first step was discussed in detail in a previous paper[11]. Extraction of 5-FU as an ion pair with TPeA$^+$, yielding a high recovery, is possible only by using a cellulose column. In this instance the dichloromethane eluate (HFU$^-$TPeA$^+$) should be used instead of the aqueous solution of 5-FU. From eqn. 2 the formation of the mono-methyl derivative seems obvious. However, spectrometric analysis proved the formation of the dimethyl derivative. This is explained by assuming a pK_a value for CH$_3$HFU that is lower than the pK_{a_2} value of the underivatized base. Under these circumstances the monomethyl derivative should be extractable as an ion pair with TPeA$^+$ and further methylated to the dimethyl derivative. To prove this hypothesis, the monomethyl derivative should be synthesized, and its pK_a value determined, a task which appears to be difficult.

Fig. 2 shows a gas chromatogram obtained after six repetitive injections of the dimethyl derivative of 5-FU synthesized according to the extractive methylation procedure. The large tailing solvent peak and fluctuations of the baseline are due to thermodegradation of tetraalkylammonium iodide formed during the derivatization reaction[15]. As shown in Fig. 3, this effect is successfully eliminated by extracting the organic phase with a saturated solution of silver sulphate in 0.1 M sulphuric acid. The sulphate is the preferred anion because of the low extraction constant of sulphate ion pairs[16]. For the THeA$^+$ reaction, however, the sulphate ion pair possesses still too high an extraction constant owing to the more lipophilic character of the hexyl in comparison with the pentyl chain. Therefore, under these circumstances a successful

TABLE I

IONS FORMED BY MASS SPECTROMETRIC FRAGMENTATION OF 5-FU, 5-ClU AND SOME OF THEIR ALKYL DERIVATIVES

Substituent	5-FU		5-ClU		N,N'-Dimethyl-5-FU		N,N'-Dimethyl-5-ClU		N,N'-Dibutyl-5-FU		N,N'-Dibutyl-5-ClU		N,N'-Dihexyl-5-FU		N,N'-Dihexyl-5-ClU	
X	F		Cl		F		Cl		F		Cl		F		Cl	
R_1	H		H		CH_3		CH_3		C_4H_9		C_4H_9		C_6H_{13}		C_6H_{13}	
R_2	H		H		CH_3		CH_3		C_4H_9		C_4H_9		C_6H_{13}		C_6H_{13}	
Fragment ion [*]	m/z	I(%) [**]	m/z	I(%) [**]	m/z	I(%) [**]	m/z	I(%) [**]	m/z	I(%) [**]	m/z	I(%) [**]	m/z	I(%) [**]	m/z	I(%) [**]
$M^{+\cdot}$	130	100.0	146	88.4	158	78.1	174	70.0	242	20.4	258	6.5	298	7.1	314	9.2
I	87	48.0	103	100.0	101	31.8	117	62.1	143	14.4	159	17.5	171	9.3	187	7.8
I + H	88	5.7	104	10.8	102	3.9	118	7.1	144	80.0	160	57.0	172	7.1	188	4.1
II	59	10.0	75	6.6	73	41.9	89	45.0	115	7.0	131	6.0	143	7.0	159	4.3
VI					59	0.8	75	1.4	59	6.2	75	2.5	59	3.6	75	2.1
III	86	2.6	102	1.6	100	5.3	116	4.3	142	2.5	158	2.5	170	1.8	186	1.1
IV	58	6.5	74	10.8	72	15.3	88	3.6	114	51.0	130	26.0	142	1.4	158	1.2
V	28	93.0	28	116.3	42	100.0	42	100.0	84	6.0	84	7.0	112	2.8	112	2.3
VII	60	46.0	76	82.5	74	3.6	90	3.6	116	5.3	132	8.7	144	46.4	160	43.9

[*] Roman figures refer to the fragment ions presented in Fig. 1.

[**] I = relative intensity of fragment ions (70 eV).

retro Diels Alder

$R_2-N=C=O$

I

− CO

− ·H

− X−C=C=O

− HCN

$R_i-N=CH$

V

II

III

$[X-C=C-O-R_i]$

VII
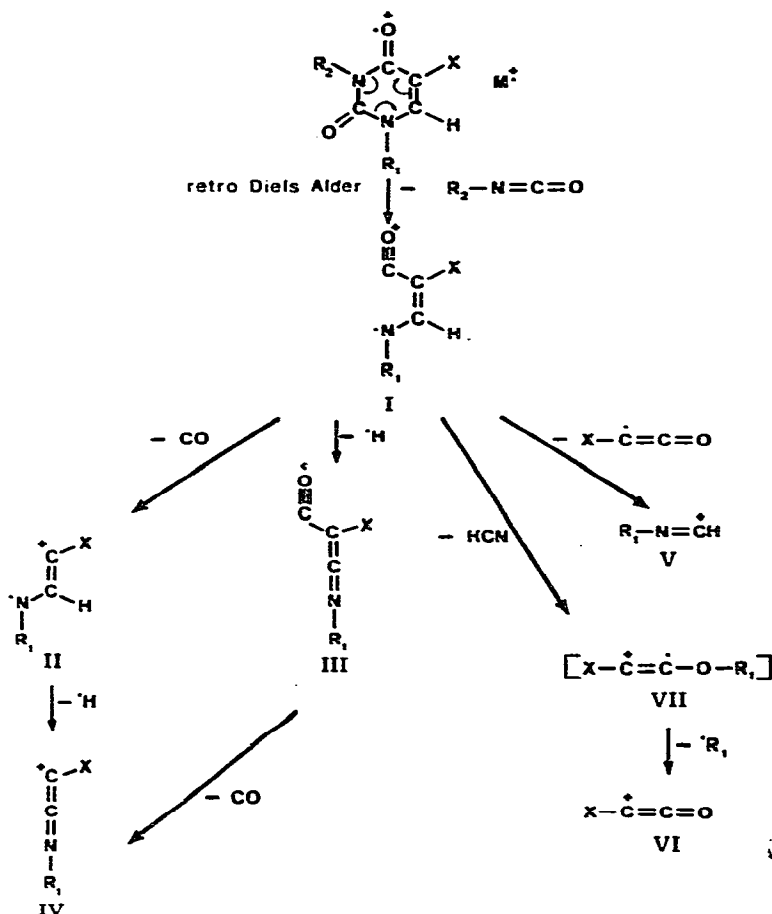
− CO

− ·R_i

$X-C=C=O$

VI

IV

Fig. 1. Proposed mass fragmentation pattern of 5-FU, 5-ClU and some of their alkyl derivatives.

clean-up can only be performed by an additional back-extraction step with diethyl ether.

To optimize the reactions conditions quantitative gas chromatography is performed using n-C_{24} as the internal standard. The effects of the amount of methyl iodide and the reaction time are illustrated in Figs. 4 and 5, respectively. From these experiments we conclude that extractive methylation of 5-FU in the range from 0 to 100 μg yielding optimal recovery is achieved by using 5 μl of methyl iodide and a reaction time of 1 h.

Wet alkylations

Methylation

Reaction conditions were studied using N,N'-dimethyl-5-ClU as the internal standard. As we always used the same batch of methylsulphinyl carbanion reagent, we investigated the influence of the amount of methylsulphinyl carbanion by diluting the stock reagent (Fig. 6). Tubes in which the methylsulphinyl carbanion or methyl iodide reaction time was varied yielded the same recovery for 5-FU. It also was found that the amount of methyl iodide is not critical provided that a large excess is
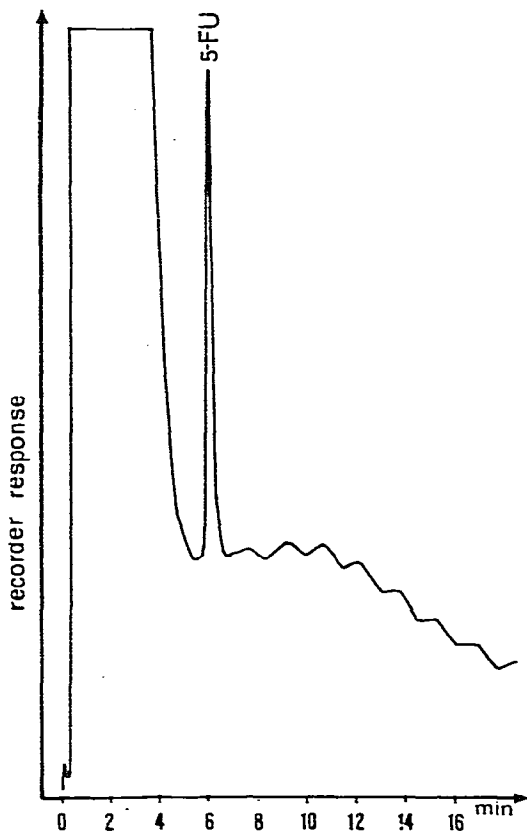
Fig. 2. GLC (FID) of the dimethyl derivative of 0.5 μg of 5-FU prepared by extractive methylation obtained after six repetitive injections of the reaction mixture (2% SP-2250; oven temperature, 135°; linear velocity of nitrogen, 7 cm/sec).
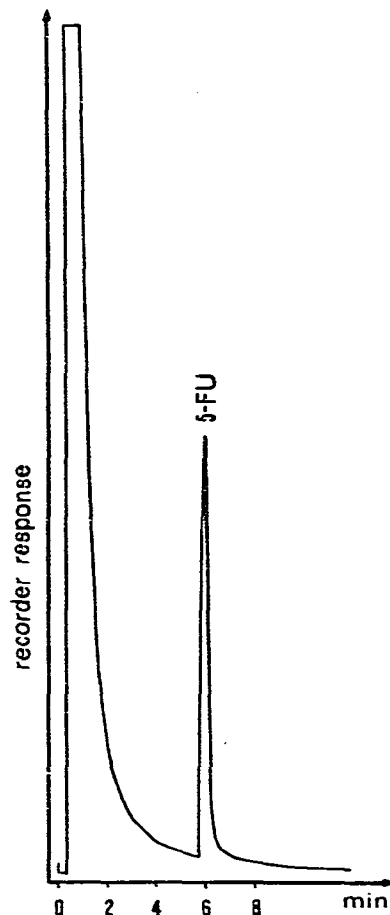
Fig. 3. GLC (FID) of the dimethyl derivative of 0.5 μg of 5-FU prepared by extractive methylation after clean-up with silver sulphate (2% SP-2250; oven temperature, 135°; linear velocity of nitrogen, 7 cm/sec).

used. Moreover, the latter is easily removed by evaporation and does not give rise to interferences in the gas chromatographic process itself.

In summary, the recovery was shown to be optimal with a 1:4 dilution of the methylsulphinyl carbanion (proton abstractor) stock reagent, and the chemical reactions occurred almost immediately.

Butylation

In this method, the acidic compound 5-FU was converted into a soluble salt (ion pair) with the organic base TMeAOH. The reaction was carried out in methanolic DMA, a highly polar solvent system. The salt thus formed reacted further with an excess of n-butyl iodide, yielding the corresponding dibutyl derivative. TMeAI precipitated slowly as a side-reaction product. Both 5-FU and 5-ClU were butylated and
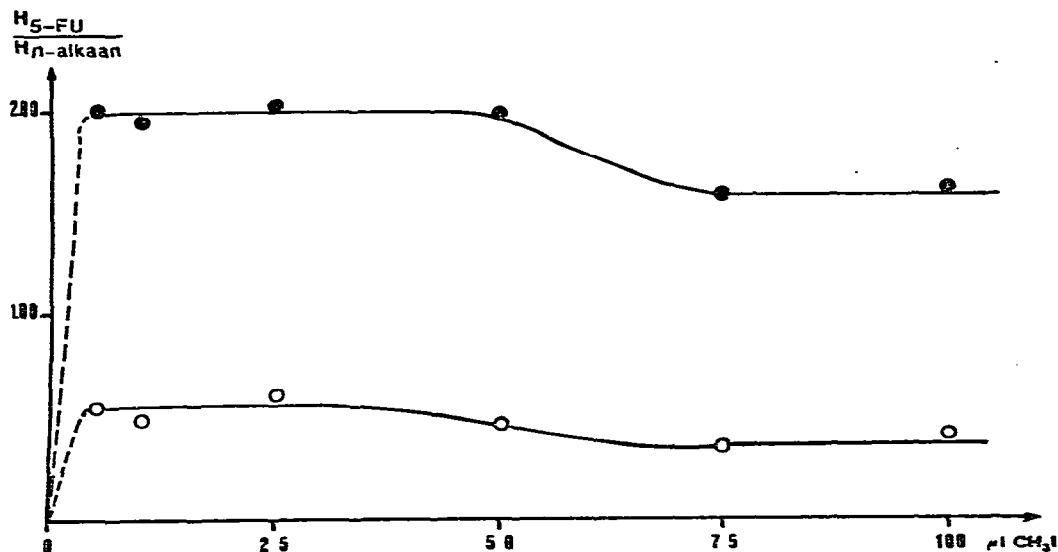
$$\frac{H_{5-FU}}{H_{n-alkaan}}$$

Fig. 4. Influence of the amount of methyl iodide using the extractive methylation procedure (5% XE-60; oven temperature, 165°; linear velocity of nitrogen, 7 cm/sec). ●, 100 μg of 5-FU, TPeA⁺ as counter ion; ○, 25 μg of 5-FU, THeA⁺ as counter ion.

a longer period for the precipitation of TMeAI was observed for 5-ClU. The reaction conditions were optimized for 5-ClU, and N,N'-dibutyl-5-FU was used as the internal standard. From these experiments, we conclude that under the circumstances outlined a reaction time of about 10 min is satisfactory (Fig. 7).

Flash hexylation
The hexylation reaction occurring in the injection port of the gas chromato-
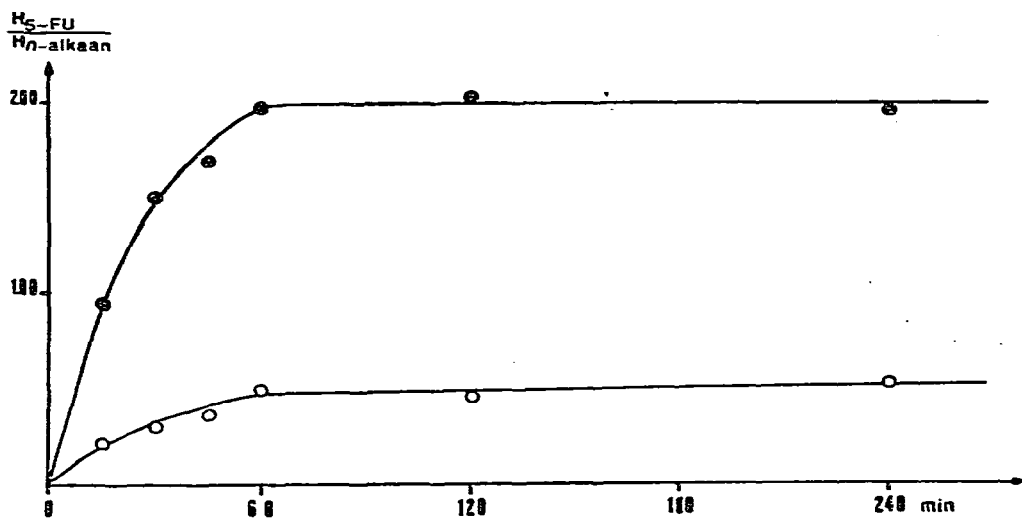
$$\frac{H_{5-FU}}{H_{n-alkaan}}$$

Fig. 5. Influence of the reaction time using the extractive methylation procedure (5% XE-60; oven temperature, 165°; linear velocity of nitrogen, 7 cm/sec). ●, 100 μg of 5-FU, TPeA⁺ as counter ion; ○, 25 μg of 5-FU, THeA⁺ as counter ion.
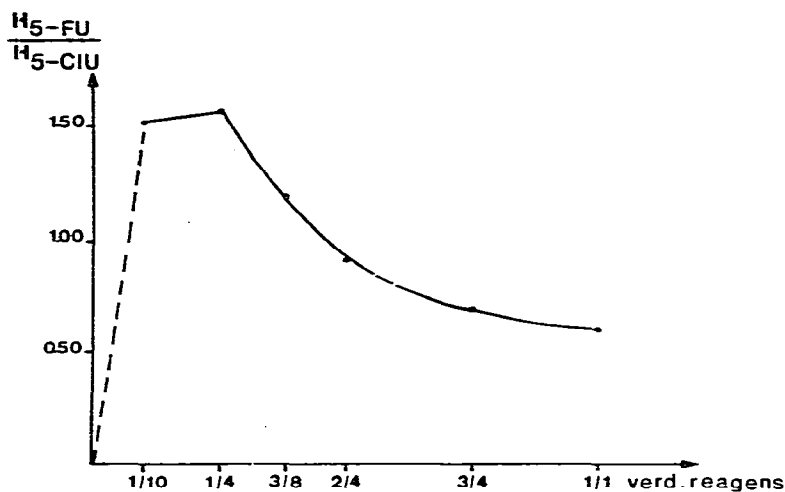
$$\frac{H_{5-FU}}{H_{5-ClU}}$$

Fig. 6. Influence of the amount of methylsulphinyl carbanion on the N-permethylation reaction (5% XE-60; oven temperature, 165°; linear velocity of nitrogen, 7 cm/sec).

graph is based on the Hofmann degradation[17]. A typical gas chromatogram is shown in Fig. 8. We originally thought that peaks 1 and 1' correspond to the monohexyl derivatives of 5-FU and 5-ClU, respectively. However, mass spectrometry did not confirm this assumption. Some fragment ions characteristic of N-alkylamines were found but no further identification could be achieved.

Reproducibility of the hexylation reaction was demonstrated by repeated injections of 5-FU and 5-ClU (internal standard), into the gas chromatograph under the conditions described. Measurement of peak-height ratios of 5-FU to 5-ClU afforded
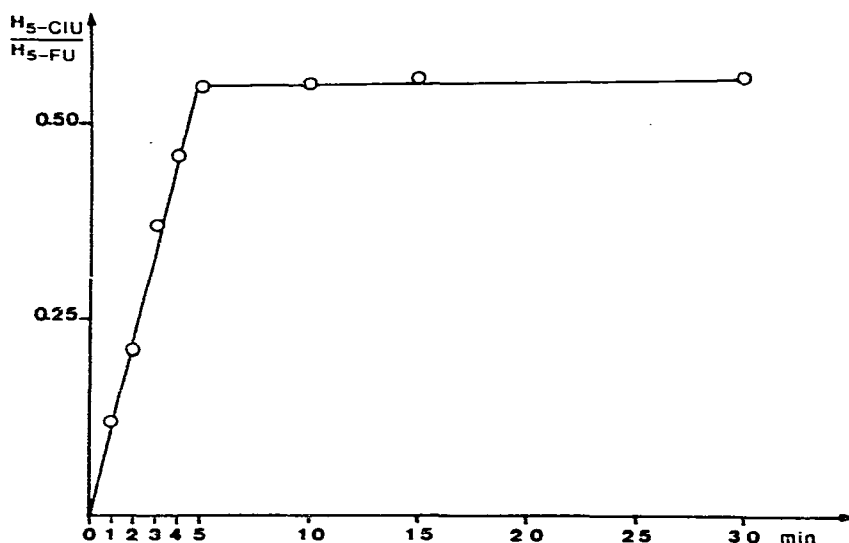
$$\frac{H_{5-ClU}}{H_{5-FU}}$$

Fig. 7. Influence of the reaction time on the butylation reaction (3% FFAP; oven temperature, 200°; linear velocity of nitrogen, 7 cm/sec).
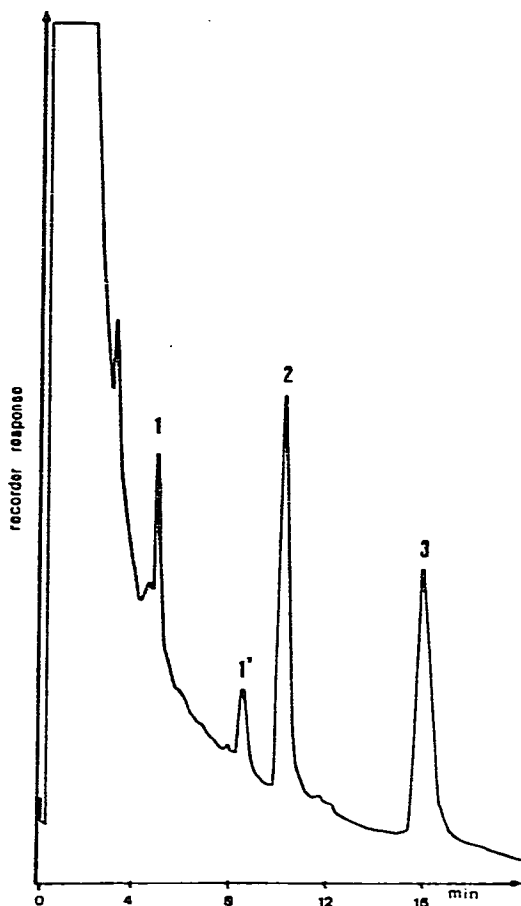
Fig. 8. GLC (FID) of the hexyl derivative of 5-FU and 5-ClU (5% XE-60; oven temperature, 210°; linear velocity of nitrogen, 7 cm/sec). 1 and 1′, N-alkylamines; 2, 0.4 µg of derivatized 5-FU; 3, 0.6 µg of derivatized 5-ClU.

a coefficient of variation (CV) of 3.97% ($n = 10$, 160 ng of 5-FU and 200 ng of 5-ClU injected).

Gas–liquid chromatography

All derivatives were tested for gas chromatographic behaviour on different stationary liquid phases, as reported in Table II. The best results in terms of a small theoretical plate height and peak symmetry were obtained on the 2% or 3% SP-2250 and 5% XE-60 column systems. For the dibutyl derivatives 5% OV-1 also gave satisfactory results.

For quantitative purposes it was obvious to choose a uracil derivative as an internal standard. In view of their possible application, dimethyl derivatives of 5-chloroucacil (5-ClU), 5-bromouracil (5-BrU) and 5-iodouracil (5-IU) were synthesized and chromatographed on a 3% OV-17 column (1.8 m × 2 mm I.D.; oven temperature, 145°; linear velocity of nitrogen carrier gas, 7 cm/sec). These compounds had retention times of 15.0, 23.8 and 35.6 min, respectively, compared with 6.3 min

TABLE II

GAS-LIQUID CHROMATOGRAPHIC PROPERTIES OF SOME ALKYL DERIVATIVES OF 5-FU AND 5-ClU

t_R = retention time; r = relative retention time (t_R of 5-FU/t_R of 5-ClU; H = column plate height; asym. = peak asymmetry factor.

Liquid phase	Gas-Chrom Q: mesh size	Column length (m)	Oven temperature (°C)	N,N'-Dimethyl-5-FU			N,N'-Dibutyl-5-FU			N,N'-Dibutyl-5-ClU			N,N'-Dihexyl-5-ClU			N,N'-dihexyl-5-FU		
				t_R (min)	H (mm)	Asym.	t_R (min)	H (mm)	Asym.	r	H (mm)	Asym.	t_R (min)	H (mm)	Asym.	r	H (mm)	Asym.
3% OV-1	80–100	1.8	150	1.64	1.44	1.09	—	—	—	—	—	—	—	—	—	—	—	—
5% OV-1	100–120	2.3	200	—	—	—	12.80	0.48	1.09	0.54	0.53	1.05	—	—	—	—	—	—
2% SP-2250	80–100	1.8	135	6.01	0.43	0.97	—	—	—	—	—	—	—	—	—	—	—	—
3% SP-2250	100–120	2.3	210	—	—	—	—	—	—	—	—	—	12.13	0.53	1.09	0.55	0.62	1.00
3% OV-17	80–100	1.8	140	8.00	0.45	0.94	—	—	—	—	—	—	—	—	—	—	—	—
	80–100	1.8	205	—	—	—	—	—	—	—	—	—	17.50	0.56	1.16	0.56	0.64	1.09
3% OV-25	100–120	2.3	150	6.27	0.93	1.09	—	—	—	—	—	—	—	—	—	—	—	—
2% QF-1	100–120	2.3	135	10.29	0.55	1.13	—	—	—	—	—	—	—	—	—	—	—	—
3% QF-1	80–100	1.8	140	9.92	0.59	1.15	—	—	—	—	—	—	—	—	—	—	—	—
			210	—	—	—	—	—	—	—	—	—	5.39	0.88	1.14	0.77	1.04	1.07
3% XE-60	80–100	1.8	165	5.40	0.97	1.24	—	—	—	—	—	—	—	—	—	—	—	—
5% XE-60	100–120	1.8	165	8.90	0.47	1.07	—	—	—	—	—	—	—	—	—	—	—	—
			210	—	—	—	—	—	—	—	—	—	15.70	0.46	1.06	0.63	0.53	0.97
3% Dexsil-300	80–100	1.8	180	—	—	—	13.13	0.47	1.15	0.58	0.56	1.09	—	—	—	—	—	—
			230	—	—	—	—	—	—	—	—	—	14.45	0.78	1.17	0.61	0.79	1.26
3% FFAP	100–120	1.8	210	—	—	—	10.54	0.45	1.71	0.51	0.51	1.45	—	—	—	—	—	—
3% FFAP	100–120	2.3	200	—	—	—	17.80	0.70	1.23	0.63	0.82	1.17	—	—	—	—	—	—
1% FFAP	80–100	1.8	180	—	—	—	8.67	1.60	2.20	0.65	1.21	0.53	—	—	—	—	—	—

for N,N'-dimethyl-5-FU as a reference. From these results it was apparent that only the chlorine-substituted substance 5-ClU is acceptable as the others (for reasons of too drastic an increase in molecular weight) possess too long retention times. Crystallized N,N'-dimethyl-5-ClU, N,N'-dibutyl-5-ClU and 5-ClU were used as internal standards to examine the linearity of the methylation, butylation and hexylation derivatization reactions, respectively. We always found a linear relationship between the peak-height or peak-area ratios of 5-FU to 5-ClU *versus* the amount of 5-FU submitted to the alkylation. As an example, Fig. 9 shows the linearization for the butyl derivatives covering the range 0–10 μg of 5-FU.
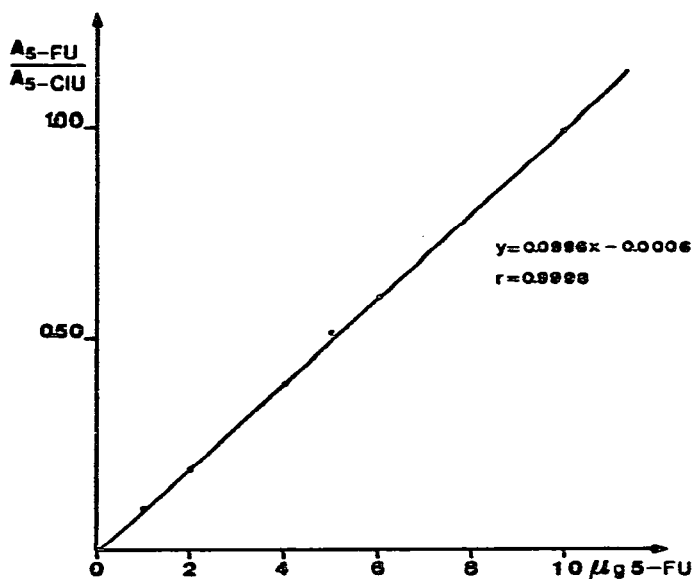

Fig. 9. Linearization of the butylation reaction in the range from 0 to 10 μg of 5-FU (5% OV-1; oven temperature, 200°; linear velocity of nitrogen, 7.5 cm/sec).

Biological applicability

Direct application of ion-pair extraction or extractive methylation to plasma samples in combination with GLC seemed impossible because of the interfering effect of the tetraalkylammonium iodide formed during the derivatization reaction[11]. Fig. 10 shows a gas chromatogram from a methylated plasma extract using the methylsulphinyl carbanion reagent and methyl iodide. From this we conclude that the methyl derivative of 5-FU is unsuitable for the quantitative analysis of plasma samples as many co-extracted compounds co-chromatograph with N,N'-dimethyl-5-FU. However, this problem was solved by increasing the retention of 5-FU by lengthening the alkyl chain in its derivative. Thus, hexylation of 5-FU and 5-ClU gave rise to compounds whose retention volumes are favourable for quantitative analysis of 5-FU in biological samples[18]. On the other hand, the reagent used for the flash hexylation was found to be extremely deleterious to the gas chromatographic column. In fact, this technique tends to strip the liquid phase from the solid support, whereas the high recommended injection block temperature of 270° causes

rapid deterioration of the column packing near the injection port. When operated daily, the latter needs to be replaced after about 1 month. In addition, as the hexyl derivatives are formed in the injection port of the gas chromatograph, preliminary purification by solvent extraction is impossible.
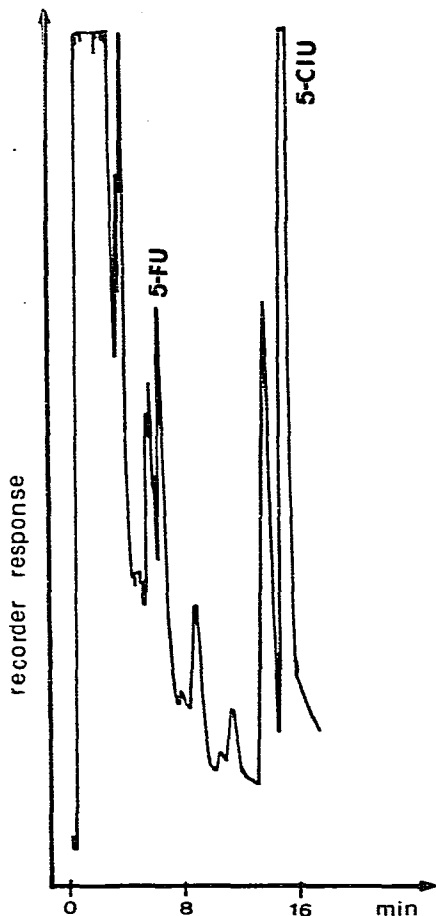

Fig. 10. GLC (FID) of a methylated extract of 1.0 ml of plasma spiked with 4 μg of 5-FU (3% OV-25; oven temperature, 150°; linear velocity of nitrogen, 7 cm/sec).

Another derivatization procedure for the sensitive detection of 5-FU in plasma samples[10] is compulsory. Derivatization with TMeAOH and n-butyl iodide as outlined above is more successful as the destructive quaternary ammonium salt (TMeAI) is removed by precipitation during the derivatization itself. The derivative can be purified further before chromatographic analysis by an additional clean-up with diethyl ether.

Mass fragmentography based on the detection of the dibutyl derivative of 5-FU allows quantitation of nanogram amounts of 5-FU in plasma[10].

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