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GAS CHROMATOGRAPHIC DETERMINATION OF 5-ALKYL-URACILS AND 5-ALKYL-DEOXYURIDINES USING FLASH METHYLATION AND GLASS CAPILLARY COLUMNS

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SUMMARY

A sensitive analytical method has been developed for 5-alkyl-substituted deoxyuridines and their main metabolites, the corresponding uracils in body fluids. The compound to be determined is methylated by the "flash" methylation technique, *i.e.*, by injecting a mixture of the compounds and trimethylanilinium hydroxide (TMAH) into the hot injector of a gas chromatograph, followed by separation of the derivatives with a suitably deactivated glass capillary column. The optimal conditions for methylation were found by studying the effect of injector temperature, residence time and TMAH/compound molar ratio on the yield of the reaction. The optimal residence time of the sample in the injector could be set by a "semi-splitless" injection method.

INTRODUCTION

The anti-neoplastic and antiviral activity of some purine and pyrimidine nucleotide bases and the corresponding nucleosides is well known. Among the most important of these agents are the 5-substituted deoxyuridines, and the effects of various 5-alkyl-substituted deoxyuridines (5-alkyl-dU) were recently demonstrated[1].

The availability of a suitable analytical method for the title compounds in various body fluids would be of great assistance in expanding knowledge of their pharmacological activity and metabolism. The most advanced methods in the analysis of nucleotide bases and nucleosides are based on gas–liquid chromatography (GLC)[2–8]. Other methods (*e.g.*, spectrophotometry, fluorimetry) lack the sensitivity and selectivity of GLC.

The highly polar nature of both the purine and pyrimidine bases and of the sugar portion of nucleosides means that, for their chromatography, the formation of thermally stable and more volatile derivatives is obligatory. Several methods are available for derivatization of these compounds. Trimethylsilylation[5–8], a generally applicable method, has been successfully used prior to GC analysis, but the trimethylsilyl derivatives are of limited stability and very sensitive to traces of moisture[2]. This makes the reaction difficult to carry out. Methyl derivatives of purine and pyrim-

idine bases and related compounds can be prepared in several ways of which the most important are: (i) "permethylation" by methyl iodide with methylsulphinyl carbonion as catalyst, the latter being generated using sodium hydride[3] or potassium *tert.*-butoxide[4] and dimethyl sulphoxide; (ii) "flash" methylation[2,9]. While both methods in (i) involve moisture-sensitive chemicals and a multi step procedure, "flash" methylation seemed much simpler and has been found useful in the analysis of fatty acids and barbiturates[10].

The "flash" methylation method is based on the fact that quaternary ammonium ions form a salt or salt-like adduct with several classes of compounds in an instantaneous reaction on mixing the reagents at room temperature. By injecting the reaction mixture into the hot injection port of a gas chromatograph, the adduct thermally decomposes producing the methylated derivative and a tertiary amine. This technique, because of its simplicity and the promising results of previous works[2,9], seemed to be the most suitable for the present compounds. As for the reagent, we chose the quaternary ammonium base trimethylanilinium hydroxide (TMAH) because of the good GLC characteristics of its by-product, dimethylaniline, in contrast with that of the previously favoured tetramethylammonium hydroxide[10].

The conventional tool for GLC separations of mixture of compounds is the packed column. The efficiency of such separations can be considerably increased by the use of a capillary (open tubular) column. This improvement in performance (which can be as large as two orders of magnitude expressed in numbers of theoretical plates) helps the analyst in several indirect ways.

(i) The large number of impurity compounds in the biosample create less difficulties. A purification step is unavoidable, but it can be simpler than if the separation were carried out by a packed column.

(ii) The phase ratio (β-value) of a capillary column is *ca.* 10–50 times higher than that of a packed column; *i.e.*, the former contains much less stationary liquid per unit volume. This has the consequence that the retention times of the separated compounds can be made considerably shorter, or the same retention can be achieved at a lower temperature; both of these possibilities decrease the danger of thermal degradation during analysis.

(iii) The peaks emerging from a capillary column are narrower and therefore higher than in the case of a packed column; this lowers the detection limit of the compounds analyzed.

(iv) The splitting injection technique usually associated with capillary columns provides unique possibilities in "flash" methylation derivatization (see below).

Although the advantages of capillary columns are well known in GLC, the use of such columns in the analysis of thermally sensitive polar compounds has been restricted by the catalytic and adsorptive activity of the column wall, either metal or glass. The rapid development of glass capillary column technology[11] in recent years indicated that such columns might be used in GLC of the title compounds. The aim of this work was to study the "flash" methylation reaction of 5-alkyl-dUs and their main metabolites the corresponding uracils (5-alkyl-Us) and to find the optimal conditions for the reaction and for their GLC analysis.

EXPERIMENTAL

Model compounds and reagents

The model compounds used are listed in Table I. They were synthesized* according to Szabolcs *et al.*[1]. No further purification was made. A stock solution of each compound was prepared with a concentration of 1 mg/ml in methanol. In some cases, dissolution had to be promoted by treatment in an ultrasonic bath. Methyl stearate (1 mg/ml) (E. Merck, Darmstadt, G.F.R.) was used as internal standard.

TABLE I

5-ALKYL-URACILS AND 5-ALKYL-DEOXYURIDINES USED AS MODEL COMPOUNDS

	Alkyl substituent	*Symbol*
Uracils	Ethyl	EtU
	n-Propyl	n-PrU
	Isopropyl	i-PrU
	Butyl	BuU
	Pentyl	PeU
	Hexyl	HeU
2'-Deoxyuridines	Ethyl	EtdU
	Isopropyl	i-PrdU
	Butyl	BudU
	Hexyl	HedU

For the "flash" methylation reaction, TMAH was used. Two batches of reagent were applied: (i) a 0.2 *M* solution in methanol ("Methelute"; Pierce, Rotterdam, The Netherlands); (ii) a 0.1 *M* methanolic solution made by ourselves according to the method of Brockmann-Hanssen and Olawuyi-Oke[10].

Derivatization

Equal amounts (usually 10 μl) of one or more of the stock solutions of the uracils, uridines and internal standard were placed into a small conical vessel, and a calculated amount of TMAH was added. After mixing, 1 μl was injected into the gas chromatograph. In all steps of TMAH preparation and sample manipulation, care should be taken to avoid any contamination by detergents and/or lipids, which produce artifact peaks on the chromatograms due to fatty acid methyl esters.

Gas chromatography

All the analyses were performed on a Perkin-Elmer Type F 22 gas chromatograph. The instrument was equipped with a flame ionization detector (FID) and a splitter type injection port.

Since the retention times and retention temperatures of the compounds should be kept as low as possible, a short column with a non-polar stationary phase was applied. A 10 m $\times$ 0.23 mm I.D. column was used, coated with the methylsilicone

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OV-101. The column was prepared by the method previously described for nonpolar silicones[12]. For deactivation, a non-extractable layer of polyethylene glycol was applied to the column wall[13], after a thorough rinsing with HCl solution[14,15]; this method has been found to be the best among many others[16]. Coating was made by the static method; the phase ratio was set to 600.

RESULTS AND DISCUSSION

Sample introduction: a "semi-splitless" method

Sample introduction has always been a critical operation in capillary column GLC. The difficulties are created by the large internal volume of the injector (*i.e.*, its insert where the evaporation of the sample takes place) compared to the volumetric flow-rate through the column. If the sample vapour left the injector through the column only, the sampling would take much longer than the time needed to get the desired sharp peaks at the column outlet. To overcome this problem (while maintaining the conventional injector configuration) some combination of the following two principles is generally used[11].

(i) A second flow of the carrier gas is drawn from the injector to the atmosphere through an adjustable valve ("splitter"). This second flow can be set to a much higher value than the column flow, therefore the injection port can be cleared of sample (solvent and compounds) in as short a time as desired. A consequence of the technique is that a large portion of the sample will leave the injector through the splitter valve instead of entering the column; the splitter device has long been used to get rid of the excess quantities of sample.

(ii) During injection, the temperature of the column is lowered considerably below the analysis temperature. In this way the first short section of the column will act as a cold trap and the sample vapour will condense there forming a sharp plug despite the prolonged injection period ("splitless injection"[17]). Even so, a ventilation operation (*e.g.*, through a splitter valve) is required to remove the last traces of the solvent from the injector, otherwise severe tailing of the solvent peak may occur. This preconcentration type of sampling has the advantage of full utilization of the sample.

In this work the problems of injection and methylation interfere. In the "flash" methylation procedure the derivatization reaction is carried out in the hot injector. The sample is pyrolyzed while in the hot zone of the insert. Therefore the residence time of the sample in the injector has to be varied independently of other circumstances. This is achieved by the combination of the two sampling techniques mentioned above.

The carrier gas was supplied by a pressure regulator, which maintained a constant column head pressure, even when the flow through the splitter valve changed. Thus the residence time of the sample could be adjusted by setting the split flow.

The sampling procedure was as follows.

(a) With the help of the splitter valve, the split flow was adjusted to get the required residence time in the injector. Knowing the internal volume of the glass injector insert, the head pressure and the flow-rate of the column, this time could easily be calculated.

(b) The column was cooled to room temperature.

(c) The sample was injected.

(d) After a certain period (usually 30 sec, longer than any of the intended residence times), the split valve was opened to get a high split flow. In this way the last traces of the solvent were removed, and a solvent peak free from tailing was produced.

(e) The oven was heated rapidly to 100°, and after the solvent had left the column a temperature program of 8°/min was started, up to 250°.

This injection method, which can be called "semi-splitless", is useful when the residence time of the sample in the injector needs to be adjustable. It should be emphasized that only the capillary column, with its high flow resistance and simultaneously low flow-rate, makes possible such an injection–derivatization technique.

Effect of reaction temperature and residence time on the yield of the methylation reaction

In previous studies the pyrolytic decomposition of the TMAH adduct producing the methylated derivative and dimethylaniline has been considered instantaneous, or at least no attention has been paid to whether the usual residence time of the sample in the hot injector was long enough to complete the pyrolysis.

As discussed above, the application of a splitter-type injector together with the capillary columns results in a flexible injection procedure which is more or less independent of the column variables. This means that the glass insert of the injector can be considered as a tube reactor. Its parameters (temperature and gas velocity) can then be varied to find their optimum range.

i-PrdU was chosen as model compound for this study: the results are given in Table II and Fig. 1. An uridine was considered as the best model for studying the residence time effect, because it was found in our preliminary experiments that uridines (in contrast to the corresponding uracils) require a longer reaction (residence) time in the hot injection port to complete the methylation reaction. In a conventional injection operation, high apparent losses occur with the uridines.

TABLE II

YIELD OF METHYLATION REACTION OF ISOPROPYL-DEOXYURIDINE AS A FUNCTION OF TEMPERATURE AND HEATING TIME

Molar ratio TMAH/i-PrdU was 15. Column flow, 1.0 cm³/min; internal volume of the injector, 0.85 cm³.

Split flow (cm³/min)	*Residence (heating) time (sec)*	*Yield (%)* at*			
		220°	*240°*	*260°*	*280°*
50	2.0	44	52	53	53
23.8	4.1	52	68	64	63
13.4	7.1	58.5			
9.0	10.2		22	70	73.5
4.0	20.4	73.4	82	83	85
2.5	29.2	74.5	79	78	78

* $\text{Yield}\ (\%) = \frac{A_{\text{i-PrdU}}}{A_{\text{st}}} \cdot \frac{g_{\text{st}}}{g_{\text{i-PrdU}}} \cdot 100$

where A = peak area, g = injected amount and st = internal standard. The difference between FID responses of i-PrdU and the methyl stearate internal standard was neglected.

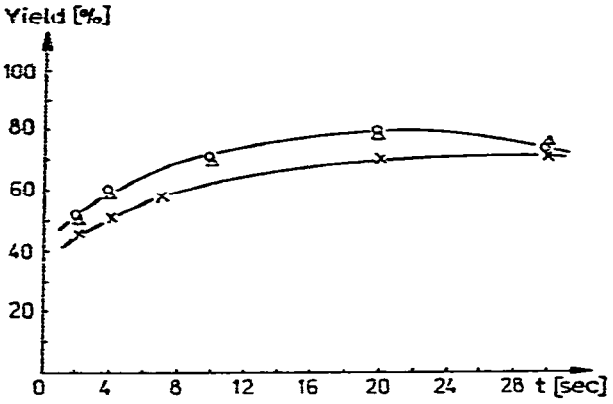

Fig. 1. Yield of methylation reaction of i-PrdU as a function of reaction time, temperature and residence time. Injector temperature: ×—×, 220°; △—△, 240°; ○—○, 280°.

The yields in Table II obviously contain an error since the difference between the FID response factors of the compound and the internal standard was neglected. However, an approximate calculation according to Halász and Schneider[18] permits an estimate of the relative FID response factors of i-PrdU compared to methyl stearate. The former compound contains higher numbers of heteroatoms O and N, both of which tend to decrease the specific FID response. However inaccurate the estimation, the relative response of uridine to methyl stearate should be considerably higher than unity. It may be concluded that the methylation reaction is complete and proceeds with an almost 100% yield under optimal conditions. The curves in Fig. 1 show that a flat maximum occurs around 20 sec; in the range 10–30 sec the yield is only slightly dependent on residence time.

No similar results have yet been reported; however, the fact that the pyrolysis reaction is not instantaneous agrees well with results of Osiewicz *et al.*[19] who suggested that a similar methylation mixture should be injected over a prolonged (10 sec or longer) period.

From Fig. 1 it is evident that if capillary column is used for separation, the conventional split ratios (1:50–1:500, corresponding to split flows of *ca.* 50–500 cm^3/min) cannot be used, because the residence time in the injector would be very

TABLE III

YIELD OF METHYLATION REACTION OF EtdU, BudU, i-PrdU AND HedU AS A FUNCTION OF TMAH/COMPOUND MOLAR RATIO

Reaction temperature, 260°; reaction time, 18 sec. Yield as defined in Table II.

TMAH/compound molar ratio	*Yield (%)*			
	EtdU	*BudU*	*i-PrdU*	*HedU*
3	52	80	70	60
6	66	85	79	67
9	68	86	82	69
15	68	83	84	71
30	62	72	72	62

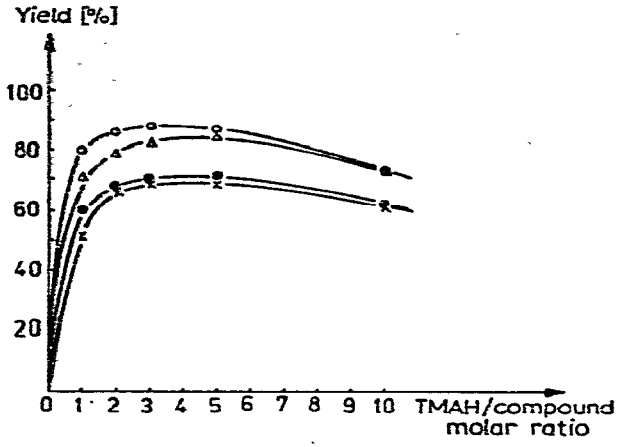

Fig. 2. Yield of methylation reaction of 5-alkyl-deoxyuridines as a function of TMAH/compound molar ratio. Compounds: ×—×, EtdU; △—△, i-PrdU; ○—○, BudU; ●—●, HedU.

short, *i.e.*, in the range 0.2–2 sec. When split injection is used with the aim of decreasing the amount of sample to be injected onto the column, the sample should be diluted rather than the split ratio increased.

The effect of reaction temperature is also shown in Fig. 1. There is no difference in yield between 240° and 280°, but a 10% loss is found at 220°. Temperatures higher than 280° are not recommended because of sample loss.

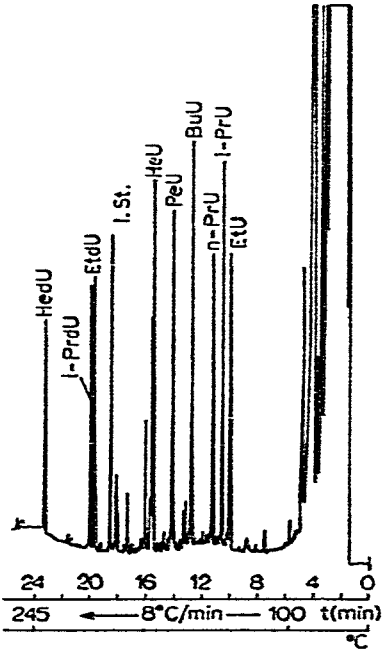

Fig. 3. Chromatogram of model compounds. I.st. = methyl stearate internal standard. Carrier gas: N_2. Split flow, 5 cm³/min. Injector temperature, 260°. Attenuation, 1 × 8. For other conditions, see text.

Effect of TMAH/substance ratio

This effect needed a more thorough study because of contradictions in the literature: suggested TMAH/substance molar ratio vary from 1.5[10] to 30,000[2]. Our results for four model substances are given in Table III and Fig. 2. Again a broad maximum was found: at molar ratios less than 6, the yield decreases rapidly; at ratios greater than 18, the decrease is not so sharp. The shape of the curves for the four model substances are quite similar, the differences being due to the FID responses and/or to impurities in the samples.

Gas chromatography

Fig. 3 shows the chromatogram of nine model compounds and the internal standard. The mixture contained roughly equal amounts of each substance; each peak corresponds to *ca.* 20 ng substance. All the peaks are sharp and free of tailing, which provides a good separation of the compounds from possible impurities in the biosample, mainly fatty acids, transesterified by the TMAH. Because of the good peak shapes, the lower detection limits of compounds are in the 1–2 ng range.

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