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SIMULTANEOUS DETERMINATION OF THYMINE AND 5-BROMOURACIL IN DNA HYDROLYSATES USING GAS CHROMATOGRAPHY—MASS SPECTROMETRY WITH SELECTED-ION MONITORING

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SUMMARY

The gas chromatographic—mass spectrometric method using selected-ion monitoring (GC—MS—SIM) described here quantitatively determines the amount of DNA thymine replacement by 5-bromouracil (BU) after exposure to 5-bromo-2'-deoxyuridine (BUDR) in as few as 10^5 cells DNA is extracted, enzymatically hydrolyzed, the nucleic acid bases (with added internal standards, 5-iodouracil and 5-chlorouracil) are extracted into ethyl acetate, concentrated and derivatized with bis(trimethylsilyl)trifluoroacetamide Thymine and BU are then quantitated by GC—MS—SIM Response is linear to thymine over the range of 100—2000 ng per sample and BU of 1 3—52 ng per sample with a coefficient of variation of less than 10% and an accuracy for seeded samples within 8% of theoretical value With V79 cells in culture, exposure to increasing BUDR concentrations (0 03– 1 0 μM) results in increasing thymine substitution by BU over a range of 1—28% Other important applications of this technique are mentioned

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INTRODUCTION

5-Bromo-2′-deoxyuridine (BUDR) is an analogue of thymidine and is incorporated solely into the DNA of replicating cells [1, 2]. As a result of this substitution, the effected cells become sensitized to toxic effects of ultraviolet [3] and X-irradiation [4]. This radiosensitization of cells induced by exposure to BUDR has been shown to be directly related to the amount of DNA thymine replaced by 5-bromouracil (BU), and with sufficient incorporation radiation sensitivity can be increased from two- to three-fold [4—6]

Quantitation of DNA thymine replacement by BU using high-performance liquid chromatography (HPLC) [7], $CsCl—Cs_2SO_4$ isopycnic gradients [3] and monoclonal antibody with fluorescence microscopy and fluorescence activated cell sorting techniques [8, 9] has been reported The HPLC method lacks sensitivity (requires 10^7 cells), the isopycnic gradient technique lacks both sensitivity and precision, and it has not been adequately demonstrated that the monoclonal antibody procedures give a quantitative measure of the replacement of DNA thymine by BU over a broad range of values [8, 9].

Presented here is a gas chromatographic—mass spectrometric method using selected-ion monitoring (GC—MS—SIM) which quantitatively determines the amount of DNA thymine replacement by BU in as few as 10^5 cells.

EXPERIMENTAL

Reagents

The reagents and suppliers were as follows Ethyl acetate and methanol (Burdick & Jackson Labs., Muskegon, MI, U S A), monobasic ammonium phosphate (J.T Baker, Phillipsburg, NJ, U S A.), ammonium sulfate (Mallinckrodt, St Louis, MO, U S.A); BUDR, thymine, 5-chlorouracil (CU), BU, 5-iodouracil (IU), DNAse I, phosphodiesterase, alkaline phosphatase, and thymidine phosphorylase (Sigma, St. Louis, MO, U S A.), bis(trimethylsilyl)-trifluoroacetamide (BSTFA) (Pierce, Rockford, IL, U S A).

Stock solutions

Thymine solution (0.1 mg/ml in methanol), BU solution (0.13 mg/ml in methanol), CU solution (0 1 mg/ml in methanol) and IU solution (0 1 mg/ml in methanol) were prepared and stored at −20°C. Saturated ammonium sulfate solution was prepared and stored at room temperature.

Standards

The BU stock solution was diluted 1.100 with water to yield the BU standard solution (1.3 µg/ml BU) The IU stock solution was diluted with water to yield the IU standard solution containing 50 µg/ml IU. The thymine and CU stock solutions were used directly as standard solutions.

Quality-control samples at three BU·thymine concentrations (2 6 200, 10 4:1000 and 20.8·1500 ng BU.thymine per 0.10 ml) were prepared by spiking water with the appropriate volumes of the BU and thymine standard solutions. After mixing, 0.1-ml aliquots were transferred to glass tubes, tightly capped and stored frozen at −30°C. Two quality-control samples of each concentration were included with every group of experimental samples to be analyzed.

Chromatographic conditions

A Hewlett-Packard 5987A gas chromatograph—mass spectrometer equipped with a 25 m × 0.32 mm I D , fused-silica, capillary column (cross-linked 5% phenylmethylsilicone, 0.52 μm film thickness) was used in these determinations. The HP 5880 gas chromatograph oven temperature was programmed to hold at 100°C for 2 min, then increased at 15°C/min to 300°C The gas chromatograph injection port temperature was 250°C, the gas chromatograph—mass spectrometer interface heaters were set to 275°C and the mass spectrometer ion source temperature was 200°C The mass spectrometer was run in the electron-impact (EI) mode, electron energy of 70 eV and a multiplier voltage of 2200 V SIM at the following *m/z* positive ions was used to detect and quantitate the thymine and BU levels thymine, *m/z* 271, CU, *m/z* 276, BU, *m/z* 319 and 321, and IU, *m/z* 367.

Sample preparation

For analysis of DNA thymine replacement by BU, cell pellets were lysed and DNA isolated using standard techniques After twice washing the DNA precipitate with 2 vols. of 95% ethanol and vacuum-drying the pellet, the DNA was dissolved in 10 m*M* Tris buffer (pH 7 4) and digested with DNAse I, phosphodiesterase, alkaline phosphatase and thymidine phosphorylase (total volume 0 1 ml). The hydrolysis was quenched with 3 vols of saturated ammonium sulfate, the precipitate centrifuged and the supernatant containing the hydrolysed base mixture assayed for its thymine and BU content

To glass tubes (15 ml capacity) were added 0.1 ml DNA hydrolysate, 0 3 ml saturated ammonium sulfate solution (if not already added to quench the enzymatic hydrolysis), 0 1 ml of 0 1 *M* ammonium phosphate buffer (pH 6 7), 0 010 ml CU internal standard solution (1000 ng CU), 0 010 ml IU internal standard solution (500 ng IU) and 4.0 ml ethyl acetate. The tubes were tightly capped and shaken for 15 min at room temperature. After centrifugation (room temperature) for 10 min at 1200 *g*, the ethyl acetate phase was transferred to clean glass tubes and the ethyl acetate extraction was repeated. The combined organic extracts were concentrated to approximately 0 5 ml by evaporation in a heated vacuum centrifuge and then transferred to 3 0 ml capacity conical-bottomed reaction vials. The final evaporation to dryness was aided by a stream of air and heating to 45°C To each sample were added 0.040 ml BSTFA and the vials were sealed and heated to 85°C for 1 h. Up to 3 μl of the reaction mixture were injected for GC—MS—SIM analysis

Standard calibration curve

Calibration samples (0.10 ml distilled water) were spiked in duplicate with the appropriate volumes of thymine and BU standard solutions to concentrations ranging from 0 to 2000 ng per 0.10 ml thymine and from 0 to 52 ng per 0.10 ml BU, and subjected to the sample preparation procedure described above. Calibration curve samples were run with each set of experimental samples.

Calculations

Calibration curves were constructed by plotting the ratio of the peak area of thymine and BU to that of their respective internal standards (CU and IU) as a

function of the sample thymine or BU content. The best-fit straight line was determined using the method of least squares. The thymine and BU concentrations of unknown samples were calculated from the least-squares regression line of the calibration curve

RESULTS AND DISCUSSION

Under the described conditions, the retention times of the bis(trimethylsilyl) (bis-TMS) derivatives of thymine, CU, BU and IU were 6.77, 7 34, 8 01 and 8.82 min, respectively Fig. 1 shows the total-ion chromatographic (TIC) profile of a mixture of nucleic acid bases and 5-halouracils derivatized with BSTFA. Complete resolution of all peaks has been achieved Fig. 2 depicts the mass spectra of the bis-TMS derivatives of thymine and its internal standard, CU. Fig 3 depicts the mass spectra of the bis-TMS derivatives of BU and its internal standard, IU. As shown in these figures, the EI mass spectra of the bis-TMS derivatives display the typical fragmentation pattern a small but significant molecular ion $(M)^+$ peak, the base peaks at $(M - 15)^+$ representing loss of a methyl group, and the characteristic TMS fragments at m/z 73 and 147 [10, 11].

To achieve maximum sensitivity in BU quantitation, we monitored in the SIM mode m/z at 319 and 321, the isotopic (and base) peaks of the $(M - 15)^+$ complex for BU-bis-TMS (M^+ = 336). The $(M + 1)^+$ ions of thymine (m/z 271)

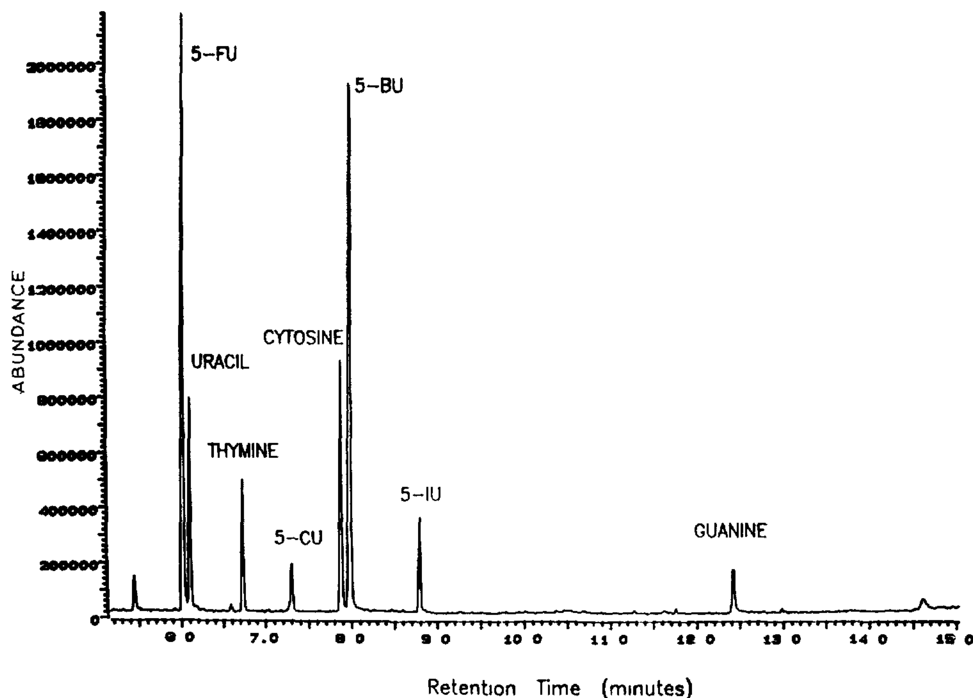

Fig 1. Total-ion chromatogram of a mixture of nucleic acid bases and 5-halouracils derivatized with BSTFA GC—MS conditions were as described in Experimental MS in scan mode, 70—500 (m/z), electron-impact ionization

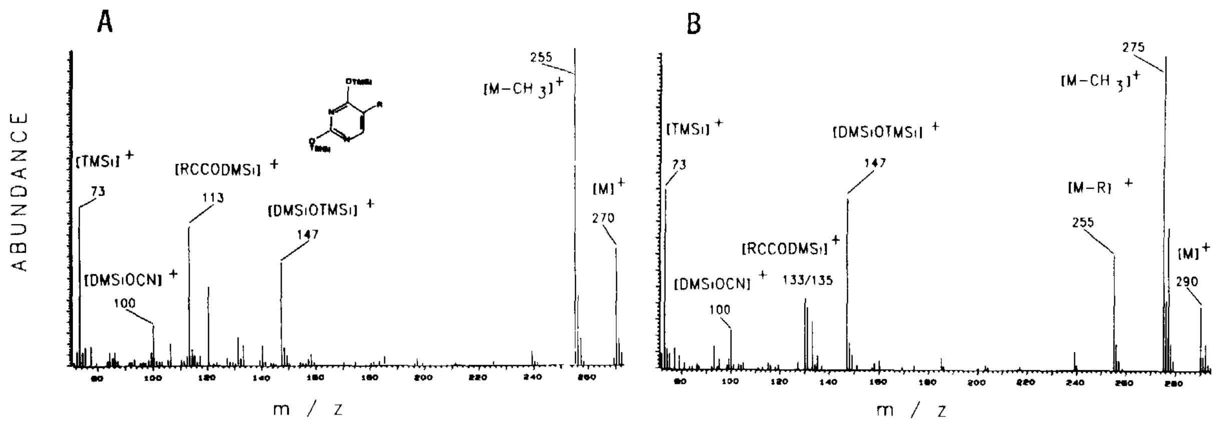

Fig. 2 Mass spectra of (A) thymine-bis-TMS (R = CH_3) and (B) its internal standard, 5- chlorouracil-bis-TMS (R = Cl)

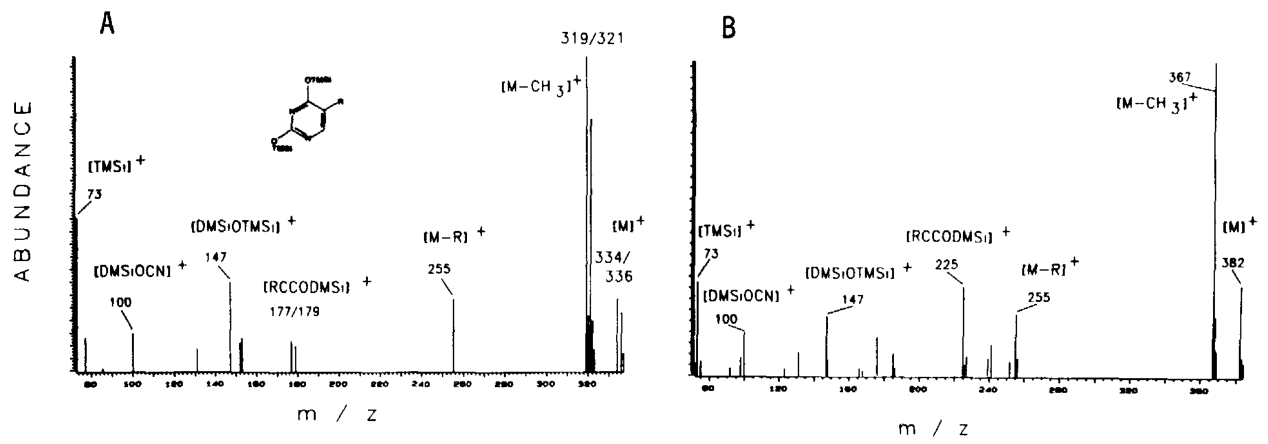

Fig 3 Mass spectra of (A) 5-bromouracil-bis-TMS (R = Br) and (B) its internal standard, 5-iodouracil-bis-TMS (R = I)

and its internal standard, CU, (m/z 276, see Fig. 2) were monitored to purposely decrease the thymine assay component peak areas to levels in the range of those elicited by BU and IU. This results in an increase in the upper linear range for thymine quantitation and allows the simultaneous determination of thymine and BU in a single injection.

The validity of the assay procedure was established through careful study of the linearity of response, reproducibility, accuracy and precision The peak-area ratios (thymine/CU and BU/IU) were directly proportional to the thymine and BU concentrations over a range of 100—2000 ng/ml and 1.3—52 ng/ml, respec-

TABLE I

THYMINE CONTENT OF SEEDED CONTROL SAMPLES (0 1 ml) ASSAYED DURING A THREE-DAY PERIOD

Day	Content found (ng)		
	200 ng Seeded	1000 ng Seeded	1500 ng Seeded
1	234 5	881 1	1673 4
	216 5	1052 7	1590 6
	236 5	1106 6	1535 7
2	206 4	1013 0	1496 2
	220 6	1013 0	1520 2
	222 8	955 1	1519 1
3	197 0	983 6	1552 5
	195 2	1013 4	1499 8
	204 0	920 4	1450 6
Mean ± S D	214 8 ± 15 2	993 2 ± 68 0	1537 6 ± 64 1
Coefficient of variation (%)	7 1	6 8	4 2
Difference from theoretical value (%)	+7 4	—0 7	+2 5

TABLE II

5-BROMOURACIL CONTENT OF SEEDED CONTROL SAMPLES (0 1 ml) ASSAYED DURING A THREE-DAY PERIOD

Day	Content found (ng)		
	2 6 ng Seeded	10 4 ng Seeded	20 8 ng Seeded
1	2 71	9 73	22 63
	2 85	12 54	19 64
	2 75	10 38	19 96
2	2 59	9 85	18 26
	2 56	9 12	19 13
	2 79	9 62	19 28
3	2 77	11 11	20 51
	2 77	11 13	20 24
	2 98	11 21	19 28
Mean ± S D	2 75 ± 0 13	10 52 ± 1 07	19 88 ± 1.23
Coefficient of variation (%)	4 6	10 2	6 2
Difference from theoretical value (%)	+5 8	+1 2	—4 4

tively. The best-fit lines were obtained using linear regression analyses. The results of the regression analyses for thymine and BU were $y = 0.000570x + 0.00283$ ($r = 0.9995$) and $y = 0.00377x + 0.000569$ ($r = 0.9977$), respectively.

The accuracy and precison of the method were assessed by seeding water at thymine and BU concentrations of 200, 1000 and 1500 ng per 0.10 ml thymine and 2.6, 10.4 and 20.8 ng per 0.10 ml BU. Triplicate quality-control samples at each concentration were assayed on each of three consecutive days. Tables I and II give the results of this experiment. The precision of the assay was found to have coefficients of variation (C.V.) ranging from 4.2 to 7.1% and from 4.6 to 10.2% for thymine and BU, respectively. The concentration means for the seeded control samples were found to be within −0.7 to 7.4% and −4.4 to 5.8% of the theoretical values for thymine and BU, respectively.

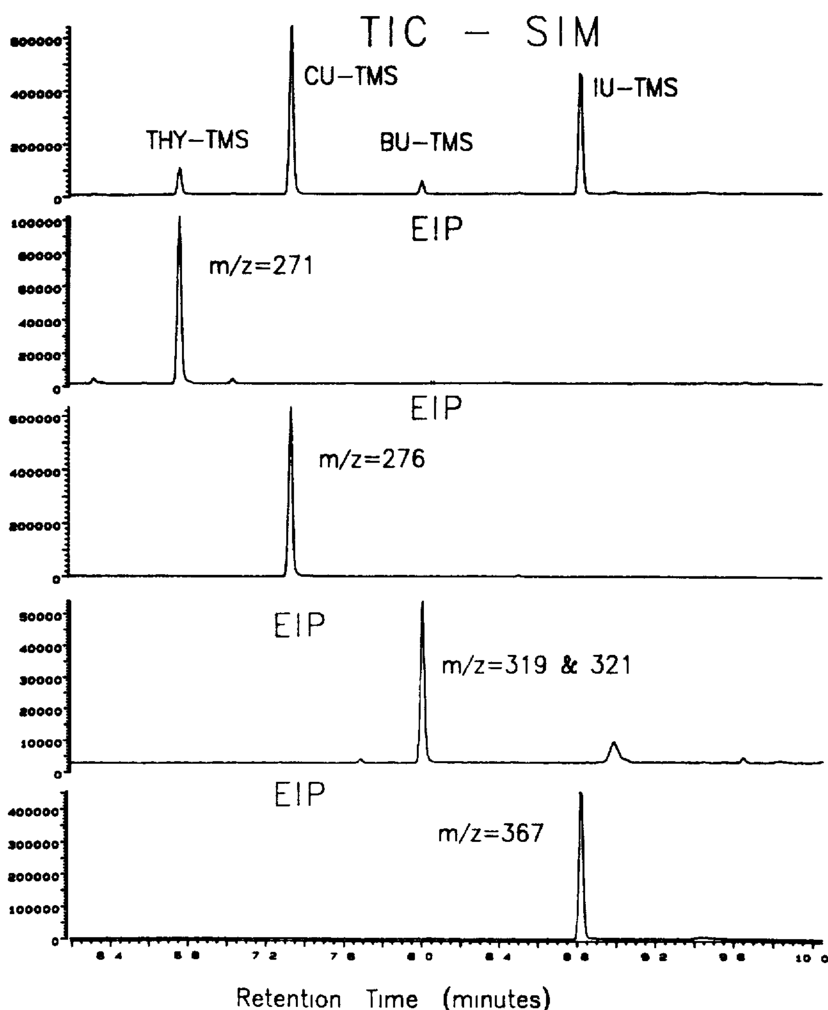

Fig. 4. Total-ion chromatogram and the extracted-ion profiles (EIP) of a sample prepared from DNA isolated from V79 cells in culture exposed to 1.0 μM BUDR for 40 h.

TABLE III

SUBSTITUTION OF CELLULAR DNA THYMINE BY 5-BROMOURACIL IN V79 CELLS EXPOSED TO MEDIUM CONTAINING BUDR

BUDR exposure (μM)	Content				Substitution (%)
	Thymine		5-Bromouracil		
	ng	nmol	ng	nmol	
0 00	560 4	4 45	0 0	0 000	0 00
0 03	375 3	2 98	6 01	0 031	1 03
0 10	287.7	2 28	27 5	0 144	5 94
0 30	364 3	2 89	57 2	0 299	9 38
1 00	310 6	2 47	185 4	0 971	28 22

Application of the method developed is demonstrated in Fig. 4 and Table III. V79 cells in culture were exposed for a period of 40 h to medium containing 0, 0.03, 0.10, 0.30 or 1.0 μM BUDR. After isolation and hydrolysis of the DNA, the samples were analysed as to their thymine and BU content Fig. 4 illustrates the TIC profile and the extracted-ion profiles (EIP) of the sample prepared from DNA isolated from cells exposed to 1 0 μM BUDR No interfering peaks were seen in the DNA hydrolysate preparations. Table III summarizes the results of this experiment. As shown in Table III, exposure to BUDR under the experimental conditions resulted in increasing percent thymidine substitution by the 5-halo-2′-deoxyuridine, BUDR, ultimately reaching 28%.

Since the average DNA content of a diploid mammalian cell is on the order of 6 μg per 10^6 cells, with a guanine/cytosine content of about 40% [12], quantitative recovery of thymine from the hydrolyzed DNA of 10^5 cells should yield 69 5 ng (0 55 nmol). In experiments not shown here were recovered 44.4 ng (0.35 nmol) thymine from 10^5 V79 cells. Based on a minimum detectable level of BU of 1.2 ng (0.006 nmol) we should, therefore, be able to measure replacement on the order of 2% using 10^5 V79 cells.

As previously mentioned, the GC—MS—SIM method described here can quantitatively determine the amount of DNA thymine replacement by BU in as few as 10^5 cells. In confirmation of prior studies, we have found, using external beam radiotherapy, that the enhancement factor for radiosensitivity in the ν79 cell line is a direct function of the percent thymine replacement by BU. This procedure can also be used in conjunction with the monoclonal antibody/fluorescence activated cell sorting (FACS) techniques to correlate the FACS distributions with DNA thymine replacement by BU. And finally, in as much as 10^5 cells can be readily obtained by needle biopsy techniques, this method should be applicable to multiple sampling in animal and patient studies. This will allow the correlation of routes and schedules of BUDR administration with appropriate target effect, i.e BU replacement of thymine in tumor and normal tissues (such as bone marrow).

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REFERENCES

1 B Djordjevic and W Szybalski, J Exp Med , 112 (1960) 509
2 E H Simmon, Exp Cell Res Suppl , 9 (1963) 263
3 G H Thomas, M A Maloney and J E Cleaver, Radiat Res , 91 (1982) 145
4 W Szybalski, Cancer Chemother Rep , 58 (1974) 539
5 T Kinsella, J B Mitchell and A Russo, Int J Radiat Oncol Biol Phys , 10 (1984) 1399
6 T Kinsella, J B Mitchell and A Russo, J Clin Oncol , 2 (1984) 1144
7 G P Raaphorst, J A Vadasz and E I Azzam, Radiat Res , 98 (1984) 167
8 F Dolbeare, H Gratzner, M G Pallovicini and J W Gray, Proc Natl Acad Sci U S A , 80 (1983) 5573
9 G Morstyn, S M Hsu and T Kinsella, J Clin Invest , 72 (1983) 1844
10 C Finn and W Sadee, Cancer Chemother Rep , 59 (1975) 279
11 D B Lakings, R H Adamson and R B Diasio, J Chromatogr , 146 (1978) 512
12 J N Davidson, The Biochemistry of Nucleic Acids, Academic Press, New York, 7th ed , 1975