Journal of Chromatography, 190 (1980) 107–112
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CHROM. 12,491

ANALYSIS OF PYRIMIDINE BASES IN BIOLOGICAL MATERIALS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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(Received October 9th, 1979)

SUMMARY

Simultaneous detection by a combination of gas chromatography–mass fragmentography and gas chromatography–mass spectrometry for total ion monitoring was developed for determination of uracil, thymine and cytosine present in biological materials as pyrimidine bases, as their silylated derivatives. The detection limits for uracil, thymine and cytosine in the first method were 0.001 μg/ml for plasma and 0.001–0.005 μg/g wet weight for tissues. Those for uracil and thymine in plasma and tissues and for cytosine in plasma in the second method were 0.2 μg/ml or g wet weight, and that for cytosine in tissues was 2.5 μg/g wet weight.

An accurate and sensitive assay for determination of pyrimidine bases was established.

INTRODUCTION

Many methods have been reported for analysis of pyrimidine bases, including microbiological methods, titrimetry and fluorimetry. In addition, methods employing paper and thin-layer chromatography, gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) have recently been developed. Of these, GLC and HPLC are the most accurate and sensitive.

Authentic samples of pyrimidine bases and of the bases in hydrolyzates of nucleic acids have been analyzed by GLC as their silylated derivatives[1–5]. HPLC has been applied to the analysis of authentic compounds, hydrolyzates of RNA and pools of pyrimidine bases in urine, yeast and liver[6–9]. In the latter method, however, pyrimidine bases are eluted in early fractions with poor separation, and there are no reports of the clear separation of all the pyrimidine bases in biological materials.

We examined various methods for the determination of the concentrations of uracil, thymine and cytosine present in biological materials as pyrimidine bases, and established an accurate and sensitive analytical method for their measurement. Their simultaneous detection is possible by a combination of gas chromatography–mass fragmentography (GC–MF) and gas chromatography–mass spectrometry for total ion monitoring (GC–MS–TIM). This report describes the method.

EXPERIMENTAL

Materials

Uracil, thymine and cytosine were purchased from Sigma (St. Louis, Mo., U.S.A.) 5-chlorouracil was synthesized and purified in our laboratory and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine, used as solvents for silylation, were obtained from Pierce (Rockford, Ill., U.S.A.). All other reagents used were analytical grade materials purchased from Wako (Osaka, Japan).

Plasma samples and organs were obtained from H-30 nude mice. Each organ was removed by the freeze-clamping method.

Instruments

For GC–MF and GC–MS–TIM, a JEOL JMS D-300 mass spectrometer (electron impact source) connected with a JEOL JGC-20KP gas chromatograph (Tokyo, Japan) was used.

The coiled glass column (1 m × 2 mm I.D.) of the gas chromatograph was packed with 3% OV-17 on Chromosorb W AW (80–100 mesh) (Gaschro Kogyo, Osaka, Japan) and conditioned at 280° for 24 h. The temperature of the injector and the detector was set at 220° and analyses were carried out with an initial column temperature of 150° and a temperature rise of 10°/min. Helium was used as the carrier gas at a flow-rate of 30 ml/min.

The mass spectrometer was operated under the following conditions: ionization energy, 70 eV; inozation current, 300 μA; acceleration voltage, 3.0 kV; and ion multiplier voltage, 1.4 kV. Determinations by GC–MS–TIM were carried out at the same time as those by GC–MF. The fragment ions selected were the molecular ion peaks (M^+) of *m/e* 256 and 270 for the respective silylated derivatives of uracil and thymine and the base ion peaks ($M-CH_3$) of *m/e* 240 and 275 for the silylated derivatives of cytosine and the internal standard 5-chlorouracil.

Analytical procedure

Samples of 1.0 ml of plasma were diluted to 2.0 ml with distilled water. Samples of 0.5–1.0 g of each organ were homogenized with three volumes of physiological saline solution in an ice bath and centrifuged at 2000 *g* for 20 min and 1.0 ml of the supernatant was diluted to 2.0 ml with distilled water. The diluted preparations were adjusted to pH 4.0 with 5 *M* HCl and extracted with chloroform (20 ml × 2, 10 min). The aqueous layer was separated, neutralized with NaOH solution, adjusted to pH 6.0 by addition of 0.2 ml of 0.5 *M* NaH_2PO_4 solution and deproteinized by centrifugation at 2000 *g* for 15 min. The resulting supernatant was shaken vigorously for 20 min with 40 ml of ethyl acetate. The ethyl acetate layer was separated, evaporated at 40°, transferred to 1.0 ml reaction vial with methanol and concentrated to dryness at 40° under nitrogen gas. Then the residue was mixed with methanol containing 1.0 μg of 5-chlorouracil as an internal standard, concentrated to dryness again and stored overnight under reduced pressure over phosphorus pentoxide. The residue was silylated by heating it at 80° for 20 min with 100 μl of freshly prepared BSTFA–pyridine (1:3 v/v). Analyses by GC–MF and GC–MS–TIM were carried out on 0.1–1.0 μl samples of the resultant solution.

Calibration curves

Calibration curves for the pyrimidine bases analyzed by GC-MF and GC-MS-TIM were obtained by plotting the ratio of the peak heights of the respective silylated derivatives to that of the silylated derivative of the internal standard 5-chlorouracil in GC-MF against the concentration. All these calibration curves were linear (Fig. 1).

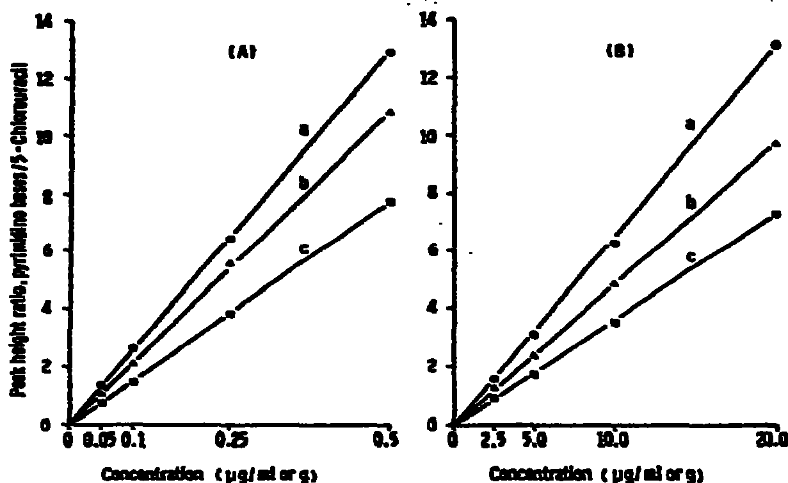

Fig. 1. Calibration curves of uracil (a), thymine (b) and cytosine (c) obtained by GC-MF (A) and GC-MS-TIM (B) methods. Ratios of the peak height of pyrimidine bases to that of 5-chlorouracil (internal standard) as their silylated derivatives are plotted against concentration.

RESULTS AND DISCUSSION

The extraction and separation of pyrimidine bases from biological materials gave good reproducibility and recovery when extraction was conducted as follows: protein was denatured by extraction with chloroform in HCl and the supernatant was adjusted to pH 6.0 with 0.5 *M* NaH_2PO_4 and extracted with ethyl acetate. The recoveries of uracil, thymine and cytosine were all *ca.* 80% from plasma and *ca.* 70% from various tissues. Extraction could also be achieved with perchloric acid, but it interfered with subsequent silylation. 5-Chlorouracil was chosen as an internal standard for multiple ion detection by GC-MF. The fragment ions detected were the molecular ion peaks in the mass spectra of the silylated derivatives of uracil and thymine (Fig. 2), and the base ion peaks for $M-CH_3$ of the silylated derivatives of cytosine and 5-chlorouracil (Fig. 2). Consequently, good separation and satisfactory sensitivity were achieved. Fig. 3 shows the separations by GC-MF of authentic samples of uracil, thymine and cytosine, and materials in an extract from the liver of H-30 nude mice. The retention times were 1.7, 2.1, 3.7 and 2.5 min for the silylated derivatives of uracil, thymine, cytosine and 5-chlorouracil, respectively, and their corresponding peaks were confirmed by mass spectrometry and mass chromatography. The detection limits for uracil, thymine and cytosine in this GC-MF method were 0.001 µg/ml for plasma and 0.001-0.005 µg/g wet weight for tissues.

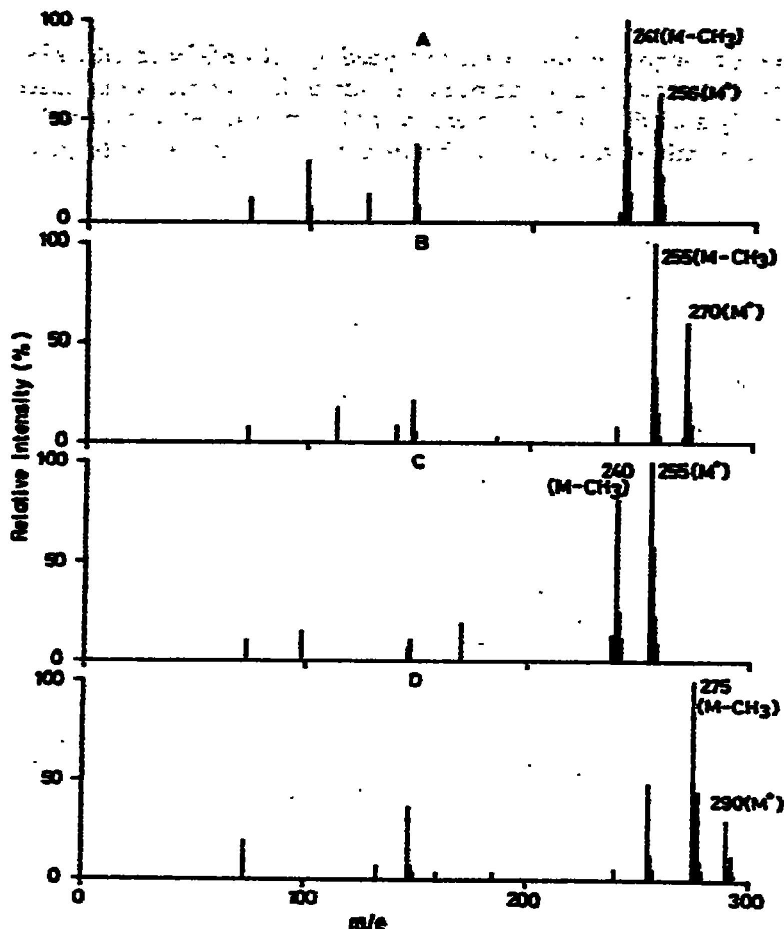

Fig. 2. Mass spectra of uracil (A), thymine (B), cytosine (C) and 5-chlorouracil (D) as their silylated derivatives.

The ion multiplier voltage of the mass spectrometer was set at 1.4 kV for determination of uracil, thymine and cytosine by GC–MF. The pools of pyrimidine bases in tissues, particularly that of uracil, exceeded the upper detection limit of 2.5 µg/g in some samples. This fact suggested the necessity for two analytical runs at different values of the ion multiplier voltage or a combination of this method with GLC employing flame ionization detection. In this study, however, we adopted the method of simultaneous detection by GC–MF and GC–MS–TIM, and developed conditions for simultaneous determinations of uracil, thymine and cytosine in any biological material. Fig. 4 illustrates the separations by GC–MS–TIM of authentic samples of uracil, thymine and cytosine and materials in an extract from the liver of H-30 nude mice. The detection limits for uracil and thymine in plasma and tissues, and for cytosine in plasma, in this GC–MS–TIM method were all 0.2 µg/ml or g wet weight. The detection limit for cytosine in tissues was 2.5 µg/g wet weight, because its retention time was very similar to that of biological constituents eluted just after it.

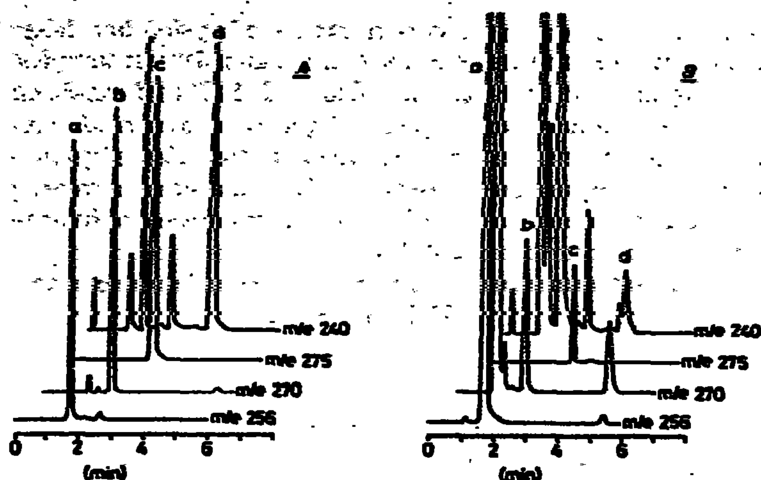

Fig. 3. Separation by GC–MF of (A) authentic samples of uracil (a), thymine (b), cytosine (d) and 5-chlorouracil (c), as an internal standard, and (B) compounds in a liver extract from H-30 nude mice. Results are for the silylated derivatives.

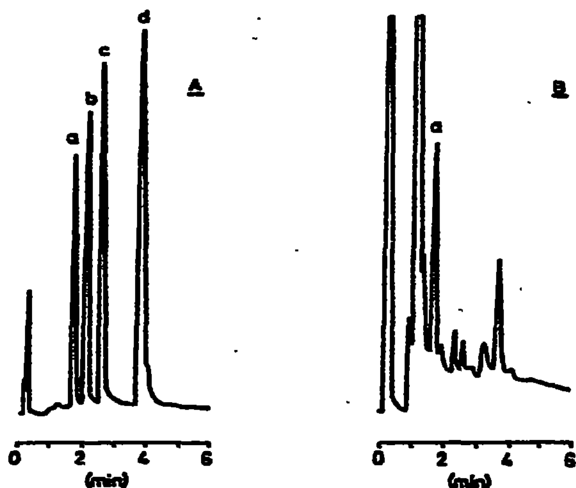

Fig. 4. Separation by GC–MS–TIM. Details as in Fig. 3.

Finally, we studied the influences of nucleosides and nucleotides of pyrimidine on the determination of uracil, thymine and cytosine by the present method: for examination of the effects of decomposition of pyrimidine nucleosides and nucleotides, authentic samples of these compounds were dissolved in plasma and tissue homogenate of liver, and then analyzed for the concentration of pyrimidine bases detected. The results showed that the decomposition of nucleosides and nucleotides had no appreciable influence on the measurement of pyrimidine bases with the present method. Changes in the concentration of pyrimidine bases were examined by incubation of plasma and tissue homogenate of liver with shaking at 5° for 12–24 h before measuring

the concentration of pyrimidine bases. But, the concentration of pyrimidine bases caused no significant difference between the value of the control and the test samples. Next, plasma and tissue homogenate of liver were acidified with HCl and extracted with chloroform, then the supernatant of the aqueous layer was incubated with shaking for 24 h at 37°. No significant difference between the concentration of pyrimidine bases in control and test samples was observed. Thus, as well as causing deproteinization, extraction with chloroform in HCl inactivated enzymes that hydrolyzed the glycoside bonds of nucleoside and nucleotides.

Table I summarizes the results obtained by the present method on the uracil, thymine and cytosine pools in various organs of H-30 nude mice.

TABLE I

PYRIMIDINE BASE POOLS IN TISSUES OF NUDE MICE (H-30)

Each value is the mean of five determinations. ND = Not detectable.

Tissue	*Concentration (per ml or g)*		
	Uracil	*Thymine*	*Cytosine*
Plasma	2.81 ± 0.78 nmol	ND	ND
Tumour	0.47 ± 0.08 μmol	8.48 ± 1.86 nmol	1.11 ± 0.11 nmol
Liver	0.20 ± 0.05 μmol	0.17 ± 0.09 nmol	0.47 ± 0.11 nmol
Kidney	0.48 ± 0.03 μmol	0.75 ± 0.45 nmol	1.37 ± 0.61 nmol
Lung	0.18 ± 0.03 μmol	1.58 ± 0.11 nmol	0.89 ± 0.20 nmol
Spleen	0.38 ± 0.78 μmol	9.56 ± 2.48 nmol	1.02 ± 0.11 nmol

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