Journal of Chromatography, 224 (1981) 423—429
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 891

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETERMINATION OF PLASMA 5-FLUOROURACIL AFTER ADMINISTRATION OF 1-HEXYLCARBAMOYL-5-FLUOROURACIL TO DOGS AND HUMANS

HIROMI ISOMURA, SABURO HIGUCHI* and SHIGEO KAWAMURA

Department of Drug Metabolism, Institute of Research and Development, Yamanouchi Pharmaceutical Co. Ltd., No. 1—8, Azusawa-1-chome, Itabashi-ku, Tokyo (Japan)

(First received January 13th, 1981; revised manuscript received March 9th, 1981)

SUMMARY

A simple, sensitive and specific method for determining 5-fluorouracil (5-FU) in plasma after the administration of 1-hexylcarbamoyl-5-fluorouracil (HCFU) was developed using gas chromatography—mass spectrometry. Thymine was used as the internal standard. After removal of interfering substances with chloroform, diethyl ether and Amberlite XAD-2 resin, 5-FU and thymine were extracted with 16% *n*-propanol in diethyl ether and methylated with trimethylanilinium hydroxide. Fragment ions at *m/e* 158 and 154, the molecular ion of the dimethyl derivatives of 5-FU and thymine, respectively, were used to monitor 5-FU and thymine. The sensitivity of the method is 10 ng/ml, which is sufficient to determine the 5-FU levels in plasma after the administration of therapeutic doses of HCFU to patients.

INTRODUCTION

1-Hexylcarbamoyl-5-fluorouracil (HCFU), synthesized as a masked form of 5-fluorouracil (5-FU) [1], has been found to be more active than 5-FU against experimental solid and ascites tumours in mice [2]. The anti-tumour activity of HCFU is thought to be due to 5-FU, one of its metabolites. Previously, microbiological [3] and high-performance liquid chromatographic methods [4] have been reported for the determination of plasma 5-FU after the oral administration of HCFU.

Although the microbiological method is highly sensitive (10 ng/ml in plasma) and facilitates the measurement of plasma 5-FU after the administration of therapeutic doses of HCFU, in practice it is inconvenient for analysing large numbers of samples because it involves lengthy procedures. Further, if patients simultaneously receive antibacterial agents, the antibiotics may interfere with the assay.

On the other hand, although the high-performance liquid chromatographic

0378-4347/81/0000—0000/$02.50

method is highly selective and simple, its sensitivity is only about 100 ng/ml, which is not sufficient for determining low plasma 5-FU levels.

Therefore, a simple, specific and sensitive method was needed to deal with large numbers of samples. We describe here such a method, which utilizes gas chromatography—mass spectrometry for the determination of plasma 5-FU after the administration of HCFU.

EXPERIMENTAL

Chemicals

5-FU, HCFU, 1-(5-carboxypentylcarbamoyl)-5-fluorouracil (CPEFU) and 1-(5-carboxypropylcarbamoyl)-5-fluorouracil (CPRFU) were kindly supplied by Mitsui Pharmaceutical (Tokyo, Japan). Thymine and 0.2 *M* trimethylanilinium hydroxide in methanol (Methelute®) were obtained from Kohjin (Tokyo, Japan) and Pierce (Rockford, IL, U.S.A.), respectively. The test organism for microbiological assay was *Staphylococcus aureus* (ATCC 6538P); the medium used was Mueller Hinton medium (Eiken, Tokyo, Japan). Other reagents were commercial products and were of analytical-reagent grade.

Gas chromatography—mass spectrometry

A JMS D-300 mass spectrometer (JEOL, Tokyo, Japan) and a Hewlett-Packard 5710A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) were used. Separations were carried out on a glass column (1.8 m × 1.8 mm I.D.) packed with 3% OV-17 on Chromosorb W (80—100 mesh). The column temperature was maintained isothermally at 185°C for approximately 4 min, then increased at 32°C/min to 260°C, and held there isothermally for 2 min. The injector, separator and ion source temperatures were 300, 250 and 200°C, respectively. The flow-rate of the carrier gas (helium) was 30 ml/min. The ionization potential and total current were 70 eV and 300 μA, respectively. The multiplier voltage supply was set at 1.6—2.2 kV.

Extraction procedure

To each plasma sample (0.5 ml), thymine (1.5 μg) was added as the internal standard (I.S.). After adding 0.1 *M* hydrochloric acid (2.5 ml), the plasma sample was extracted with chloroform (4 ml). The aqueous layer was then shaken with Amberlite XAD-2 resin (0.5 ml) for 1 h. After removal of XAD-2 resin, the aqueous layer was saturated with sodium hydrogen carbonate and extracted with an equal volume of diethyl ether. A 2-ml volume of the aqueous layer was added to 30 ml of 16% *n*-propanol in diethyl ether and shaken for 20 min. The organic layer was collected and evaporated in a water-bath at 45°C. Residual *n*-propanol was evaporated to dryness under reduced pressure at 40°C. Methelute (30 μl) was added to the residue immediately before measurement; 3-μl aliquots were injected into the column for gas chromatography—mass spectrometry and the fragment ions at *m/e* 158 and 154 were used to monitor 5-FU and thymine, respectively.

Calibration graph

The calibration graph was prepared by subjecting human plasma samples, to which known amounts of 5-FU had been added, to the above procedures. The peak height ratio of 5-FU to the I.S. was plotted against 5-FU concentration.

Extraction recoveries

Control plasma samples (0.5 ml) containing 5-FU (150 ng) were carried through the above procedure without adding I.S. Thymine (1.5 μg) was added to the 16% *n*-propanol—diethyl ether extracts and the solutions were evaporated to dryness. The subsequent procedures were as described above. Recoveries were calculated by comparing the peak height ratios with those obtained when 5-FU and I.S. were processed without the extraction procedure.

Recoveries of 5-FU from the mixtures of 5-FU, HCFU, CPEFU and CPRFU were obtained as follows: (1) 5-FU (150 ng) and I.S. (1.5 μg) were added to control plasma samples (0.5 ml) and carried through the above procedures; (2) the same procedures were followed with control plasma samples (0.5 ml) containing 5-FU (150 ng), I.S. (1.5 μg), HCFU, CPEFU and CPRFU (500 ng each). The percentage recovery of 5-FU was calculated as 100 (peak height ratio obtained with procedure 1/peak height ratio obtained with procedure 2).

Microbiological method

The microbiological method of Watanabe et al. [3] was used.

Animal studies

After overnight fasting, three male beagle dogs were given a 100-mg tablet of HCFU. Blood samples (10 ml) were obtained from the antecubital vein with heparinized syringes, immediately cooled with ice and, within 15 min of sampling, centrifuged for 15 min at 1000 *g* in a refrigerated centrifuge (4°C). Plasma samples were collected, 1 *N* hydrochloric acid (0.2 ml) was added to each sample and they were stored at −20°C until taken for assay.

Human studies

We were requested to measure the plasma 5-FU concentrations in patients who had been administered HCFU by clinicians; these samples were used for human studies.

RESULTS AND DISCUSSION

HCFU is rapidly and extensively metabolized in laboratory animals and humans. Its major metabolites are 5-FU, CPEFU, CPRFU, α-fluoro-β-guanidopropionic acid, α-fluoro-β-ureidopropionic acid and α-fluoro-β-alanine [5]. The potent anti-tumour activity of HCFU is thought to be due to the metabolite 5-FU, and in order to determine the optimal dose for safety and efficacy the 5-FU plasma concentration after oral administration of HCFU was previously determined by microbiological assay [3] and high-performance liquid chromatography [4, 6]. However, as neither of these methods was

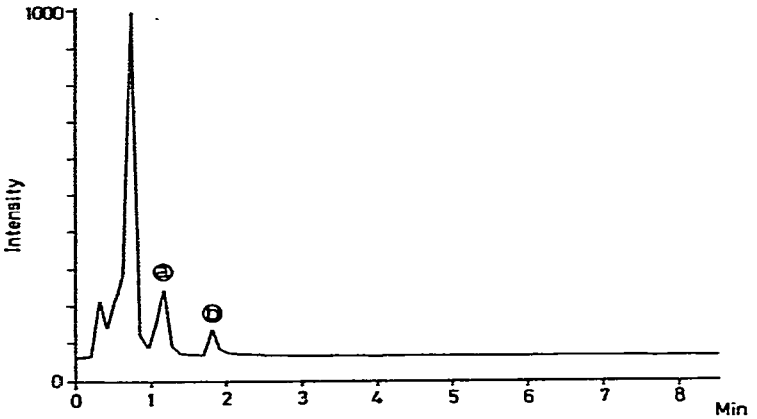

Fig. 1. Gas chromatogram obtained by total ion current detection of HCFU in the presence of trimethylanilinium hydroxide.

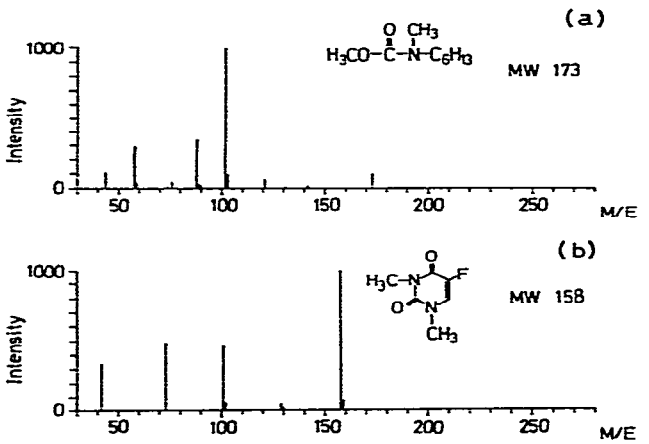

Fig. 2. Mass spectra of peaks (a) and (b) in Fig. 1.

completely satisfactory, we developed a simple, specific and sensitive gas chromatographic—mas spectrometric method for processing large numbers of samples.

Several gas chromatographic—mass spectrometric methods have been reported for determining plasma 5-FU after the administration of 5-FU [7—10] and 1,3-bis(tetrahydro-2-furanyl)-5-fluorouracil [11, 12]. In these methods, 5-FU was converted into its methyl, butyl or silyl derivative, which was subjected to gas chromatography—mass spectrometry. In the present study, trimethylanilinium hydroxide was used as the reagent for methylation [7].

HCFU, CPEFU or CPRFU, injected directly with trimethylanilinium hydroxide in methanol into the column, decomposed into two products, resulting in two peaks. The total ion chromatogram and mass spectra of the products from HCFU are shown in Figs. 1 and 2, and the compounds were identified as N-methoxycarbonyl-N-methylhexylamine (Fig. 2a) and dimethyl derivatives of 5-FU (Fig. 2b) from the high-resolution mass spectrometric data. CPEFU and CPRFU were also decomposed to dimethyl derivatives of

5-FU and 6-(N-methoxycarbonyl-N-methyl)aminohexanoic acid or 4-(N-methoxycarbonyl-N-methyl)amino-*n*-butanoic acid, respectively. These results indicate that the degradation product, 5-FU, interfered in the assay of plasma 5-FU. Further, HCFU, CPEFU and CPRFU are labile at neutral and alkaline pH and hydrolyse easily to 5-FU [3].

The previous methods [7—12] were not suitable for determining 5-FU in plasma after the administration of HCFU, because the physico-chemical properties of HCFU and its metabolites were unsuitable. These difficulties were overcome by removing the HCFU, CPEFU and CPRFU at acidic pH before gas chromatography. HCFU was completely removed and CPEFU and CPRFU were partly removed by extraction with chloroform. The remaining CPEFU and CPRFU in the aqueous layer were removed by shaking with Amberlite XAD-2 resin [3].

Endogenous interfering peaks, which appeared within 3 min on the gas chromatogram, could not be removed by the above extraction procedures, and were removed by extraction with an equal volume of diethyl ether [7].

To confirm the specificity of the assay, the recovery of 5-FU (300 ng/ml) from plasma to which HCFU, CPEFU and CPRFU (1 μg/ml of each) had been added, was determined. The recovery was 98.4 ± 2.6% (mean ± standard error, n = 6), demonstrating that these compounds were completely removed by this procedure.

Thymine was used as an internal standard. The molecular ions at *m/e* 158 and 154 were used to monitor the dimethyl derivatives of 5-FU and thymine, respectively. Chromatograms obtained from human plasma are shown in

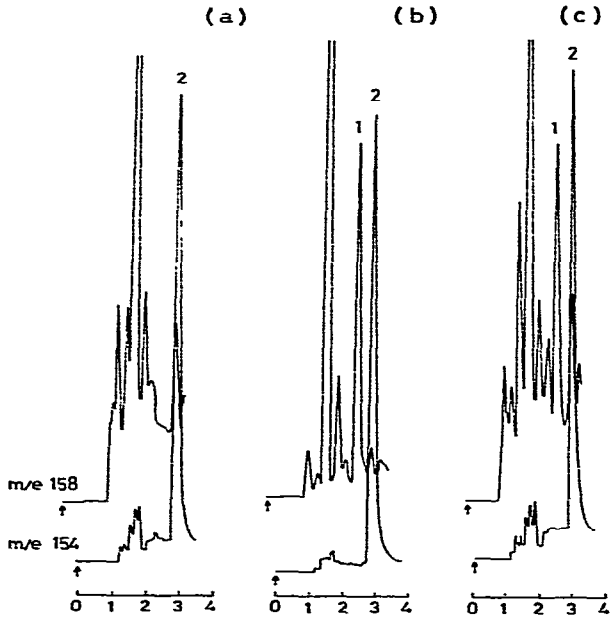

Fig. 3. Chromatograms of human plasma extract. (a) Control plasma containing 3 μg/ml of I.S.; (b) calibration standard with 500 ng/ml of 5-FU in plasma; (c) 8-h plasma sample after the oral administration of 600 mg of HCFU to a cancer patient. Peaks: 1 = 5-FU; 2 = thymine (I.S.).

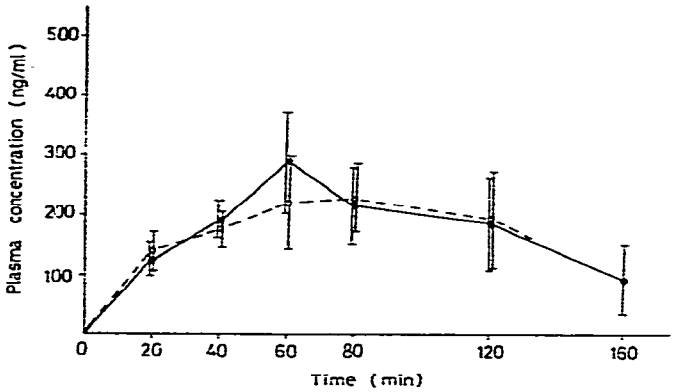

Fig. 4. Plasma 5-FU concentration after the oral administration of 100 mg of HCFU to dogs. o— — — o, Microbiological method; •———•, gas chromatographic—mass spectrometric method.

Fig. 3. The retention times of the dimethyl derivatives of 5-FU and thymine were 2.7 and 3.0 min, respectively. No endogenous peak disturbing the determination of 5-FU was found in the control plasma. About 4 min after sample injection, the column temperature was increased to 260°C and held there for 2 min. As endogenous substances derived from plasma were eluted within these times, this procedure shortened the time required for measurement. About 11 min were needed for measuring each sample. The calibration graph for 5-FU showed good linearity in the range 10—500 ng/ml. The sensitivity of the method was 10 ng/ml, which is sufficient for determining the plasma concentration of 5-FU after a therapeutic dose of HCFU. The extraction recovery of 5-FU added to plasma at a concentration of 300 ng/ml was 94.1 ± 2.1% (mean ± standard error, $n = 6$).

The existence of two new metabolites of HCFU, 1-(5'-oxohexylcarbamoyl)-

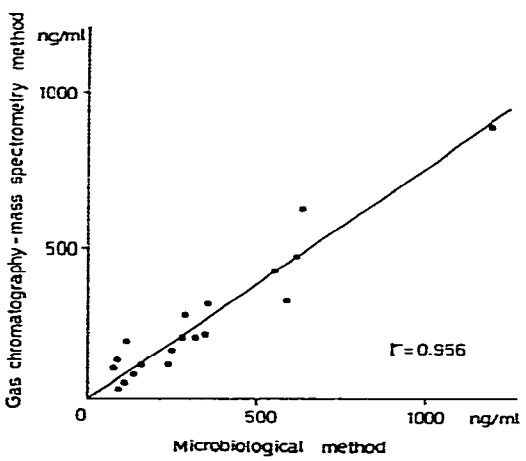

Fig. 5. Correlation between values obtained by the microbiological and the gas chromatographic—mass spectrometric method on plasma samples of cancer patients administered 200—600 mg of HCFU.

5-fluorouracil and 1-(5'-hydroxyhexylcarbamoyl)-5-fluorouracil [6], was reported while our study was in progress. Judging from their chemical constitution, these metabolites may be removed in our procedure, although we have not yet determined whether they interfere in the assay.

Plasma 5-FU levels after the oral administration of HCFU to dogs and humans were determined by our method and the results were compared with those obtained by the microbiological method. Fig. 4 shows the plasma concentration versus time curves obtained after the oral administration of 100 mg of HCFU to dogs. The values obtained by the two methods were in good agreement. Fig. 5 shows the correlation between the values obtained by the two methods after the oral administration of HCFU (200—600 mg) to cancer patients. The correlation coefficient was 0.956.

Because of its simplicity and high specificity, the proposed method should be useful for assaying large numbers of samples. Chemotherapeutic agents appear not to interfere and, owing to the high sensitivity, plasma 5-FU levels as low as 10 ng/ml can be determined using a small amount of sample.

ACKNOWLEDGEMENTS

The authors thank Dr. A. Tachibana and Mrs. Y. Noshiro (Yamanouchi Pharmaceutical Co., Ltd.) for the microbiological assays.

REFERENCES

1 S. Ozaki, Y. Ike, H. Mizuno, K. Ishikawa and H. Mori, Bull. Chem. Soc. Jap., 50 (1977) 2406.
2 A. Hoshi, M. Iigo, A. Nakamura, M. Inomata and K. Kuretani, Chem. Pharm. Bull., 26 (1978) 161.
3 S. Watanabe, K. Mori, H. Misono and M. Yokoyama, Chemotherapy, 27 (1979) 778.
4 A. Kono, M. Tanaka, S. Eguchi, Y. Hama and Y. Matsuyama, J. Chromatogr., 163 (1979) 109.
5 T. Kobari, K. Tan, M. Kumakura, S. Watanabe, I. Shirakawa, H. Namekawa and H. Yamamoto, Xenobiotica, 8 (1978) 547.
6 A. Kono, Y. Hara, S. Eguchi, M. Tanaka and Y. Matsushima, J. Chromatogr., 182 (1980) 125.
7 B.L. Hillcoat, M. Kawai, P.B. McCulloch, J. Rosenfeld and C.K.O. Williams, Brit. J. Clin. Pharmacol., 3 (1976) 135.
8 D.B. Lakings, R.H. Adamson and R.B. Diasio, J. Chromatogr., 146 (1978) 512.
9 M.-C. Cosyns-Duyck, A.A.M. Cruyl, A.P. De Leenheer, A. De Schryver, J.V. Huys and F.M. Belpaire, Biomed. Mass Spectrom., 7 (1980) 61.
10 B.H. Min and W.A. Garland, Res. Commun. Chem. Pathol. Pharmacol., 22 (1978) 145.
11 T. Marunaka and Y. Umeno, J. Chromatogr., 157 (1978) 321.
12 T. Marunaka and Y. Umeno, J. Chromatogr., 221 (1980) 382.