Journal of Chromatography, 99 (1974) 519–527

CHROM. 7633

APPLICATION OF GAS CHROMATOGRAPHY–CHEMICAL IONIZATION MASS FRAGMENTOGRAPHY IN THE EVALUATION OF BASES AND NUCLEOSIDE ANALOGUES USED IN CANCER CHEMOTHERAPY

C. PANTAROTTO, A. MARTINI, G. BELVEDERE, A. BOSSI, M. G. DONELLI and A. FRIGERIO

Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)

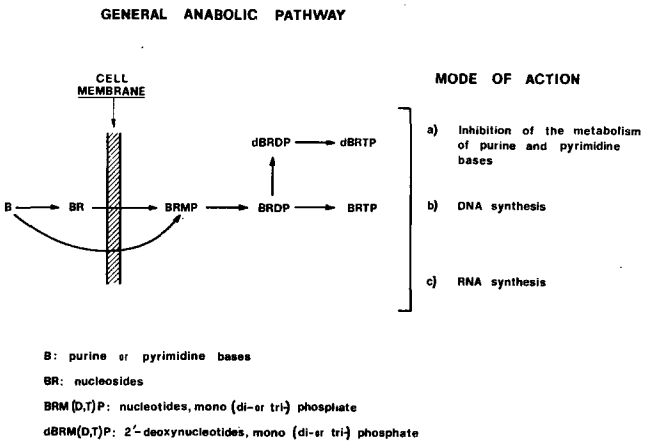
SUMMARY

Gas chromatography–chemical ionization mass fragmentography has been applied in the quantitative determination of purine and pyrimidine bases and nucleoside analogues that are widely used in cancer chemotherapy. A permethylation reaction makes them suitable for gas chromatographic analysis.

Single ion detection by focusing the mass spectrometer on the abundant "quasimolecular ions" proved to be a highly specific and sensitive procedure for the identification and evaluation of these drugs and their metabolic products present in biological fluids in amounts as low as 100 ng/ml without any interference due to endogenous substrates.

INTRODUCTION

Very little information is available on the pharmacokinetics of anti-neoplastic agents, structurally related to purine or pyrimidine bases and nucleosides, both exo-



Scheme 1. General anabolic pathway and mode of action proposed for this class of anti-metabolites.

genous or products by anabolic synthesis (Scheme 1). The classical approach in the quantitation of this type of compounds was based on the well-established microbiological, spectrophotometric, fluorimetric and, more recently, high-performance liquid chromatographic techniques[1–5]. However, they suffer from a lack of sensitivity and specificity, particularly when applied to biological problems. Labelled substrates have also been utilized, but the results obtained require validation for specificity, especially because these drugs undergo complicated metabolic pathways[6–9].

Gas chromatographic (GC) analysis of nucleosides and related compounds has never been applied in quantitative studies because of the thermal lability and facile hydrolysis of the trimethylsilyl derivatives[10,11].

For the present work, the permethylation reaction was selected because of its ease and because of the formation of low-molecular-weight derivatives; in contrast, the trimethylsilyl derivatives were difficult to form and often a mixture of high-molecular-weight compounds was present.

In the last 2 years, chemical ionization mass spectrometry (CI-MS) has been employed in the identification of drugs and in metabolism studies[12–15]. The greatest advantage of CI over the classical electron impact MS is a reduced fragmentation pattern accompanied by a prominent "quasimolecular ion" $(M+1)^+$.

The possibility of focusing the instrument on the $(M+1)^+$ ion has prompted us to apply this technique to mass fragmentographic detection and measurement of the permethylated derivatives of the anti-metabolites, thus satisfying the requirements of high specificity and sensitivity. To test the applicability and versatility of this analytical procedure, 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP), 5-fluoro-2′-deoxyuridine (5-FUDR), 6-mercaptopurine ribonucleoside (6-MPR) and arabinosylcytosine (ARA-C) were chosen as model bases and nucleosides for our investigation.

MATERIALS AND METHODS

Standards and reagents

5-FU, 6-MP, 5-FUDR, 6-MPR and ARA-C were kindly supplied by the N.I.H. (Bethesda, Md., U.S.A.) and imipramine (IMI) used as internal marker for the quantitation of ARA-C by Ciba-Geigy (Basel, Switzerland).

The following reagents were used: N,N-Dimethylaniline, methyl iodide and silver oxide (Merck, Darmstadt, G.F.R.); and methanol, ethanol, *n*-butanol and ethyl acetate (Carlo Erba, Milan, Italy). A 1 *M* solution of trimethylbenzylammonium hydroxide in methanol was prepared according to the procedure described by Brochmann-Hanssen and Olawuyi Oke[16] with minor modifications.

Gas chromatography–chemical ionization mass fragmentography

A Finnigan Model 3100 quadrupole mass spectrometer equipped with a gas chromatograph and a computer system, Model 6000, programmed for mass fragmentography (MF) was used. The chromatographic conditions were as follows. The column was a glass tube, 1 m long and 2 mm I.D., packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, Pa., U.S.A.), conditioned for 1 h at 250° (30 ml/min helium flow), 4 h at 310° (no helium flow) and 24 h at 275° (30 ml/min helium flow). The operating conditions for 5-FU and 6-MP analysis were: column temperature, 160°; injector port temperature, 230°; and the flow-

rate of carrier gas (helium for electron impact and methane for chemical ionization analysis) was 20 ml/min. The operating conditions for 5-FUDR, 6-MPR and ARA-C analysis were: column temperature, 250°; injector port temperature, 270°; and the flow-rate of carrier gas (helium for electron impact and methane for chemical ionization analysis) was 20 ml/min.

The mass spectrometer was set to the following conditions: molecular separator temperature, 250°; ion source temperature, 100°; energy of the ionization beam, 70 eV; ionization current, 200 μA.

CI-MF measurements were performed by focusing the instrument on the "quasimolecular ions" $(M+1)^+$ of the permethylated derivatives of the drugs under investigation.

General extraction procedure and derivatives formation

To a 0.1-ml volume of serum (in 10-ml glass-stoppered test-tubes) were added 0.4 ml of distilled water and 2 ml of *n*-butanol. The tubes were gently shaken in the horizontal position for 20 min on an automatic shaker and then centrifuged at 9000 *g* at 4° for 10 min. Then 1.5-ml volumes of the alcoholic phase were transferred to conical glass tubes and brought to dryness at 40° in a water-bath under a gentle stream of nitrogen, and 50 μl of the 1 *M* alcoholic solution of trimethylbenzylammonium hydroxide was added to the dry residues. The tubes were capped, passed for 20 sec over a mixer and 2 or 3 μl injected on to the GC column.

Quantitative determinations

A range of standard solutions, each containing 10–200 ng of these drugs, were made up in 50 μl of the 1 *M* alcoholic solution of trimethylbenzylammonium hydroxide. Standard calibration curves were prepared by injecting 2- or 3-μl aliquots of these solutions prior to determining these drugs in the test samples.

The peak area ratios of bases were, in general, found to be linear over the range 400 pg to 5 ng on injection. The peak area ratios of nucleosides were found to be linear over the range 500 pg to 10 ng on injection. The standard solutions were stable for several months when stored at 4° in a refrigerator.

Recovery studies

Separate and combined additions of 5-FU, 6-MP, 5-FUDR, 6-MPR and ARA-C to drug-free serum of mice at concentrations ranging from 100 ng/ml to 10 μg/ml resulted in the overall recoveries shown in Table I.

TABLE I

EXTRACTION RECOVERIES FROM SERUM OF 5-FLUOROURACIL (5-FU), 6-MERCAPTOPURINE (6-MP), 5-FLUORO-2′-DEOXYURIDINE (5-FUDR), 6-MERCAPTOPURINE RIBONUCLEOSIDE (6-MPR) AND ARABINOSYLCYTOSINE (ARA-C)

Each value is the mean of five determinations.

Drug	*Recovery from serum (%)*
5-FU	78 ± 6
6-MP	74 ± 5
5-FUDR	58 ± 8
6-MPR	54 ± 6
ARA-C	60 ± 5

RESULTS AND DISCUSSION

The "flash methylation" reaction of 5-FU, 6-MP, 5-FUDR, 6-MPR and ARA-C makes these substances suitable for GC, as only one peak was observed for each compound.

The identities of these GC peaks were checked by means of GC–MS and the structures were also elucidated by comparing the mass spectra obtained by using electron impact and CI ion sources. The mass spectra of the permethylated derivatives of these drugs are reported in Figs. 1–5. The upper spectra correspond to those recorded by electron impact and the lower spectra to those recorded by CI.

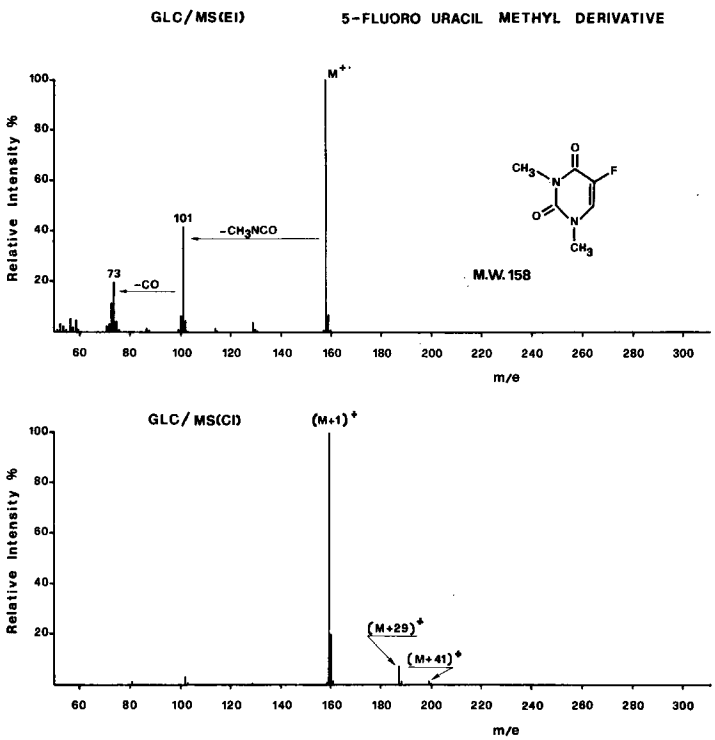


Fig. 1. Mass spectra of dimethyl-5-fluorouracil. Upper spectrum obtained by electron impact (EI); lower spectrum obtained by chemical ionization (CI) using methane as reagent gas.

It can be seen that the two types of mass spectra are clearly different in the degree of fragmentation and significantly in relation to the intensity of the molecular ion (in the case of electron impact) and the "quasimolecular ion" (in the case of chemical ionization). According to the principles of the MF technique, in order to attain higher sensitivity it is generally advisable to focus on ions with high relative abundance (preferably the base peak). In the case of the nucleosides (Figs. 3, 4 and 5), the fragmentation by electron impact is not useful for quantitative determinations as the main fragments are related to the substituted sugar moiety of the mole-

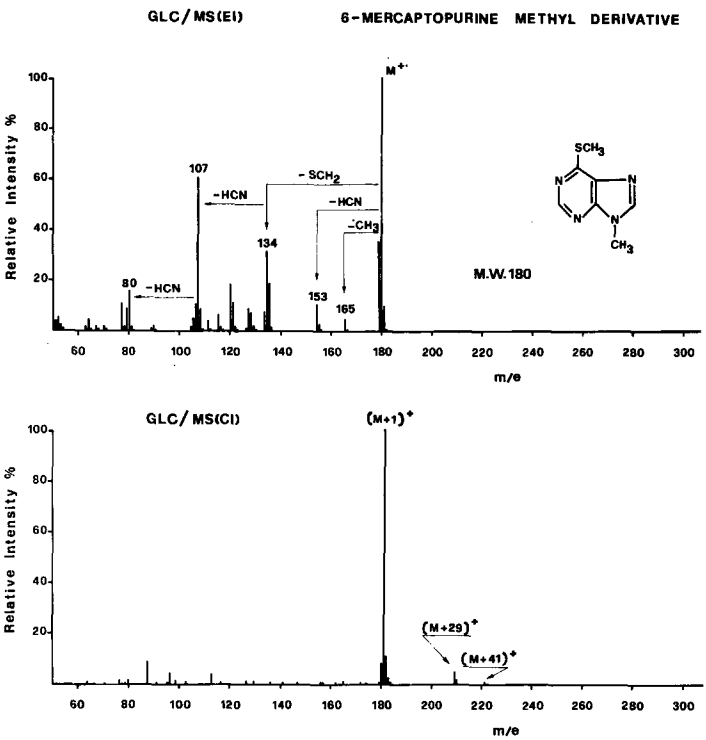


Fig. 2. Mass spectra of dimethyl-6-mercaptopurine. Upper spectrum obtained by electron impact; lower spectrum obtained by chemical ionization using methane as reagent gas.

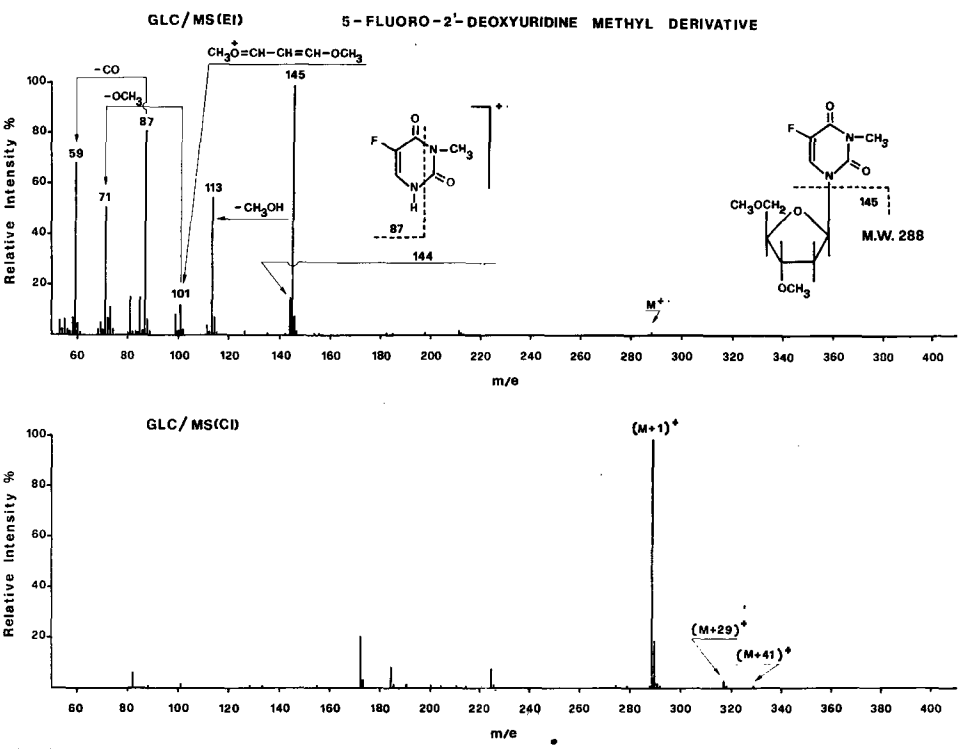


Fig. 3. Mass spectra of trimethyl-5-fluoro-2′-deoxyuridine. Upper spectrum obtained by electron impact; lower spectrum obtained by chemical ionization using methane as reagent gas.

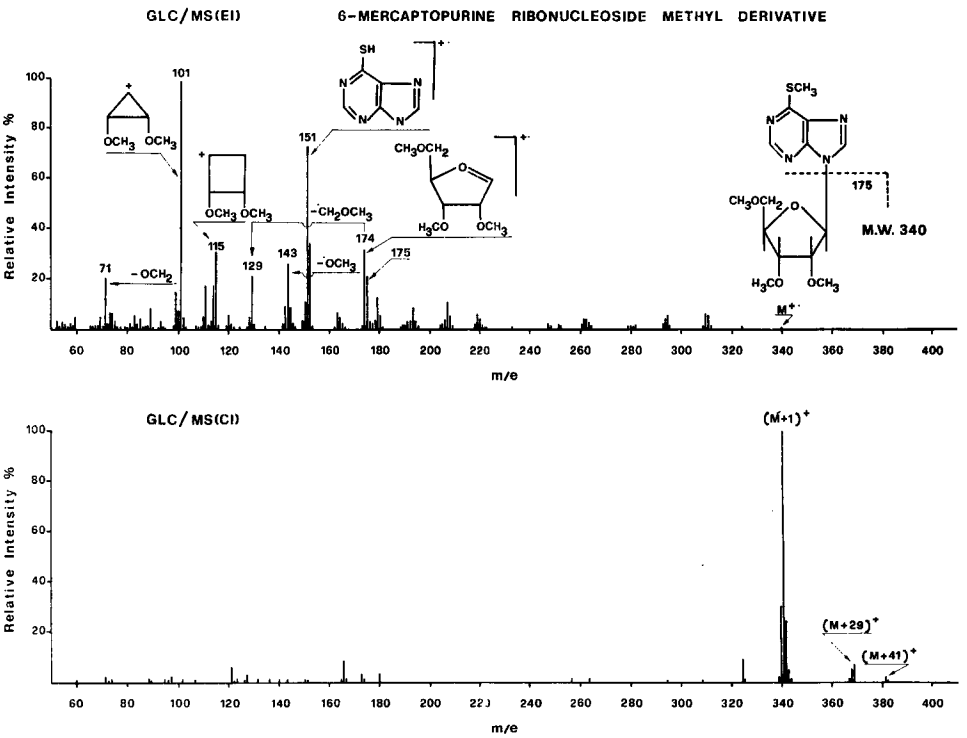


Fig. 4. Mass spectra of tetramethyl-6-mercaptopurine ribonucleoside. Upper spectrum obtained by electron impact; lower spectrum obtained by chemical ionization using methane as reagent gas.

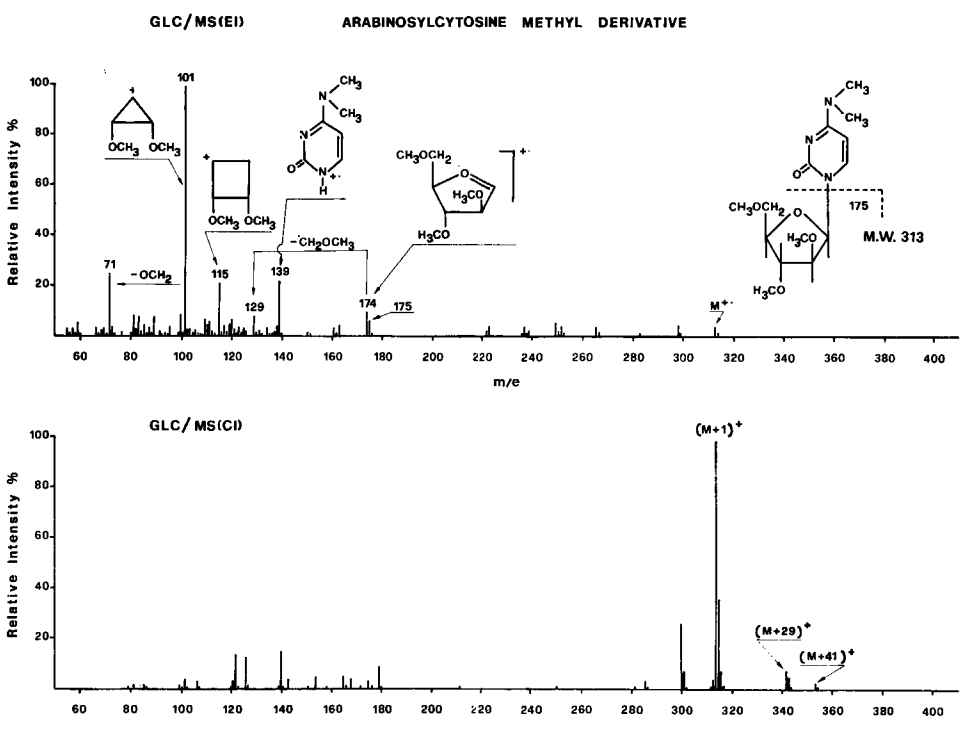


Fig. 5. Mass spectra of pentamethylarabinosylcytosine. Upper spectrum obtained by electron impact; lower spectrum obtained by chemical ionization using methane as reagent gas.

cules. These fragments may also be derived from many endogenous substrates and therefore electron impact MF detection and quantitation will not be able to achieve the level of specificity required to apply this technique to pharmacological problems. By contrast, in CI-MS the base peak of the permethylated bases and nucleosides corresponds to the "quasimolecular ion", which is highly specific and satisfies the requirements of mass fragmentographic detection (Figs. 1–5).

Figs. 6 and 7 show the MF separations of bases and nucleosides extracted from mice serum according to the general extraction procedure described above.

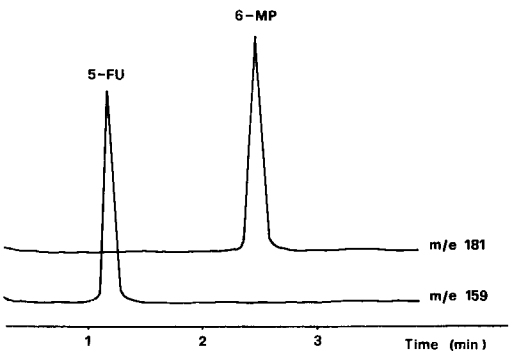


Fig. 6. Mass fragmentogram showing the separation of 5-fluorouracil (5-FU) and 6-mercaptopurine (6-MP) as their dimethylated derivatives extracted from serum of mice.

Detection was performed by focusing on the $(M+1)^+$ ion for each substance and no interference from endogenous substrates was observed. The possibility of applying this technique to the quantitative evaluation of these anti-metabolites in biological specimens was investigated by measuring the disappearance curve of ARA-C in serum of mice.

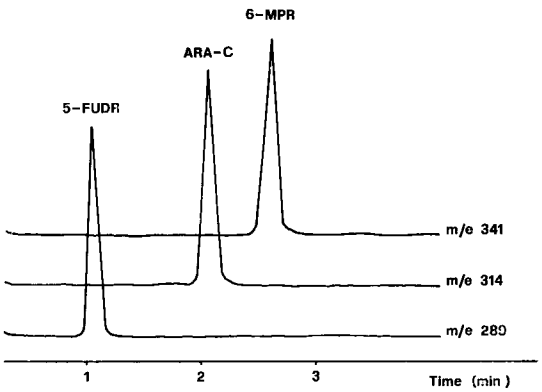


Fig. 7. Mass fragmentogram showing the separation of 5-fluoro-2'-deoxyuridine (5-FUDR), 6-mercaptopurine ribonucleoside (6-MPR) and arabinosylcytosine (ARA-C) as their permethylated derivatives extracted from serum of mice.

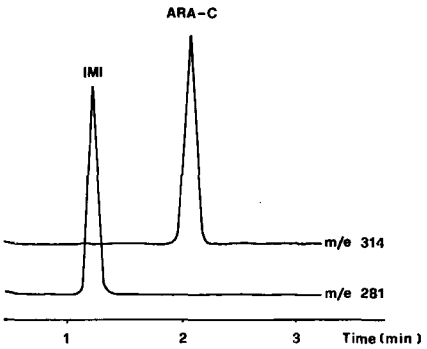


Fig. 8. Mass fragmentogram of arabinosylcytosine extracted from serum of mice and of imipramine used as internal marker for quantitation. The ion at *m/e* 281 corresponds to the "quasimolecular ion" of imipramine.

Male C57 Bl/6J inbred mice (25 ± 2 g) were injected intravenously with a single dose of 50 mg/kg of ARA-C prepared immediately prior to administration by dissolving the compound in distilled water. Groups of three animals were sacrificed and serum samples collected at 1, 5, 10, 15, 30, 60 and 120 min after drug injection. Quantitation was performed by using imipramine as internal marker, added after the extraction in an amount of 2 μg per sample.

The MF separation of ARA-C and IMI is shown in Fig. 8 and the calibration curves used for quantitation of the drug are given in Fig. 9.

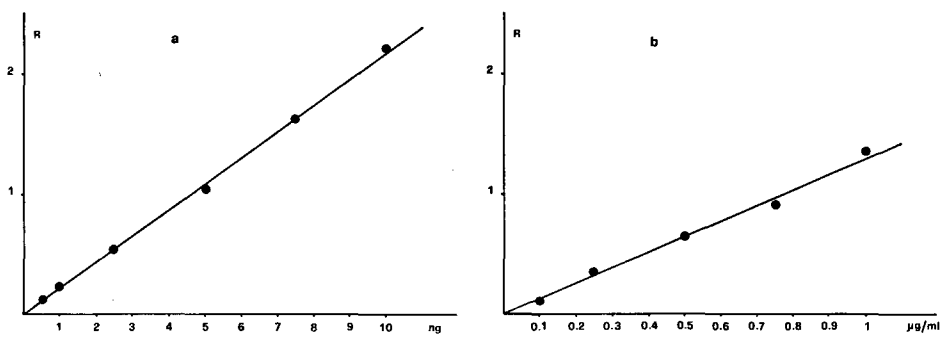


Fig. 9. (a) External calibration curve of arabinosylcytosine. (b) Calibration curve obtained adding known amounts of arabinosylcytosine to 0.1 ml of mice serum and then processed as indicated in the extraction procedure. R = ratio of the peak areas of arabinosylcytosine and its internal marker (imipramine).

In Fig. 10, the disappearance curve in serum of ARA-C shows that biological measurements are possible without interference in the range from nanogram to microgram amounts.

Besides the pharmacokinetic approach, the possibility of applying this procedure to biochemical studies is very useful. It will be possible to investigate the complex metabolic pathways of the different anti-metabolites and to confirm their level of ac-

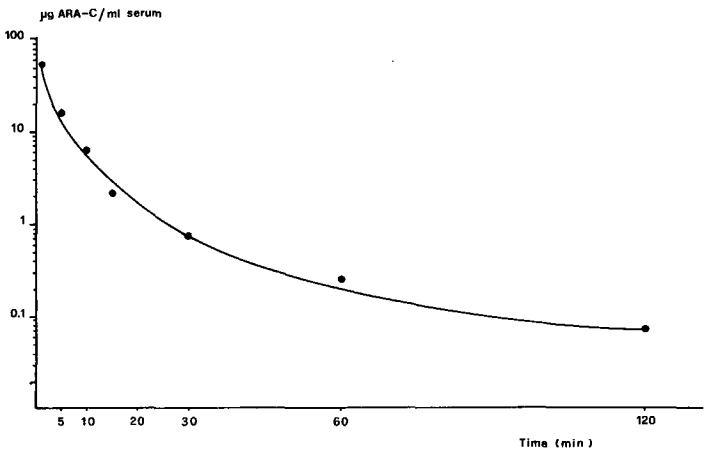


Fig. 10. Arabinosylcytosine levels in serum at different times after intravenous injection. Each point is the average of three determinations.

tion in the various steps of the synthesis of nucleotides. The procedure described can be extended to endogenous bases, nucleosides and nucleotide monophosphates (unpublished work in this laboratory).

ACKNOWLEDGEMENT

This work was supported by Contract NIH-NCI-C-72-3242 and Euratom 088–72–1–BIA C.

REFERENCES

1 C. G. Smith, J. E. Grady and F. P. Kupiecki, *Cancer Res.*, 25 (1965) 241.
2 D. E. Hunt and R. F. Pittillo, *Cancer Res.*, 28 (1968) 1095.
3 R. F. Pittillo and C. Woolley, *Cancer Chemother. Rep.*, 57 (1973) 275.
4 J. M. Finkel, *Anal. Biochem.*, 21 (1967) 362.
5 J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971.
6 K. L. Mukherjee, J. Boohar, D. Wentland, F. J. Ansfield and C. Heidelberger, *Cancer Res.*, 23 (1963) 49.
7 R. L. Dixon and R. H. Adamson, *Cancer Chemother. Rep.*, 48 (1965) 11.
8 G. B. Elion, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 26 (1967) 898.
9 V. P. Popovic, R. Massironi and R. Lehr, *Cryobiology*, 6 (1969) 87.
10 J. A. McCloskey, A. M. Lawson, K. Tsuboyama, P. M. Krueger and R. N. Stillwell, *J. Amer. Chem. Soc.*, 90 (1968) 4182.
11 J. J. Windheuser, J. L. Sutter and E. Auen, *J. Pharm. Sci.*, 61 (1972) 301.
12 R. L. Fotlz, M. W. Couch, M. W. Greer, K. N. Scott and C. M. Williams, *Biochem. Med.*, 6 (1972) 294.
13 G. W. A. Milne, H. M. Fales and R. W. Colburn, *Anal. Chem.*, 45 (1973) 1952.
14 T. T. L. Chang, Ch. F. Kuhlman, R. T. Schillings, S. F. Sisenwine, L. O. Tio and H. W. Ruelius, *Experientia*, 29 (1973) 653.
15 J. A. McCloskey, J. H. Futrell, T. A. Elwood, K. H. Schram, R. P. Pauzica and L. B. Townsend, *J. Amer. Chem. Soc.*, 95 (1973) 5762.
16 E. Brochmann-Hanssen and T. Olawuyi Oke, *J. Pharm. Sci.*, 58 (1969) 370.