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DETERMINATION OF CYTOSINE ARABINOSIDE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY WITH A NITROGEN-SENSITIVE DETECTOR AND BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A method for the determination of cytosine arabinoside in the plasma of leukemic patients being treated with this drug is described using either gas—liquid chromatography with a nitrogen-sensitive flame ionization detector or gas chromatography—mass spectrometry (GC—MS). To increase volatility, a double derivative of cytosine arabinoside was used, prepared by acetylation and subsequent methylation. Cytidine was used as internal standard for the GC procedure. GC—MS was performed with either cytidine as internal standard and detection by single-ion monitoring or by the use of [$^{2}H_{3}$] acetate-methyl derivative of cytosine arabinoside as internal standard and subsequent multiple-ion monitoring. Attempted extraction of cytosine arabinoside from plasma with various organic solvents was unsuccessful, but protein precipitation with ethanol or trichloroacetic acid followed by washing of the aqueous residue with organic solvents to remove as many of the interfering substances as possible gave satisfactory results. The minimum detectable quantity of pure cytosine arabinoside was similar for both techniques (approximately 500 pg). However, with GC using a nitrogen-sensitive detector, the lower limit of detection from plasma was found to be approximately 40—70 ng per ml plasma whilst GC—MS showed greater analytical selectivity with a detection limit in some cases as low as 1 ng per ml plasma.

INTRODUCTION

The study of the pharmacokinetics of the synthetic antitumour purine and pyrimidine nucleosides has been limited by lack of suitable assay techniques

capable of detecting and analysing small concentrations of these drugs in biological fluids free from interference from structurally related endogenous compounds. In particular, cytosine arabinoside (ara-C), which is one of the most useful agents for treatment of acute myeloid leukemia (AML), has been a difficult drug to assay in plasma and other tissues because of its high water solubility making selective extraction from plasma difficult and also because of its structural similarity to naturally occurring pyrimidine nucleosides.

The clinical pharmacology of ara-C has been principally studied using radiolabelled (tritiated) drug in selected patients [1—6]. However, because of the difficulty in using radiolabelled drugs in man, the alternative approach has been the development of a number of biological and related assay techniques for ara-C. These include microbiological [7—9], radioenzymatic [10], radioimmunoassay [11,12], and other bioassay techniques [13—16]. Whilst in some cases these techniques have a considerable degree of sensitivity (less than 50 ng/ml in plasma) they are subject to varying degrees of interference from endogenous substances.

In contrast there are few reports of physical and chemical techniques for measurement of ara-C in biological fluids. The first of these was an elegant technique by Furner et al. [17] using ultraviolet spectroscopy to determine simultaneously cyclocytidine, ara-C and uracil arabinoside in plasma or serum, but this technique lacked the detection sensitivity for plasma analysis at concentrations of ara-C resulting from therapeutic doses in man. (Detection sensitivity of this method was approximately 2 μg/ml.) In recent times, high-pressure liquid chromatographic techniques have had increasing use in separation and analysis of naturally occurring nucleosides but with only little application for determination of ara-C in biological fluids. Published reports using this technique [3,18] for ara-C determination give insufficient data as to the scope and sensitivity of the assay. Similarly, the application of gas chromatographic (GC) techniques for analysis of ara-C have received little attention, mainly due to the difficulty in applying these techniques to such highly polar compounds. One report, that of Pantarotto et al. [19] described a method using GC—chemical ionization mass fragmentography for determination of ara-C and other cytotoxic bases in mouse plasma using permethylated derivatives Although this approach was promising, only moderate detection sensitivity in plasma (0.1 μg/ml) was reported by these workers because of high background and the use of small plasma volumes.

In view of the lack of assay techniques based on physicochemical methods for direct and sensitive measurement of the free drug in plasma this report examines the application of gas—liquid chromatography (GLC) using a nitrogen-sensitive flame ionization detector (N-FID) for enhanced sensitivity, and a coupled mass spectrometer for enhanced selectivity, to the measurement of ara-C in the plasma of leukemic patients receiving this drug. Various extraction methods were investigated and derivatization was by formation of acetyl-methyl derivative as previously described [20].

EXPERIMENTAL

Materials

Ara-C was kindly supplied by Upjohn (Crawley, Great Britain). Cytidine was purchased from Sigma (St. Louis, Mo., U.S.A.). Ethereal diazomethane was prepared from Diazald (Aldrich, Milwaukee, Wisc., U.S.A.). Catalyst solution of boron trifluoride was prepared by diluting 100 μl of boron trifluoride etherate (BDH, Poole, Great Britain) with 5 ml of diethyl ether. All solvents were re-distilled before use.

Gas chromatography

GC was performed on a Hewlett-Packard 5750G gas chromatograph with a nitrogen—phosphorus-sensitive FID. The chromatograph was linked to a Hewlett-Packard 3370 digital integrator for chart presentation and peak area determination. Glass columns (2 m × 4 mm I.D.) were packed with 3% SE-30 on 80—100 mesh Chromosorb W (Pierce, Rockford, Ill., U.S.A.) or 3% OV-17 on 100—120 Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The injection port temperature was 290° and the N-FID was maintained at 400°. The column oven temperature was 225° for the SE-30 column and 275° for the OV-17 column. Flow-rates of helium carrier gas were 45 ml/min, hydrogen 28 ml/min and air 180 ml/min. The height of the rubidium bromide crystal in the detector block was adjusted to give equal response to a 2-μl injection of azobenzene (10 ng) and octadecane (5000 ng) in hexane when the instrument was in the 'normal' mode. For all subsequent analyses the instrument was used in the 'high sensitive' mode.

Gas chromatography—mass spectrometry (GC—MS)

Single- and multiple-ion monitoring were performed with a V.G. Micromass 70/70F Mass Spectrometer interfaced to a Varian 2400 GLC instrument using 1 or 2 m × 2 mm columns packed with either 3% SE-30 or OV-17. The column oven temperature was set to give a retention time of 1.5 min (in the range of 230° for 1 m SE-30, to 265° for 2 m OV-17). The mass spectrometer was operated at 70 eV with an accelerating voltage of 4 kV and a trap current of 100 μA. GLC-interface and ion-source temperatures were 300 and 260°, respectively.

Procedure for plasma extraction

Blood (5 ml) was collected by an indwelling catheter before and at various time intervals after a single intravenous bolus of ara-C (2 mg/kg) into a lithium heparin tube containing tetrahydrouridine (final concentration 10^{-4} M). The samples were kept over ice until centrifugation. The blood was centrifuged at 2000 g for 10 min and the plasma was removed and stored at −20° until required. Before extraction the plasma was thawed and re-centrifuged to remove any suspended solids. Investigation into plasma extraction using various methods showed the following to be the most useful:

Method 1. Plasma (1 ml) was treated with absolute ethanol (6 ml) in a glass test tube and mixed thoroughly on a vortex mixer for 15—20 sec. The extract was centrifuged for 5 min, the supernatant was transferred to another tube and

was evaporated to near dryness at 60° under a stream of nitrogen. The residue was then redissolved in water (3 ml) and was extracted with isoamyl alcohol (2 ml) by mixing on the vortex mixer. After centrifuging, the top isoamyl alcohol layer (containing most of the yellow pigments) was removed and discarded. The aqueous residue was again extracted with isoamyl alcohol (2 ml) and the isoamyl alcohol was discarded. The aqueous residue was then extracted twice with 3-ml volumes of ether and the ether was discarded. The aqueous phase was then evaporated to dryness under a stream of nitrogen at 60° and the residue was derivatized as described below for GLC or GC—MS analysis.

Method 2. Plasma (1 ml) was treated with cold 15% (v/v) trichloroacetic acid (3 ml), thoroughly mixed on a vortex mixer for 15 sec, and was stood in ice for 30 min to allow complete protein precipitation. The mixture was then centrifuged for 10 min and the supernatant was transferred to a separate tube. The supernatant was then extracted four times with 3-ml volumes of ether (the ether extracts were discarded), then once with isoamyl alcohol (2 ml). The isoamyl alcohol was discarded. The remaining aqueous phase was then freeze-dried and the residue was derivatized as described below.

Method 3. Plasma (3 ml) was ultrafiltered by centrifugation at 0° (250 g for 1 h) through a conical ultrafilter (Amicon, Lexington, Mass., U.S.A.). 1 ml of water was added to 1 ml of the colourless ultrafiltered plasma and the mixture was then extracted twice with 2-ml volumes of ether. The ether extracts were discarded. The aqueous phase was then freeze-dried, and the residue was derivatized as described below.

Derivatization

To the dried plasma extract, acetic anhydride (60 μl) and dried pyridine (30 μl) were added and the mixture was stood at room temperature for 40 min. The acetylation mixture was then removed by evaporation at 50° under a stream of nitrogen. The residue was dissolved in water (1 ml) and chloroform (2 ml) was added. The mixture was thoroughly mixed on a vortex mixer for 20 sec and centrifuged. The top aqueous layer was removed and discarded. The chloroform extract was washed again with water (1 ml) and the aqueous phase was removed and discarded. The chloroform residue was then evaporated to dryness under a stream of nitrogen. The residue was dissolved in ethyl acetate (50 μl) and ethereal diazomethane (200—300 μl) was added together with boron trifluoride etherate in ether (1:50, 2 μl). The yellow mixture was stood at room temperature for approximately 30 sec and then evaporated to dryness under a stream of nitrogen. The residue was dissolved in ethyl acetate (20—40 μl) and aliquots (1—3 μl) were used for GLC or GC—MS analysis.

Calibration

Stock solutions (1 mg/ml) of ara-C and cytidine were prepared in methanol and serial dilutions were made. Varying amounts of ara-C (0.075—2 μg/ml) and a constant amount of cytidine (1 μg/ml) were added to plasma from normal subjects and a standard curve was constructed following extraction and derivatization as described above.

Recovery

Recovery of ara-C from plasma was determined by adding known amounts of ara-C and [^{3}H]-labelled ara-C (5-[^{3}H]cytosine-β-D-arabinoside, 15 Ci/mmol; The Radiochemical Centre, Amersham, Great Britain) to drug-free plasma to give final concentrations of 20, 200 and 2000 ng/ml and an activity of 0.01 μCi/ml. The plasma was then extracted by each of methods 1, 2 and 3, as described. The dried extracts were redissolved in methanol (0.5 ml) and Instagel (Packard) (8 ml) and the radioactive content was determined by liquid scintillation counting.

Reproducibility and stability

Eight aliquots of a mixture of pure ara-C (100 μg) and cytidine (100 μg) were derivatized to give the acetyl-methyl derivatives, as described above. The derivatives were dissolved in ethyl acetate (100 μl) in a stoppered tube and stood at room temperature for 24 hours. Aliquots (1 μl) were chromatographed immediately and again after 24 hours.

Storage

Plasma from patients receiving ara-C or drug free plasma containing known amounts of ara-C were assayed immediately and after storage at −20° for 7 days.

Deuterated derivatives of ara-C

[$^2H_3$]acetyl and [$^2H_3$]-methyl analogues of the acetyl-methyl derivative of ara-C were prepared by substituting [$^2H_6$]acetic anhydride or [$^2H_2$]diazomethane, respectively, for the unlabelled reagents in the above preparations.

RESULTS AND DISCUSSION

Plasma extraction

Ara-C, unlike many of the clinically used drugs, presented a problem for selective extraction from plasma using conventional solvent extraction techniques. Clarke [21] has indicated that ara-C is extracted by organic solvents from aqueous alkaline solution, however repeated attempts at extraction of ara-C from aqueous solution (neutral or alkaline) with a variety of water immiscible organic solvents and mixtures of solvents gave poor recovery. This is not surprising since ara-C has a considerable degree of water solubility and is barely soluble in organic solvents. However, the recovery was greatly enhanced by saturation of the aqueous solution containing ara-C with ammonium sulphate and using polar solvents such as n-butanol or propan-2-ol, but considerable salt residues together with large quantities of undesired plasma components in the extract rendered this method unsuitable for GC work-up. Attempts using charcoal adsorption and elution with methanol or methanol—pyridine gave inconsistent recoveries. It was found that the best way to achieve sample clarification was by initial protein precipitation using ethanol or trichoroacetic acid solution, or by protein removal through centrifugation of the plasma through conical ultrafilters, and subsequent washing of the aqueous residue containing the ara-C with organic solvents to remove as many plasma compo-

nents as possible whilst leaving the ara-C in the aqueous phase. Lyophilization of the aqueous residue gave a suitable extract for derivatization.

Gas chromatography

Among the various chromatographic derivatives that were previously examined [20] the acetyl-methyl derivative was chosen because of the relative ease of preparation and because of the water stability of the nucleoside acetates enabling partial purification of the extract by extraction from the aqueous phase with chloroform. Furthermore, the acetyl-methyl derivative of ara-C could be well separated by GLC from the acetyl-methyl derivative of the major metabolite, uracil arabinoside, and from other structurally similar pyrimidine nucleosides on either OV-17 or SE-30. The N-FID gave enhanced detection sensitivity over the conventional flame ionization, with minimum detection of 500 pg for the derivative of the pure nucleoside with a 3:1 signal-to-noise ratio. Because of the close structural similarity to ara-C, cytidine (the epimeric ribonucleoside) was used as an internal standard since cytidine has similar solubility properties to ara-C and undergoes the same derivatization reaction.

The chromatograms from normal individuals or from leukemic patients prior to receiving ara-C (pre-dose plasma) contained many chromatographic peaks, mainly at earlier retention times than that of ara-C. Examples of chromatograms of drug-free plasma are shown in Figs. 1A and 1C. The chromatogram in Fig. 1B shows plasma from a normal individual to which 0.5 μg/ml ara-C was added. Analysis of plasma from normal individuals or from leukemic patients prior to receiving ara-C showed little or no response for the presence of endogenous cytidine, allowing cytidine to be used as internal standard. The chromatogram in Fig. 1D shows plasma from a leukemic patient who had received ara-C and to which cytidine (1 μg/ml) had been added as internal standard. The concentration of ara-C in that sample was 0.53 μg/ml. The tetrahydrouridine (a deaminase inhibitor) added to the blood on collection to prevent deamination of ara-C during storage did not give a chromatographic peak under the conditions used and hence did not interfere with the assay.

The percentage recovery of added ara-C from plasma by extraction methods 1, 2 and 3 is shown in Table I. For each recovery method the percentage did not vary significantly in the concentration range of 0.02—2 μg/ml. Furthermore the percentage recovery of ara-C and that of cytidine was the same since peak height ratios of extracted plasmas containing known amounts of both these compounds were the same as that of identical quantities of derivatized standards. After acetylation of the plasma extract, extraction with chloroform gave near quantitative recovery (98%) of the acetate into that solvent, hence there was little further loss of ara-C after the initial plasma extraction procedure.

To test the reproducibility of the derivatization reaction and relative stability of the derivatives, eight aliquots of 100 μg each of ara-C and cytidine were derivatized and 1-μg quantities were injected into the gas chromatograph. The mean peak height ratio of ara-C to cytidine was 1.19 ± 0.08 (standard deviation) from sixteen determinations. After standing at room temperature for 24 h the samples were re-analysed and showed little change in peak height ratio (mean 1.205 ± 0.053). An identical experiment using 0.5 μg each of ara-C and cytidine gave similar results.

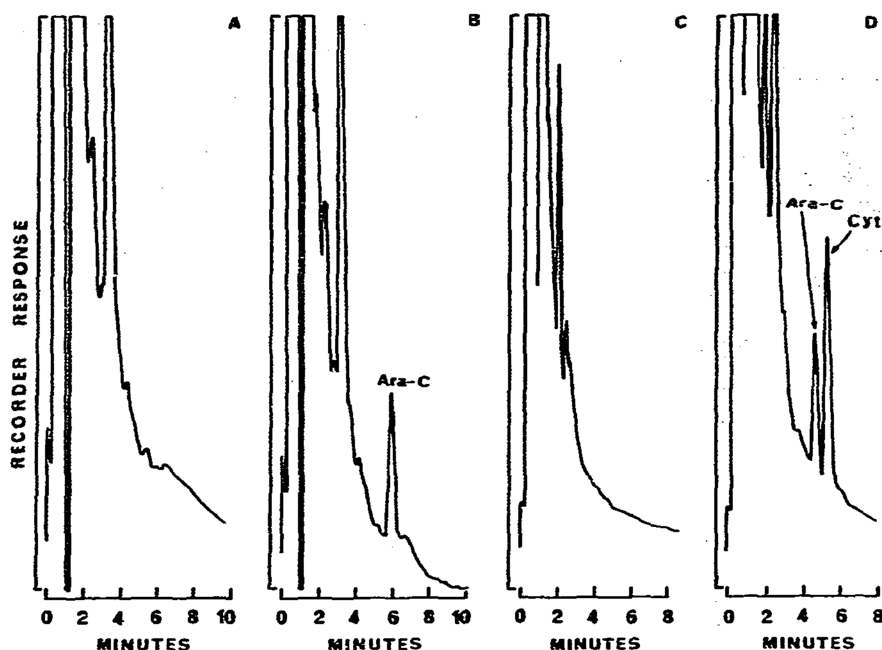

Fig. 1. Gas chromatography after derivatization of plasma extracts. Chromatograms A and B were obtained on SE-30 at 225° and were from drug-free plasma from a normal individual (A) and the same plasma after 0.5 μg/ml ara-C had been added (B). Chromatograms C and D were obtained on OV-17 at 275° and were from plasma of a leukaemic patient prior to receiving ara-C (C) and 10 min after a single dose of ara-C (2mg/kg) (D). In addition to ara-C, chromatogram D contains a peak produced by cytidine (1 μg/ml) added as an internal standard.

TABLE I

PERCENTAGE RECOVERY OF ARA-C FROM PLASMA

Extraction method	Mean percentage recovery ± S.D.	n
1	71 ± 5.4	9
2	84 ± 4	6
3	85* ± 4	6

*Recovery per ml of filtrate.

Ara-C in plasma showed no decomposition on storage in that samples of plasma from patients or drug-free plasma to which ara-C had been added showed no change in peak height response when assayed immediately or after storage at −20° for seven days or longer.

For calibration, analysis of drug-free plasma containing known amounts of added ara-C showed a linear response (peak height ratio) up to 2 μg/ml with a minimum detection from one ml of plasma of 0.04—0.07 μg/ml (twice signal strength above background). The precision of the assay at 0.5 μg/ml of added

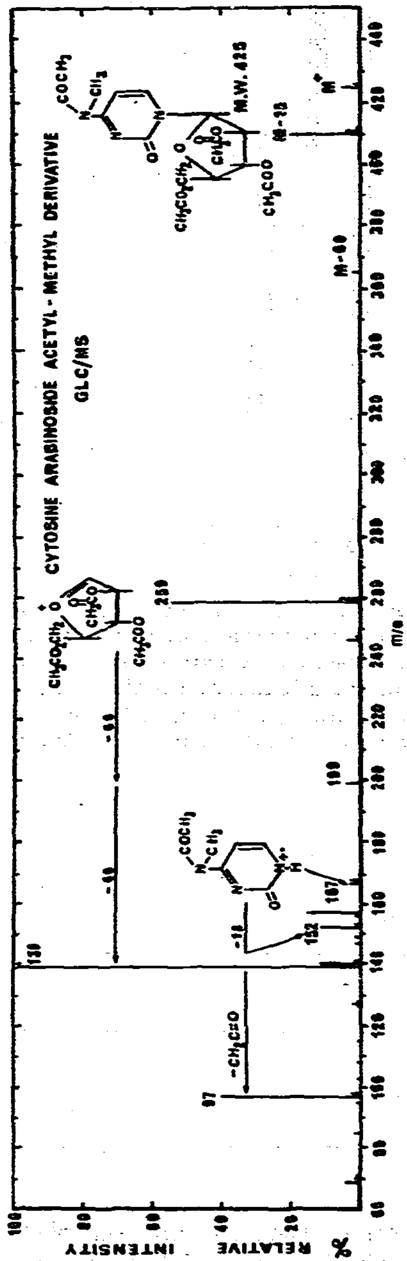

Fig. 2. 25-eV mass spectrum of the acetyl-methyl derivative of ara-C.

ara-C to drug-free plasma was 7.6% (coefficient of variation from ten determinations). This value increased at the lower concentrations. Each of methods 1, 2 and 3 for plasma extraction gave similar results, with little difference in background or sensitivity. Attempts at further clean-up of the plasma by pre-chromatography on ion-exchange columns or by thin-layer chromatography did not significantly improve the background response or increase the detection limit. Similarly use of either SE-30 or OV-17 showed no significant difference in sensitivity. Repeated injection over a period of 6—8 h into the chromatograph of plasma extracts gave diminished absolute response from the N-FID due to contamination of the rubidium crystal but the peak height ratios of ara-C to standard did not alter. Optimum detection response was readily regained by removing the rubidium crystal from the detector block and removing the accumulated deposits from the crystal.

Gas chromatography—mass spectrometry

Single-ion detection. The detection limit from plasma was improved by the use of the more selective mass spectrometer as the GC detector. Using single-ion monitoring for the $[M-15]^+$ ion it was possible to achieve a limit of 3—10 ng/ml from plasma and an absolute detection limit of 0.5 ng for the pure derivative. Although better sensitivity could be achieved with the drug alone by monitoring the more abundant ions in the lower mass range (*e.g.*, *m/e* 169; Fig. 2), when these ions were monitored in the presence of plasma extracts the increased background produced by endogenous plasma constituents at these masses resulted in poorer overall performance. At the mass of the $[M-15]^+$ ion (*m/e* 410) there was a dramatic improvement in the background at the retention time of ara-C with OV-17 compared with SE-30 (Fig. 3). However, a small residual peak was present and this limited the sensitivity of the assay to about 3—10 ng/ml. The nature of the compound producing this peak is unknown. More polar columns such as OV-25 and OV-210 were also investigated but these produced broad peaks with ara-C and no improvement in sensitivity [20]. OV-17 was thus used for measurement of ara-C in plasma. As with GLC, cytidine could still be used as an internal standard at levels of about 50 ng/ml as its spectrum also contained an ion at *m/e* 410. Fig. 4 shows the single-ion trace (*m/e* 410) from 0.5 μg/ml ara-C and 2 μg/ml of cytidine extracted from plasma. Fig. 5 shows a comparison between the plasma levels of ara-C found using an N-FID detector and by MS monitoring of *m/e* 410 in the plasma of an AML patient who had received a single dose of the drug (2 mg/kg). The ara-C was extracted using method 2. The limit of detection using N-FID was approximately 40 ng/ml whilst single-ion monitoring could achieve greater sensitivity (detection limit 20 ng/ml) and enabled measurement of ara-C for a longer period of time (60 min) after drug administration.

Multiple-ion monitoring. Below about 50 ng/ml, cytidine proved to be a poor internal standard for monitoring ara-C levels because of interference by endogenous compounds (probably cytidine itself) at the same retention time. At this level it was thus necessary to subtract the contribution of the endogenous substances. Also, as only one ion was monitored, the selectivity of the assay, in the presence of interfering compounds was poor at low levels. Thus

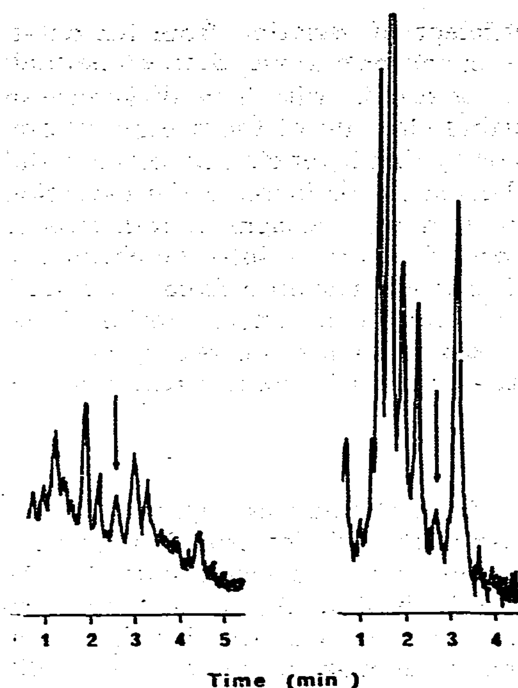

Fig. 3. Comparison of the single-ion traces of m/e 410 recorded from drug-free plasma on 3% SE-30 (left) and 3% OV-17 (right). The position at which ara-C elutes is indicated by the arrow. The amplifier gain was different during the recording of each trace; the compounds producing the major peak and the peak at the retention time of cytidine are of equal concentrations.

multiple-ion monitoring using a deuterated standard was used to overcome these difficulties. Deuterated ara-C itself was not available but the derivative incorporating four [$^2H_3$]acetate groups was readily prepared and was added as an internal standard and carrier to the plasma samples after conversion of the plasma ara-C to its unlabelled acetate derivative. An attempt to prepare the deuterated methyl analogue by reaction of ara-C with deuterodiazomethane give a mixture of [$^2H_1$]-, [$^2H_2$]-, and [$^2H_3$]- derivatives and was not investigated further. The recovery of ara-C from plasma before derivatization was measured radiochemically (Table I) and the plasma values found by multiple-ion monitoring were corrected accordingly. Monitoring was performed using four ions, the molecular and $[M-15]^+$ ions from both normal and deuterated derivatives m/e 425, 410, 437 and 419 ($M-CD_3$), respectively. Fig. 6 shows a typical trace recorded from a 1-m 3% OV-17 column; the deuterated standard can be seen eluting just ahead of the unlabelled derivative. The calibration curve of peak height ratio against concentration was linear over the range 1 ng to at least 100 ng.

Although ara-C could be detected in plasma at levels of around 1 ng/ml, the presence of endogenous compounds made quantitation difficult at levels below about 3—10 ng depending on the background level in the patient's plasma. When measuring the plasma levels following a single dose, a value for the background was obtained before administration of ara-C and this was then subtrac-

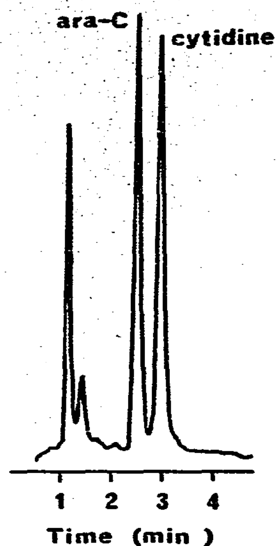

Fig. 4. Single-ion chromatogram (*m/e* 410) of ara-C (0.5 μg) and cytidine (2 μg) extracted from plasma and separated on 3% OV-17.

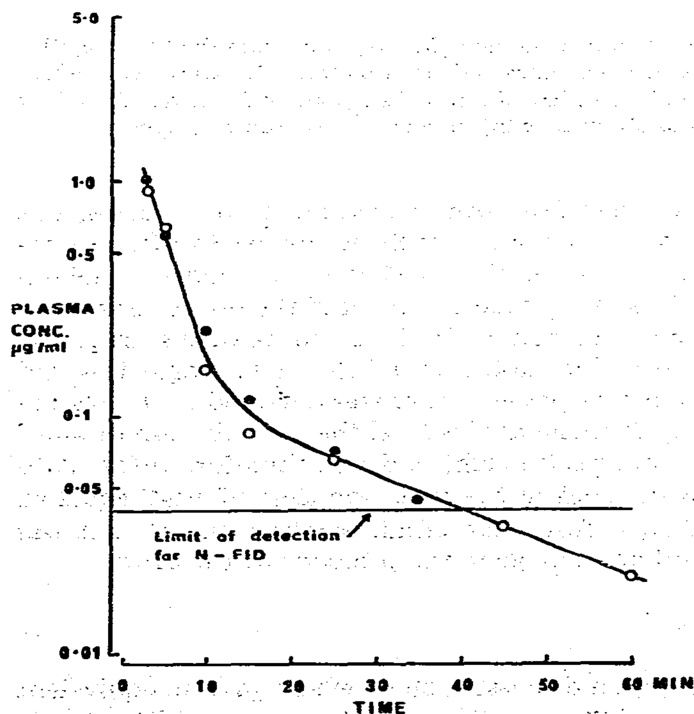

Fig. 5. Comparison of the plasma levels of ara-C from a patient who had received single dose (2 mg/kg) of the drug as measured by the N-FID (•) and by mass spectrometry (○, single-ion monitoring of *m/e* 410).

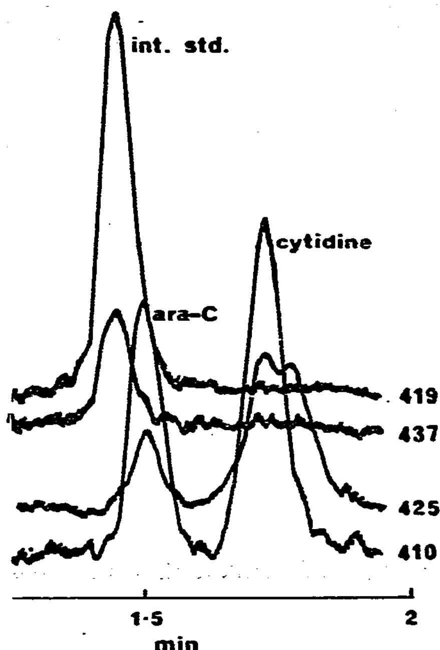

Fig. 6. Typical multiple-ion trace of the molecular and $[M-15]^+$ ions from the acetyl-methyl and $[^2H_3]$acetyl-methyl derivatives of ara-C extracted from plasma. The trace represents a plasma level of 47 ng/ml of ara-C and 50 ng/ml of internal standard and was recorded from the equivalent of 0.04 ml of plasma or about 2 ng injected into the chromatograph.

ted from the total value found after drug administration. In most cases, this method seemed to give a good value for ara-C levels as the height of the background ion was generally unchanged when the ara-C could no longer be detected. However, it was not possible at this stage to check if the concentration of the interfering compound remained unchanged in the presence of the drug. Fig. 7 shows a typical plasma level curve for ara-C found following a single dose and extraction using method 2. From this patient ara-C was detected in the plasma to 5 h after the dose was given, although at this level the contribution of ara-C present was only 20% of the total peak height and was therefore difficult to quantitate. At 6 h the height of the peak at the retention time of ara-C equalled that of the plasma sample taken before drug administration and thus it was assumed that the background did not change in the presence of the drug.

CONCLUSIONS

Of the three methods of detection discussed, all of which gave an equivalent detection limit of approximately 500 pg with pure drug, the N-FID was the least sensitive for measuring N-FID in plasma because of the presence of interfering substances rendering detection below 40—70 ng/ml difficult. MS considerably reduced the signal produced by these endogenous compounds at the retention time of ara-C giving an enhanced detection limit (1 ng/ml) in plasma.

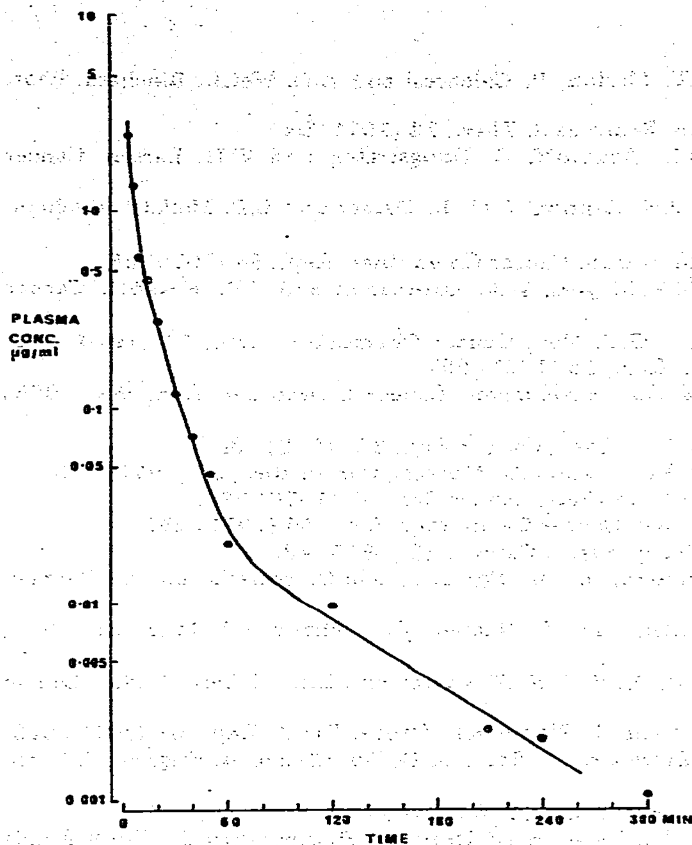

Fig. 7. Plasma levels of ara-C found using multiple-ion monitoring from the plasma of a patient who had received a single intravenous dose (2 mg/kg) of the drug.

With single-ion monitoring, problems arose with the choice of an internal standard at low concentrations of ara-C, multiple-ion detection on the other hand with the [$^2H_{12}$]acetyl-methyl derivative as internal standard and carrier enabled ara-C to be measured to a level of 3—10 ng/ml and detected at the 1-ng/ml level. This is a significant increase in the sensitivity of detection over the biological assay techniques and because of the specificity of GC—MS this method is suitable for pharmacokinetic studies of ara-C and for plasma monitoring of long-term infusion of this drug.

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