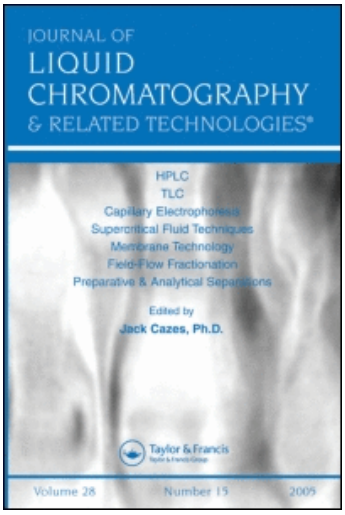


Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Reverse Phase HPLC Determination OF 5,6-Dihydro-5-azacytidine in Biological Fluids

Philipp N. Huguenin[a]; Hiremagalur N. Jayaram[a]; James A. Kelley[a]
[a] Laboratory of Medicinal Chemistry and Biology National Cancer Institute, National Institutes of Health, Bethesda, Maryland

To cite this Article Huguenin, Philipp N. , Jayaram, Hiremagalur N. and Kelley, James A.(1984) 'Reverse Phase HPLC Determination OF 5,6-Dihydro-5-azacytidine in Biological Fluids', Journal of Liquid Chromatography & Related Technologies, 7: 7, 1433 — 1453
To link to this Article: DOI: 10.1080/01483918408074056
URL: http://dx.doi.org/10.1080/01483918408074056

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(7), 1433-1453 (1984)

REVERSE PHASE HPLC DETERMINATION OF 5,6-DIHYDRO-5-AZACYTIDINE IN BIOLOGICAL FLUIDS

Philipp N. Huguenin, Hiremagalur N. Jayaram and James A. Kelley*
Laboratory of Medicinal Chemistry and Biology
National Cancer Institute, National Institutes of Health
Bethesda, Maryland 20205

ABSTRACT

A sensitive and specific reverse phase HPLC method which allows measurement of the new antitumor agent 5,6-dihydro-5-azacytidine (DHAC) in biological fluids at concentrations as low as 50 ng/ml (2×10^{-7} M) has been developed. After addition of 5'-chloro-5'-deoxy-5,6-dihydro-5-azacytidine as an internal standard, sequential ultrafiltration, boronate gel affinity chromatography and cation exchange chromatography are employed to isolate DHAC from plasma or urine. DHAC is then reacted with N,N-dimethylformamide diethylacetal to form a dimethylaminomethylene derivative with enhanced UV detectability (λ_{max} = 264 nm, log ε = 4.3) and better retention on a reverse phase column. Isocratic separation is then accomplished on a fully loaded and end-capped ODS column with 0.050 M formic acid in 20% acetonitrile/water. This assay has been used to determine the plasma pharmacokinetics of DHAC in rats given a single i.v. bolus dose of 50 mg/kg. Analysis of the drug in human plasma indicates that this method is suitable for determining DHAC disposition and pharmacokinetics in human subjects.

INTRODUCTION

5-Azacytidine (5-AC, Figure 1) was synthesized in 1963 (1) and has since been found to be clinically useful in the treatment

*Author to whom correspondence and reprint requests should be addressed.

 0148-3919/84/0707-1433$3.50/0

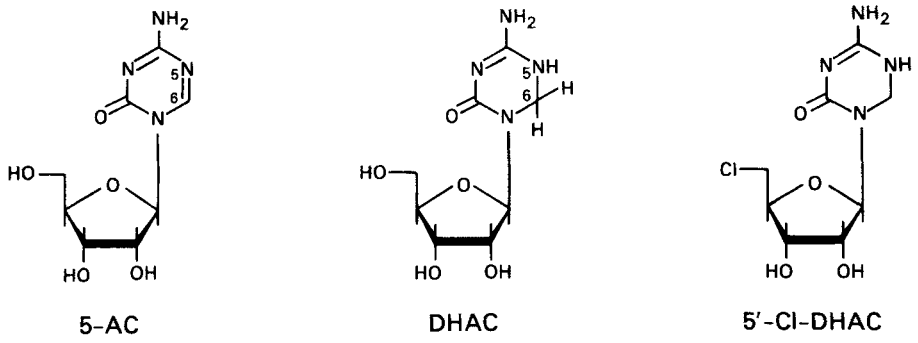


FIGURE 1. Structure of 5-AC, DHAC and the Internal Standard 5'-Cl-DHAC.

of acute myelocytic leukemia (2,3). Unfortunately, when this drug is given as a bolus injection, severe nausea and vomiting occur. These dose-limiting toxicities can be reduced or eliminated by administering 5-AC slowly via constant infusion (4,5). However, 5-AC decomposes in aqueous solution via opening of the triazine ring to produce compounds with limited antitumor activity (6). This aqueous instability makes strict dosage control for infusional therapy very difficult to achieve. To circumvent this problem the hydrolytically susceptible 5,6-imino double bond of 5-AC was reduced to produce 5,6-dihydro-5-azacytidine (DHAC, Figure 1) (7). This reduced nucleoside is not susceptible to hydrolytic attack, has good solubility, and is stable in aqueous solutions for weeks over a broad pH range.

DHAC is currently undergoing Phase I clinical trial. This potential new antitumor agent shows a broad spectrum of activity in murine model tumor systems. Good reproducible activity has been demonstrated against murine L1210 and P388 leukemias. In the L1210 system maximum activity of DHAC was observed at a dose level which was 33 times the optimum dose of 5-AC. The antitumor efficacy of DHAC at this particular dose was about 80% of that shown by 5-AC (7). Screening in solid tumor models has also

shown activity against the human MX-1 mammary xenograft, the murine CD8F mammary and the subcutaneously implanted colon 38 tumors (8).

Like 5-AC, DHAC is cell-cycle-specific with S-phase cells being the most sensitive (9). This provides a rational basis for infusional therapy over an extended time period. This also means that although the administered dose of DHAC will be relatively large, it will be administered over the course of 24 or more hours. Thus, because of this potentially extended schedule, determination of DHAC pharmacokinetics in humans will require both a sensitive and specific analytical method to measure this compound in plasma and other physiological fluids. The following report details the development of a reverse phase HPLC assay for DHAC and demonstrates its suitability for use in animal and human pharamacokinetic studies.

MATERIALS

DHAC (4-Amino-5,6-dihydro-1-β-D-ribofuranosyl-1,3,5-triazin-2(1H)-one, NSC 264880) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI, and used as received. The drug (Lot AP-02-03) was supplied as its hydrochloride salt and was greater than 99% pure. Hexamethylene-phosphoramide (HMPA), N,N-dimethylformamide diethylacetal (DMF-DEA) and thionyl chloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Both HMPA and DMF-DEA were vacuum distilled from CaH_2. N,N-Dimethylformamide (DMF, "distilled in glass" grade, Burdick and Jackson, Muskegon, MI) was mixed with benzene (J.T. Baker Chemical Co., Phillipsburg, NJ) and water in a 125:15:6 ratio by weight and the water removed by azeotropic distillation. The DMF fraction was then redistilled from CaH_2 at reduced pressure. Pyridine (J.T. Baker) was redistilled from potassium hydroxide. All purified reagents and solvents were stored over activated 4 Å molecular sieves (4-8 mesh, Aldrich)

under argon. Acetonitrile and methanol were HPLC grade (Fischer Scientific Co., Fairlawn, NJ) solvents, and together with distilled water were filtered through the appropriate 0.45 µm solvent-resistant filters (Millipore Corp., Bedford, MA) before mixing to make the mobile phase. Acetic acid, ammonium acetate (Mallinckrodt, Paris, KY), ammonium hydroxide (J.T. Baker), ammonium phosphate, formic acid (MCB Manufacturing Chemists, Inc., Gibbstown, NJ) and phosphoric acid (Fisher) were employed in making buffers.

The internal standard, 5'-chloro-5'-deoxy-5,6-dihydro-5-azacytidine (5'-Cl-DHAC, Figure 1), was synthesized by reacting DHAC with thionylchloride. DHAC hydrochloride (200 mg, 0.71 mmol) was added to a solution of thionylchloride (0.3 ml, 2.5 mmol) in 2.0 ml HMPA. This reaction mixture was stirred for 16 hr at room temperature and then maintained at 4°C for 24 hr. The solution was then diluted with 18 ml H_2O and applied directly to a 5 x 6 cm cation exchange column in the H^+ form (Dowex AG 50W-X8, 100-120 mesh; Bio-Rad, Richmond, CA). The column was washed with 1.0 ℓ H_2O and the product eluted with an equal volume of 1N NH_4OH. The eluate was evaporated at 45°C to yield a solid residue, which showed a major spot (R_f = 0.43) and a minor spot (R_f = 0.89) after TLC on silica gel with 5% 1N NH_4OH in 1:1 $CHCl_3$:CH_3OH with visualization at 254 nm (DHAC R_f = 0.18). A portion of this product was purified further by semi-preparative HPLC on a 10µm µBondapak C_{18} column (7.8 mm x 300 mm; Waters Associates, Milford, MA) using a mobile phase of 6% methanol in pH 5 0.010 M $(NH_4)_2PO_4$ buffer (v/v) at a flow rate of 2.0 ml/min. 5'-Cl-DHAC was monitored by UV detection at 234 nm and the appropriate fractions were collected. Buffer was removed by passing the HPLC eluate through an 8 x 70 mm cation exchange column in the NH_4^+ form. The column was washed with 30 ml H_2O and the product eluted with 30 ml pH 10 NH_4OH. Evaporation produced a crystalline residue, mp 204° (decomposition); NMR (D_2O) δ5.80 (d,1H,

$J_{1',2'}$ = 6Hz), 4.74 (s,2H), 4.34 (m,1H,C_4'-H), 4.26 (m,2H, C_2'-H and C_3'-H), 3.87 (m, 2H,C_5'-H); UV λ_{max} 0.01 M K_3PO_4, pH = 7.1) 231 nm, log ε = 3.67; GC/MS (tetrakis-trimethylsilyl derivative) m/z (rel intensity) 552 (M^+, 7.9), 551 (M-H, 15), 537 (M-CH_3,8.2) 517 (M-Cl,18), 388 (13), 294 (s-H,10), 287 (b^+30, 11), 257 (b,100) 217 (69), 73 (81); MS (FAB,Xe) 267 (^{37}Cl MH^+, 37), 265 (^{35}Cl MH^+, 100).

METHODS

Proton NMR spectra were recorded on a Varian XL-200 spectrometer (Varian Associates, Palo Alto, CA). Chemical shifts are reported in δ ppm downfield from deuterated sodium 3-(trimethylsilyl)proprionate which was used as an internal standard in D_2O solutions. A Beckman Model 34 spectrophotometer (Beckman Instruments, Irvine, CA) was used to obtain UV spectra. Mass spectra were obtained on a VG Micromass 7070E mass spectrometer (VG Analytical, Altrincham, England) operated at an accelerating voltage of 6kV and dynamic resolution of 2000. For gas chromatography-mass spectrometry (GC/MS) analyses, nucleosides were derivatized on a microscale with 2:1 acetonitrile:bis(trimethylsilyl)-trifluoroacetamide (Aldrich) at room temperature. Separations were effected on a 1.83 m x 2 mm i.d. glass column packed with 3% OV-17 on 120/140 mesh Gas-Chrom Q (Applied Science Laboratories, State College, PA). The Hewlett-Packard 5710A gas chromatograph (Hewlett-Packard, Avondale, PA) was interfaced to the mass spectrometer via a single stage glass jet separator and was temperature programmed from 210° to 250° at 4°/min. GC/MS operating conditions were: GC injector, 250°; jet separator and transfer lines, 240°; ion source, 250°; and scan speed, 2 sec/decade. Positive ion fast atom bombardment (FAB) mass spectra were obtained by using a FAB source at ambient temperature. Ionization was effected by a beam of xenon atoms derived by neutralizing xenon ions accelerated through 8.6-9.4 kV. Glycerol was used

as the sample matrix and spectra were acquired at a scan speed of 10 sec/decade under the control of a VG 2035 data system.

Separations were accomplished at ambient temperature (20°C) on a 5-μm Spherisorb S5 ODS II column (Regis Chemical Co., Morton Grove, IL) using a mobile phase of 0.050 M formic acid in 20% CH_3CN/H_2O (v/v) at a flow rate of 1.5 ml/min. The analytical column was preceded by a column inlet filter with a replaceable 2-μm element. The remainder of the HPLC system was comprised of a Waters Associates Model 6000A solvent delivery system, a U6K U6K injector and a LC-85 variable wavelength detector (Perkin-Elmer, Norwalk, CT). Injections of standards and unknown samples (50 μl) were made using a 100 μl Waters gas tight syringe. Peak areas and heights were simultaneously determined on a SP4100 computing and recording integrator (Spectra-Physics, Santa Clara, CA).

Standards were made by adding 80 μl of 5'-Cl-DHAC internal standard solution (4.2 μg) to 1.0 ml aliquots of plasma in a 3-ml capacity glass conical centrifuge tube and then by spiking with the required volume of DHAC standard solution. Pooled rat plasma and outdated human plasma were used for these spiked standards. For biological samples, 1.1 ml of plasma was transferred to a 15-ml capacity glass conical centrifuge tube and centrifuged for 10 min at approximately 1000 x g on a Dynac table top centrifuge (Clay Adams, Becton Dickinson and Co., Parsippany, NJ). Exactly 1.0 ml of this plasma was transferred to a 3-ml capacity glass centrifuge tube and internal standard was added as above. All samples were briefly vortexed and transferred to a MPS-1 micro-partion system equipped with a YMP membrane (Amicon Corp., Danvers, MA) for ultrafiltration by centrifugation at 1145 x g for 40 min. Half a milliliter of the resulting ultrafiltrate was mixed with 5 ml 0.25 M NH_4OAc (pH 8.8); and this solution was transferred to an 8 x 40 mm phenylboronate affinity column (Affi-Gel 601, Bio-Rad), previously equilibrated with 10 ml of the same

buffer. The column was washed with an additional 10 ml of NH_4OAc buffer, and then the DHAC and internal standard were eluted with 10 ml 0.1 M formic acid. This eluate was directly applied to an 8 x 15 mm Dowex 50W-X8 cation exchange column in the NH_4^+ form. The ion exchange column was washed with 15 ml distilled water and the nucleosides were eluted with 2 x 7.5 ml pH 10 NH_4OH solution (approximately 30 mM). This eluate was evaporated to dryness and the resulting residue transferred to a smaller (i.e., 10 or 15 ml) flask with about 2 ml water. This solution was likewise evaporated to dryness. An 8 mm stirring bar was added to the flask, and the residue was dried *in vacuo* for 10 min. A hot air blower (Model 202, Oster Corp., Milwaukee, WI) was used to warm the flask for 2 min to ensure complete drying.

Two hundred microliters DMF and 100 µl 2M DMF-DEA in pyridine were added to the residue under argon. This solution was briefly stirred and then maintained at room temperature for 30 min. Excess reagents were then removed *in vacuo* (and trapped in a dry ice cold trap) while the solution was stirred and gently heated with a hot air blower. Heating was continued for 3 min after appearance of an apparently dry residue to ensure complete removal of all reagents. The residue was dissolved in 0.50 ml water and stored at 4°C until analysis.

For study of DHAC pharmacokinetics in rats, the drug was dissolved in 0.9% NaCl solution at a concentration of 50 mg/ml. Male Sprague-Dawley rats, weighing 260-300 g (Taconic Farms, Germantown, NY) were anesthetized with ether and a 50 mg/kg dose of DHAC was given as a single bolus injection via the tail vein. Animals were sacrificed under ether anesthesia at varying timed intervals after DHAC injection and blood from each animal's inferior vena cava was collected in separate heparinized glass tubes kept on ice. Plasma was obtained by centrifugation at 1000 x g for 10 min and was then frozen until analysis.

Recoveries of DHAC and 5'-Cl-DHAC from rat plasma were determined by comparison of the absolute HPLC peak area of standards

derivatized directly to that of comparable spiked plasma samples derivatized after workup. Ultrafiltration and protein precipitation were compared by 3 replicate analyses using each method of the same pooled plasma from a rat receiving 50 mg/kg DHAC. For deproteination by methanol precipitation, 3 x 1 ml methanol was added to each sample with shaking for 30 sec following each addition. The resulting suspension was centrifuged at 400 x g for 15 min. Isopropranol (10 ml) was added to the supernatant and this solution was evaporated to dryness under reduced pressure. The residue was dissolved in 5 ml 0.25 m NH_4OAC, pH = 8.8, and extracted with 5 ml chloroform. The aqueous layer was then removed and filtered through a 0.45 µm Millex-HA filter unit before undergoing affinity and ion exchange chromatography.

The peak area or peak height ratio of DHAC to 5'-Cl-DHAC internal standard was computed for each spiked standard and plotted against DHAC concentration to generate a calibration curve (Figure 2). A calculator least squares program (TI-55-II, Texas Instruments, Dallas, TX) was used to define the best straight line through each set of standard points. A blank was also run each time a calibration curve was constructed. Initial pharmacokinetic parameters for DHAC were calculated from the rat plasma concentration (C_p)-time curve by the method of residuals (10). The experimental data points were then fit to the biexponential function representing a two-compartment open model ($C = Ae^{-\alpha t} + Be^{-\beta t}$) by using MLAB, an on-line computer modeling laboratory utilizing an interactive, non-linear least squares program (11). Based on observed assay characteristics, each data point was weighted by $1/C_p^2$.

RESULTS

Both DHAC and the 5'-Cl-DHAC internal standard could be efficiently isolated from plasma and urine and derivatized repro-

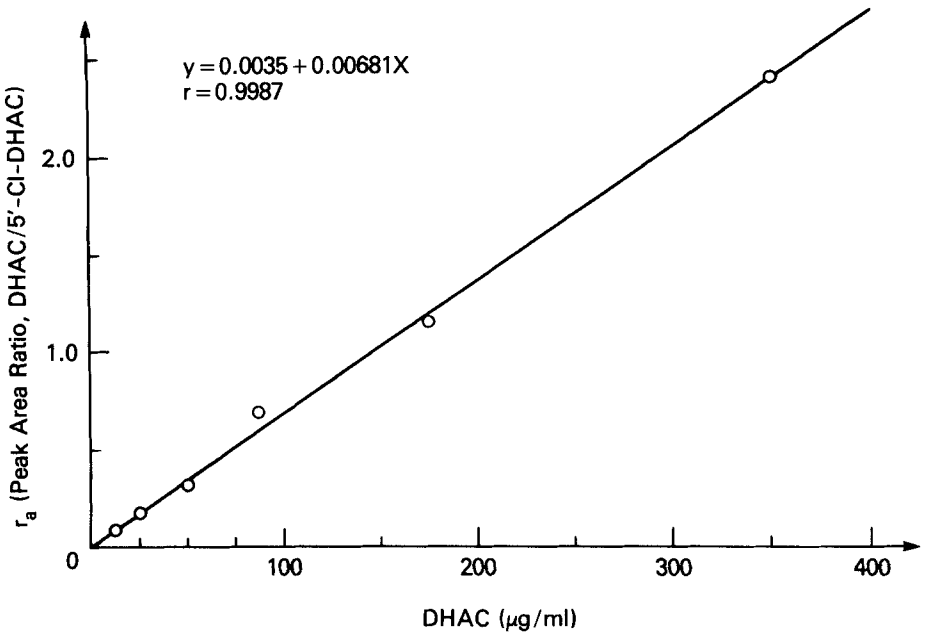


FIGURE 2. Typical Calibration Curve for Rat Plasma.

ducibly at the concentrations expected in biological samples. For DHAC spiked at concentrations of 0.1 to 10.0 μg/ml in human plasma, a recovery of 92 ± 10% (n = 5) was observed. Higher recoveries were found at the higher concentrations of DHAC with almost complete recovery (> 98%) at concentrations greater than 2.5 μg/ml. For 5'-Cl-DHAC spiked at 3.3 μg/ml plasma, 82 ± 10% (n = 6) could be recovered by the multi-step isolation procedure. Conversion to the dimethylaminomethylene (DMAM) derivative was quite reproducible since the variance in the HPLC peak areas of directly derivatized 5'-Cl-DHAC internal standard was less than 3% (n = 6). Both ultrafiltration and methanol precipitation were compared to determine DHAC protein binding and to assess their suitability for removal of these plasma proteins. No protein binding was observed since the quantitative results from the ultrafiltered and precipitated samples were equivalent; however,

greater DHAC recoveries and HPLC responses were seen after ultrafiltration. Linear and reproducible calibration curves could be constructed from spiked standards over a wide concentration range for all biological fluids examined (Figure 2):

rat plasma: $y = 0.0184 + 0.0134x$, 1.0-50 µg/ml ($r = 0.9972$)
rat plasma: $y = 0.0035 + 0.0068x$, 12.5-350 µg/ml ($r = 0.9987$)
human plasma: $y = 0.0310 + 0.3870x$, 0.07-1.4 µg/ml ($r = 0.9996$)
human urine: $y = 0.0125 + 0.0064x$, 50-500 µg/ml ($r = 0.9998$)

(Since the amount of internal standard added varied according to the concentration range examined, the slopes of the above calibration curves are different.)

HPLC analysis of derivatized samples isolated from 1.0 ml aliquots of rat plasma from animals given a bolus injection of 50 mg/kg DHAC resulted in chromatograms similar to Figure 3. The selectivity of the derivatization procedure and the resulting shift of the DHAC λ_{max} to 264 nm minimized interferences and allowed reliable peak integration. The limit of quantitation was found to be about 50 ng/ml ($< 2 \times 10^{-7}$ M) for both human and rat plasma using 1.0 ml spiked samples; the limit of detection was about half this amount. A limit of detection was not determined for urine since high concentrations of DHAC were anticipated and no interferences were encountered in sample blanks.

DISCUSSION

HPLC was chosen as a method of analysis for DHAC because of the potential problems that would have been encountered had the other applicable analytical technique, gas chromatography, been employed. Nucleosides require derivatization for vapor phase analysis (12). Silylation is probably the most widely employed method to form volatile nucleoside derivatives (13). However, silylation of DHAC results in at least partial aromatization of the base to form the corresponding derivative of 5-AC (14).

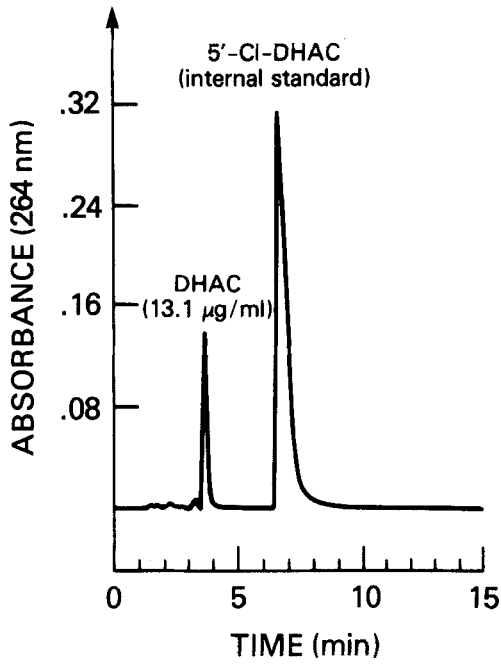


FIGURE 3. Representative HPLC Analysis of DHAC in Rat Plasma. HPLC conditions are as described in Methods. A male Sprague-Dawley rat was intravenously administered a single bolus 50 mg/kg dose of DHAC. The animal was sacrificed 63 min after drug injection and the plasma analyzed.

Since 5-AC may be a metabolite of DHAC (7), a method which does not equivocably differentiate between the two compounds is ruled out. Permethylation, an alternate technique for making volatile derivatives of nucleosides, is not straightforward with cytidine derivatives and gives multiple products (15,16). This too would be a problem since adequate sensitivity to define plasma pharmacokinetics completely is a concern.

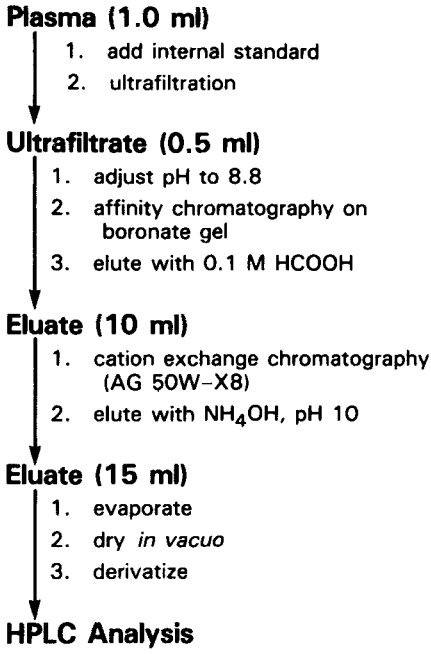
Determination of DHAC by HPLC appeared more promising since many procedures for nucleoside analysis were available (17,18). However, problems still existed. One was the isolation and con-

centration of the drug from a complex biological matrix such as plasma or urine where it was a trace component. A second problem involved enhancing the detectability of DHAC since it had a relatively low UV absorbance at a wavelength where interferences were common. This meant consideration had to be given to derivatization. As will be seen, these two problems were interrelated since derivatization was not efficient unless the DHAC was isolated from the biological matrix in an anhydrous state. Thus it was necessary to employ the rather extensive isolation procedure outlined in Scheme I.

Removal of proteins and lipids from biological samples is essential to protect the analytical column in reverse phase HPLC, especially if large sample volumes are to be injected, as is the case for the numerous samples usually encountered in a pharmacokinetics study. We found ultrafiltration of biological samples by centrifugation in a MPS-1 micropartition system to be a rapid and simple method of deproteinization. Recoveries of both DHAC and the internal standard from spiked plasma were actually greater than after protein removal by methanol precipitation. This indicated plasma protein binding was minimal and ultrafiltration could be used reliably. Plasma ultrafiltrate, however, was not amenable to direct chromatographic analysis without further cleanup.

A two-step column chromatography procedure was found to be necessary to isolate DHAC from the remaining biological matrix so that it could be reliably derivatized and analyzed. Affinity chromatography using an immobilized phenylboronic acid, which selectively binds ribosides via the 2',3'-cis-diol moiety was employed as the first step. This procedure was a modification of that reported by Gehrke et al. for the isolation of ribonucleosides from urine (18). Even though the nucleosides were eluted from the affinity column with 0.1 N formic acid, residual ammonium acetate from the initial buffer was hard to eliminate and hindered the subsequent derivatization. Partly for this reason and partly

DHAC ISOLATION PROCEDURE



because chromatographic interferences still existed, a second step employing cation exchange chromatography was employed. DHAC could be bound to the cation exchange resin at the pH of the affinity column eluant, so this step followed directly in line. After elution with ammonium hydroxide, evaporation, and drying in vacuo, a sample with no visible residue was obtained. Thus this two-step column chromatography sequence preferentially isolates only those compounds that possess both cis-diol and amino moieties like DHAC.

Derivatization was employed both to enhance the UV detectability of DHAC and to modify its retention on a reverse phase column. Zemlicka and Holy reported that treatment of cytidine with N,N-dimethylformamide diethylacetal (DMF-DEA) in dimethylformamide under mild conditions led to the N^4-dimethylaminomethyl-

ene derivative in high yield (19). Furthermore, the λ_{max} of this derivative was shifted to higher wavelength with a concomitant increase in extinction coefficient. More recently, this reagent was used to form N-dimethylaminomethylene alkyl ester derivatives of amino acids for GC and GC/MS studies (20). The fact that DMF-DEA produced these derivatives in high yield on a microscale prompted investigation of its use for DHAC quantitation. DHAC could be converted easily to its dimethylaminomethylene derivative (DHAC-DMAM, Figure 4) simply by reaction with DMF-DEA in pyridine and dimethylformamide at room temperature for 30 min. Solvent and excess reagent were simply removed in vacuo. Reproducible derivatization required complete removal of all reagents, so brief heating was employed to assist in this. Some 2',3'-O-dimethylaminomethylene derivative is also formed during this reaction, but this material is hydrolyzed to the DHAC-DMAM derivative by treatment with pH 7.4 borate buffer for 70 min at 50°. Formation of DHAC-DMAM was quantitative on a milli- to micromolar scale while yields of 70% to 80% were obtained with 1-5 nanomoles (0.28 - 1.4 µg), respectively. Because of extended conjugation in the base, this derivative exhibited a shift in its λ_{max} to 264 and a three-fold increase in extention coefficient compared to underivatized DHAC. This enhanced UV detectability, combined with better retention on a reverse phase HPLC column, resulted in good chromatographic sensitivity and lack of endogenous interferences. DHAC-DMAM was also quite stable in aqueous solution, showing only 5%, 15% and 24% decomposition after storage at 5°C for 5, 17 and 32 days, respectively. Thus standards may be used for several days and sample analysis immediately following derivatization is not mandatory.

An ideal internal standard should compensate for variations in both the isolation and derivatization procedures, act as a carrier for low levels of analyte, and have similar but not identical chromatographic properties. The choice of the proper inter-

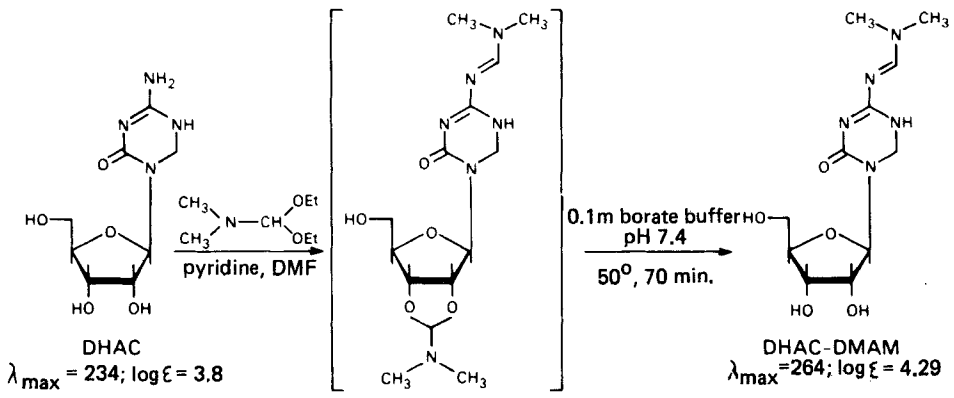


FIGURE 4. Chromogenic Derivatization of DHAC. Complete reaction conditions are described in Methods.

nal standard was especially critical for this assay because of the constraints both the isolation and derivatization procedures place on such a compound. The internal standard must bind both the phenylboronate gel and cation exchange resin, have a primary amino group to form the DMAM derivative, and possess chromatographic properties similar to those of DHAC-DMAM. Many commercially available ribosides with an exocyclic amino group in the aglycon were tested, but none proved completely satisfactory. Synthesis of 5'-chloro-5'-deoxy-5,6-dihydro-5-azacytidine (5'-Cl-DHAC, Figure 1) finally provided an internal standard that met all of the above criteria. This compound could be reproducibly isolated from plasma and its DMAM derivative formed in good yield. Furthermore, this derivative was retained on a reverse phase column so that it eluted shortly after DHAC-DMAM (Figure 3) and could likewise be monitored at 264 nm because it had exactly the same chromophore.

A column containing a high capacity octadecylsilane stationary phase that was fully capped, 5 μm Spherisorb ODS-2, was found to give the best separation under isocratic conditions. This

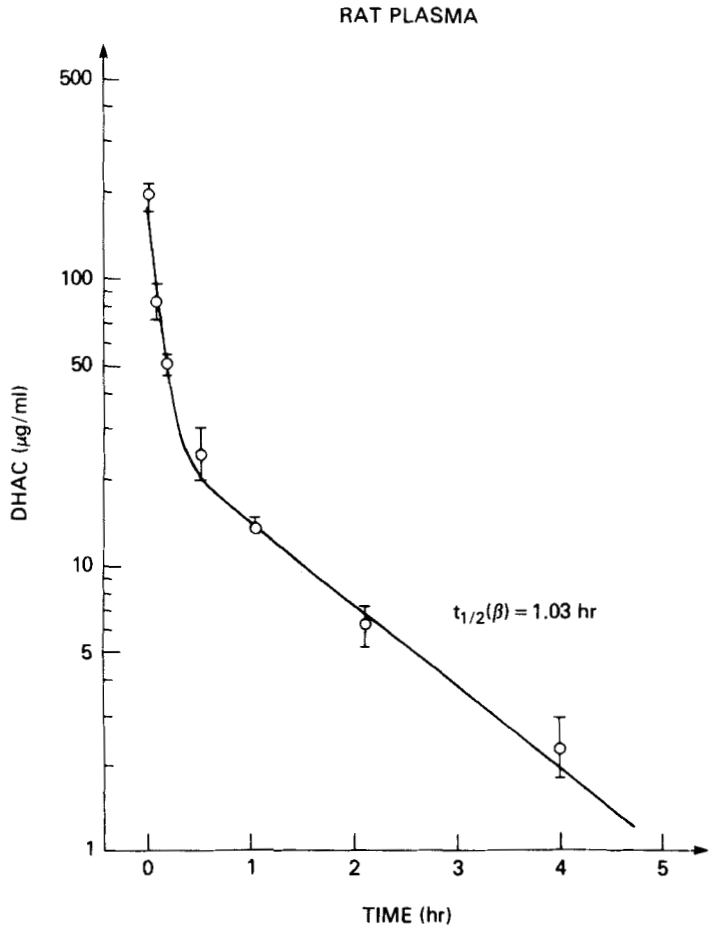


FIGURE 5. Plasma Pharmacokinetics of a Single 50 mg/kg Dose of DHAC in Male Sprague-Dawley Rats. Each point represents the mean DHAC concentration in 3 or more animals. The brackets about each point signify the range of measured DHAC concentrations.

column has also been reported to provide the best chromatographic separations of the more common deoxy- and ribonucleosides and their bases while using a pH 3.5 phosphate buffer mobile phase (21). For this analysis a mobile phase of 0.050 M formic acid in 20% acetonitrile/water gave the best chromatography and resulted

in k's of 2.6 and 4.8 for the DMAM derivatives of DHAC and 5'-Cl-DHAC, respectively.

This assay was applied to determine the single dose plasma pharmacokinetics of DHAC in rats receiving a dose (50 mg/kg) of the drug that would produce plasma concentrations equivalent to those anticipated in humans. A typical chromatogram is shown in Figure 3. The DMAM derivatives of both DHAC and the 5'-Cl-DHAC are clearly defined with no obvious interferences. For maximum sensitivity, the assay requires at least 1.0 ml of biological fluid. Such a sample size presents no problem for humans, but for 260-300 g rats where the total blood volume is only a few milliliters, serial sampling of the type necessary for pharmacokinetics would severely perturb the system. Therefore, rats of similar age, sex and weight were sacrificed at prescribed times after administration of DHAC and only a single sample was obtained from each animal. The data from these composite samples was then used to define the plasma pharmacokinetics.

DHAC exhibits biphasic behavior in the rat (Figure 5). Its plasma disappearance curve is described by a two-compartment open model where $t_{1/2}(\alpha)$ = 4.2 min and $t_{1/2}(\beta)$ = 61 min. Each point in Figure 5 represents multiple animals and the error bar represents the range of concentrations for these different animals. No problem was encountered in measuring DHAC levels 4 h after drug administration since plasma concentrations were well above the limit of sensitivity of the assay.

CONCLUSION

The data presented above show that this HPLC assay is suitable for measuring DHAC concentrations in human patients. This method possesses sufficient sensitivity and specificity to determine DHAC pharmacokinetics, even when the drug is given as a 24-hr continuous infusion. This is the case for our Phase I clinical trial where the initial dose of DHAC will be 1000 mg/m^2/day (42

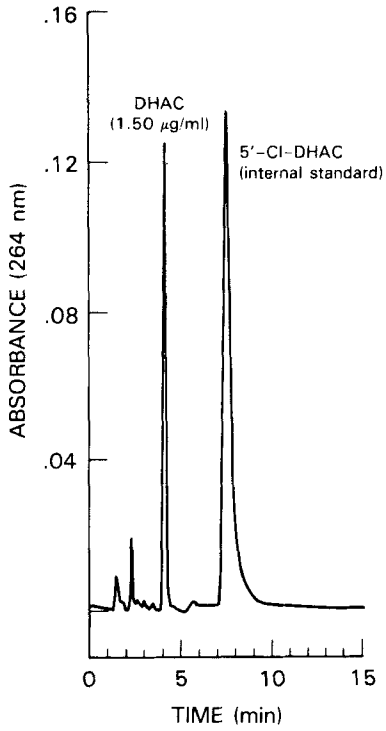


Figure 6. Representative HPLC Analysis of DHAC in Human Plasma. Chromatography conditions are as described in Methods. Normal human plasma was spiked with DHAC and incubated for 3 hr at 37°. A 0.5 ml aliquot of this plasma was diluted to 1.0 ml with distilled water and processed as indicated in Methods.

mg/m^2/hr). Based on extrapolation from single dose pharmacokinetics in the rat, steady state levels of only a few μg/ml DHAC are expected in humans at this entry level dose. Post-infusion plasma levels which will be used to determine pharmacokinetics will, of course, be much lower. Figure 6, which shows the analysis of DHAC in spiked human plasma after incubation at 37° to determine drug stability, indicates that there are no obvious interferences from endogenous plasma components and that adequate sensitivity does indeed exist. Determination of DHAC disposition

and pharmacokinetics in human patients participating in a Phase I clinical trial is currently in progress using this assay. Thus, a sample preparation method which combines the specificity of affinity and ion exchange chromatography for isolation from the biological matrix with the enhanced sensitivity of chromogenic derivatization allows the reverse phase HPLC determination of DHAC in plasma and urine.

ACKNOWLEDGMENTS

The authors thank Dr. Jerry M. Collins, Clinical Pharmacology Branch, NCI for the calculation of pharmacokinetic parameters and Dr. Louis Malspeis, the Ohio State University Cancer Center, for graciously providing prepublication details of an alternate HPLC method for measuring DHAC in plasma and urine. This assay has been used to determine the pharmacokinetics of DHAC in two male foxhounds (22). PNH also gratefully acknowledges partial support from the Swiss National Science Foundation.

REFERENCES

1. Piskala, A. and Sorm, F., Nucleic Acid Components and Their Analogs. II. Synthesis of 1-Glycosyl Derivatives of 5-Azauracil and 5-Azacytosine, Collection Czech. Chem. Commun., 29, 2060.

2. Karon, M., Sieger, L., Leimbrock, S., Finklestein, J.Z., Nesbit, M.E., and Swaney, J.J., 5-Azacytidine: A New Active Agent for the Treatment of Acute Leukemia, Blood, 42, 359, 1973.

3. McCredie, K.B., Bodey, G.P., Burgess, M.A., Gutterman, J.V., Rodriguez, V., Sullivan, M.P., and Freireich, E.J., Treatment of Acute Leukemia with 5-Azacytidine, Cancer Chemother. Rep., 57, 319, 1973.

4. Lomen, P.L., Baker, L.H., Neil, G.L., and Samson, M.K., Phase I Study of 5-Azacytidine (NSC-102816) Using 24-Hour Continuous Infusion for 5 Days, Cancer Chemother. Rep., 59, 1123, 1975.

5. Israili, Z.H., Vogler, W.R., Mingioli, E.S., Pirkle, J.L., Smithwick, R.W., and Goldstein, J.H., The Disposition and Pharmacokinetics in Humans of 5-Azacytidine Administered Intravenously as a Bolus or by Continuous Infusion, Cancer Res., 36, 1453, 1976.

6. Beisler, J.A., Isolation, Characterization, and Properties of a Labile Hydrolysis Product of the Antitumor Nucleoside, 5-Azacytidine, J. Med. Chem., 21, 204, 1978.

7. Beisler, J.A., Abbasi, M.M., Kelley, J.A., and Driscoll, J.S., Synthesis and Antitumor Activity of Dihydro-5-azacytidine, a Hydrolytically Stable Analog of 5-Azacytidine, J. Med. Chem., 20, 806, 1977.

8. Dihydro-5-Azacytidine Hydrochloride (DHAC, NSC 264880) Clinical Brochure, Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, December 1981.

9. Traganos, F., Staiano-Coico, L., Darzynkiewicz, Z., and Melamed, M.R., Effects of Dihydro-5-azacytidine on Cell Survival and Cell Cycle Progression of Cultured Mammalian Cells, Cancer Res., 41, 780, 1981.

10. Gibaldi, M. and Perrier, D., Pharmacokinetics, Marcel Dekker, New York, 1975.

11. Knott, G.D., MLAB-A Mathematical Modeling Tool, Comput. Programs Biomed., 10, 271, 1979.

12. Knapp, D.R., Handbook of Analytical Derivatization Reactions, John Wiley and Sons, New York, 1979, p. 599.

13. Poole, C.F., Recent Advances in the Silylation of Organic Compounds for Gas Chromatography in Handbook of Derivatives for Chromatography, Blau, K. and King, G., eds., Heyden, London, 1977, pp. 152.

14. Kelley, J.A., Abbasi, M.M., and Beisler, J.A., Silylation-Mediated Oxidation of Dihydropyrimidine Bases and Nucleosides, Anal. Biochem., 103, 203, 1980.

15. von Minden, D.L. and McCloskey, J.A., Mass Spectrometry of Nucleic Acid Components. N,O-Permethyl Derivatives of Nucleosides, J. Am. Chem. Soc., 95, 7480, 1973.

16. Einck, J.J., Pettit, G.R., Brown, P., and Yamauchi, K., Methylation of Pyrimdine Nucleosides, J. Carb. Nucl. Nucl., 7, 1, 1980.

17. Krstulovic, A.M., Brown, P.R., and Rosie, D.M., Identification of Nucleosides and Bases in Serum and Plasma Samples by Reverse-Phase High-Performance Liquid Chromatography, Anal. Chem., 49, 2237, 1977.

18. Gehrke, C.W., Kuo, K.C., Davis, G.E., Suits, R.D., Waalkes, T.P., and Borek, E., Quantitative High-Performance Liquid Chromatography of Nucleosides in Biological Materials, J. Chromatogr., 150, 455, 1978.

19. Zemlicka, J. and Holy, A., Preparation of N-Dimethylaminomethylene Derivatives - A New Method of Selective Substitution of Nucleoside Amino Groups, Coll. Czech. Chem. Comm., 32, 3159, 1967.

20. Thenot, J.P. and Horning, E.C., Amino Acid N-Dimethylaminomethylene Alkyl Esters. New Derivatives for GC and GC/MS Studies, Anal. Lett., 5, 519, 1972.

21. Miller, A.A., Benvenuto, J.A., and Loo, T.L., Comparison of the Reversed-Phase High-Performance Liquid Chromatography Separations of Fluoropyrimidines, Pyrimidines and Purines, J. Chromatogr., 228, 165, 1982.

22. Malspeis, L., Cheng, H. and Staubus, A.E.,, Pharmacokinetics of 5,6 Dihydro-5-azacytidine (NSC-264880) in the Foxhound, Inv. New Drugs, 1, 47, 1983.