The Disposition and Pharmacokinetics in Humans of 5-Azacytidine Administered Intravenously as a Bolus or by Continuous Infusion

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SUMMARY

The disposition of 5-[4-14C]azacytidine, administered i.v. as a bolus or continuous infusion, was studied in cancer patients. After bolus, plasma 14C levels exhibited a multiphasic disappearance pattern; half-life (t1/2, β phase) = 3.4 to 6.2 hr. Of 14C in plasma, <2% was associated with 5-azacytidine 30 min after dose. The ratios of 14C levels were: red cells/plasma, 0.8; leukocytes/plasma, 1.1 to 2.3; nucleic acids/leukocytes, 0.2 to 0.43; sputum/plasma, 0.05 to 0.17. Urinary excretion (3 days) accounted for 73 to 98% of 14C, <1% in feces. The relative concentration of 5-azacytidine in plasma with continuous infusion stayed higher than with bolus; urinary excretion was similar. Fewer side effects were observed with continuous infusion than with bolus. The stability of 5-azacytidine was determined in various media at several temperatures by thin layer chromatography and nuclear magnetic resonance. At 20°C in Ringer’s lactate (pH 6.2), the t1/2 was 94 to 100 hr. Stability increased with lowering of temperature and pH. From our data we conclude that 5-azacytidine should be given by continuous infusion rather than as a bolus.

INTRODUCTION

5-AC, a pyrimidine analog of cytidine (Chart 1) synthesized in 1964 (25), was shown to possess marked antibacterial (7, 24, 29) and cancerostatic properties (29). In mice, 5-AC decreased the number of circulating lymphocytes and mature bone marrow myeloid cells (30). The compound possesses remarkable inhibitory properties against rapidly proliferating tissues including various experimental neoplastic growths (17, 33, 34). 5-AC inhibits the synthesis of DNA (17, 27) and RNA (5, 13). The mechanism of action probably involves phosphorylation, incorporation into newly synthesized nucleic acids followed by fission of the triazine ring (26). It was found that 5-[14C]AC was phosphorylated in all leukemic tissues studied (17). Further, the drug has been shown to exhibit profound antitumor activity against murine L-1210 leukemia in vitro (17) and in vivo (30), against Walker 256 carcinoma (3), and against acute lymphoblastic leukemia in AK mice (30).

Studies of the phase specificity of 5-AC revealed that it acted predominantly in the S phase of the mitotic cycle (17) and was lethal to L-1210 cells grown in tissue culture (17). Lloyd et al. (18) noted that continuous exposure to low doses of 5-AC was more effective than was short exposure to larger doses and that the drug was relatively inactive in L-1210 cells maintained in nonproliferating state. However, there are some suggestions that the drug may not be purely cell cycle-phase specific and may impair cell structures even in the resting state (23).

5-AC has been found to be promising in the treatment of acute leukemia (1, 11, 14, 15, 35). The apparent instability of 5-AC (26) in solution has necessitated administration by rapid i.v. injection. In clinical trials (6, 15, 20, 21, 31, 32, 35, 37), this has resulted in severe nausea and vomiting. Kanon et al. (15) found that, in children, if the drug was infused over a period of 10 to 15 min or given in divided doses, gastrointestinal toxicity was reduced. Moertel et al. (21) found that, by dividing the dose, less nausea and vomiting occurred.

Thus, the in vitro data suggesting that continuous exposure was more effective and the clinical data indicating that intolerable gastrointestinal toxicity was a frequent side effect with bolus doses prompted us to reexplore the stability of 5-AC in solution. Furthermore, we compared the pharmacokinetic data obtained with continuous infusion and single injections using 5-[14C]AC in patients with metastatic cancer and leukemia.

MATERIALS AND METHODS

Patients, whose prior written informed consent was obtained, were selected for study on the basis of metastatic cancer or leukemia, life expectancy of at least 6 weeks, normal bone marrow and blood counts (except for leukemic patients), normal renal function, stable hepatic function, and no other existing disorder. Liver function tests included serum glutamate-oxalacetate transaminase, alkaline phosphatase, prothrombin time, and serum protein. Renal func-
tion tests included blood urea nitrogen and creatinine clearance. The details of the patients and the doses administered (mg/sq m and μCi) are given in Table 1. After admission, each patient was observed during a control period of 1 to 6 days. This was followed either by a treatment period of 1 day (for single bolus injection studies) or 6 days (for continuous infusion studies) and a follow-up period of 1 to 3 days. The diet was normal except that the patients were fasted from midnight until the morning of the treatment period.

The single i.v. dose of 5-[14C]AC was given at 8:00 a.m. For continuous infusion, the dose of 5-[14C]AC was started at 8:00 a.m. and continued over a 12-hr period. This treatment was then followed with 5-AC every 12 hr for 9 doses; other medications were allowed after the 1st 12-hr period. After a single i.v. dose, 10-ml samples of heparinized blood (with 1000 units of bovine lung heparin; Upjohn Co., Kalamazoo, Mich.) were obtained at predetermined intervals and immediately chilled in ice. Plasma was separated by centrifugation (500 × g, 30 min, 0-4°). Urine collections were made every 4 hr on the 1st day and every 24 hr on the 2nd and 3rd day. Daily collections of feces were made for several days. Vomitus, if any, was collected; specimens of sputum were obtained at specified times. In 1 case, leukocytes were also isolated (see below). With the continuous infusion protocol, 20-ml blood samples (heparinized) were collected at various time intervals after the beginning of infusion of 5-[14C]AC. Urine collections were made every 2 hr for the 1st 12 hr and then every 3 hr for the next 12 hr; thereafter, 24-hr collections were obtained for the next 3 days. Feces collections were made for 0 to 24 and 24 to 48 hr. In 1 case, a sample of cerebrospinal fluid was also obtained. All biological samples were stored below 0° and analyzed as soon as possible.

Dose Formulation

5-AC was obtained in a dosage form consisting of either (a) 50 mg of the drug and 100 mg of polyvinylpyrrolidone (plasdone-C-15), or (b) a 1:1 mixture of the drug with mannitol for i.v. use (Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Md.). The labeled compound (5-[14C]AC) was dissolved in distilled water and the solution was sterilized by passing through a disposable sterile filter (Swinnex 0.22 μm; Millipore Corp., Bedford, Mass.) and added to carrier 5-AC dissolved in sterile distilled water. This final solution was either injected immediately (in 8 to 10 min) as a bolus or added to 500 to 1000 ml of Ringer’s lactate (Hartmann’s McGaw Laboratories, Glendale, Calif.) for continuous infusion. The 1st 100 ml of solution were injected over a 20-min period and the remainder in the next 11 hr and 40 min.

An aliquot of the dose solution was tested in rats for the absence of pyrogens (19). Male Wistar rats (197 to 217 g, 4 in each group) were given s.c. injections (20 ml/kg) of either: Group A, sterile distilled water; Group B, a 20% suspension of brewers’ yeast in sterile water; or Group C, 1.4 × 10^-3 m 5-[14C]AC (0.3 μCi/animal) in sterile water passed through a sterile Swinnex filter. Rectal temperatures were measured by a thermocouple at 0, 4, and 24 hr after injections. The average temperatures for Groups A, B, and C were 37.1°, 37.5°, and 36.9° respectively. The stability of the solution of 5-AC in Ringer’s lactate was determined prior to infusion (see below).

TLC

TLC was carried out on: (a) Silica Gel G-coated glass plates without fluorescent indicator (Analtech Uniplate, Newark, Del.), and (b) Silica Gel G-coated plastic sheets with fluorescent indicator (Eastman Chromagram 6060). The plates were developed in System 1 (1-butanol: ethanol:water, 49:11:19, v/v/v) at room temperature. Radiochromatography was carried out on glass plates and 1-cm segments of silica gel were scraped and transferred into counting vials; 0.5 ml of methanol was added prior to counting fluid. 5-AC and related compounds (1 μl of 3.5 mM aqueous solutions) were spotted on fluorescent plates (6060) and, after development, the spots were visualized by quenching of fluorescence under UV light (254 nm). The Rf values for 5-AC, 5-azacytosine, and 5-azauracil were 0.35, 0.24, and 0.22, respectively.

Measurement of Radioactivity in Biological Materials and Extracts

Aliquots (up to 2 ml) of plasma or urine were mixed with 18 ml of counting fluid (prepared by mixing 7 g of PPO, 0.36 g of POPOP, 200 ml of Beckman Biosolv BBS-3, and 1 liter of toluene). They were counted alone or in the presence of known amounts of 5-[14C]AC in a Beckman LS-255 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) (counting efficiency, 90%). Similarly, 14C was measured in vomitus, sputum, WBC, and spinal fluid: for RBC, 0.3-ml aliquots were oxidized in a Beckman-Harvey combustion instrument, and the resulting 14CO2 was trapped in “Harvey carbon-14 cocktail” (R. J. Harvey Instrument Corp., Hillsdale, N.J.) (15 ml; counting efficiency, 70 to 75%). Recoveries (85 to 90%) of 14C after combustion were determined by oxidizing known amounts of 5-[14C]AC added to sucrose. Feces were homogenized with 100 to 500 ml of 95% ethanol. Ten-ml aliquots were centrifuged and 1 to 2 ml of the clear supernatant were counted. This procedure was found to be satisfactory since practically no radioactivity was detected in the residue upon oxidation.

Fresh plasma (3 ml) obtained from patients receiving 5-[^14]C]AC was diluted with 5 ml of 10 mM Tris buffer, pH 6.5. Then, 3 drops of 10% w/v oxalic acid were added (final pH, 6 to 6.5). After shaking, the mixtures were immediately shell frozen and lyophilized (VirTis Co., Gardiner, N.Y.). The residue was triturated with 10 ml of methanol, sonically extracted for 5 min, and centrifuged for 10 min at room temperature. The precipitate was reextracted with 10 ml of methanol. The final residue was discarded, since essentially no \(^{14}C\) was found in it. The methanol extracts were pooled and evaporated to dryness in a vacuum at room temperature. The resulting residue was redissolved in 1 ml of methanol. Aliquots of this solution were chromatographed (Silica Gel G-coated plates, without fluorescent indicator; Analtech Uniplate) using System 1.

Isolation of Leukocytes and Nucleic Acids

Leukocytes were isolated by the method of Hirsch (10), and the resulting residue was redissolved in 1 ml of methanol. Aliquots of this solution were chromatographed (Silica Gel G-coated plates, without fluorescent indicator; Analtech Uniplate) using System 1.
arm, 0.5 ml 1 N HCl; center well, paper wick plus 0.5 ml 1 N NaOH. The flask was tightly stoppered and the contents of the 1st side arm were tipped and mixed with the urea solution. After a 2-hr incubation at 37°, HCl was added to the reaction mixture to release \(^{14} \text{CO}_2\). After 24 hr, the contents of the central well were transferred into a scintillation vial. The well was washed with several small aliquots of water and the washings were also transferred into the scintillation vial and mixed with counting fluid, and \(^{14} \text{C}\) was measured.

**Measurement of Partition Coefficient and Binding of 5-\([^{14} \text{C}]\)AC**

The partition coefficient of 5-\([^{14} \text{C}]\)AC was measured at room temperature between \(\frac{1}{15}\) Sorenson buffer, pH 7.4 (28), and various organic solvents (chloroform, \(n\)-heptane, and peanut oil). An aqueous solution of 5-\([^{14} \text{C}]\)AC (10 ml, 10 \(\mu\)g, 0.01 \(\mu\)Ci) was shaken with buffer-saturated organic solvents (20 ml) for 40 min. After the phases were separated by centrifugation, \(^{14} \text{C}\) was measured in 2 ml of the buffer phase, 1 ml of peanut oil, and 2 ml of chloroform or \(n\)-heptane (after evaporation).

The binding of 5-\([^{14} \text{C}]\)AC to human albumin was measured at 37° and pH 7.4 by 2 methods: (a) equilibrium dialysis, and (b) molecular sieve (22).

**Equilibrium Dialysis.** Visking dialysis tubing (VWR Scientific, Atlanta, Ga.; 5/8 inch, size 20) was washed twice and then kept in distilled water for at least 24 hr and blotted dry. In 1 set of experiments, 2 ml of 5% w/v human serum albumin (crystallized, Pentex; Miles Laboratories, Kankakee, Ill.) were placed inside the dialysis bags. These were then placed in test tubes containing 10 ml of Sorenson buffer containing 100 \(\mu\)g, 0.01 \(\mu\)Ci of 5-\([^{14} \text{C}]\)AC (freshly prepared). In another set of experiments, 200 \(\mu\)g (0.02 \(\mu\)Ci) of 5-\([^{14} \text{C}]\)AC were added to 2 ml of albumin solution, which was then placed in the dialysis bag and dialyzed in 10 ml of Sorenson buffer. In these experiments, equilibration was achieved by shaking in air (100 cycles/min; Metabolyte Bath, New Brunswick, N. J.) at 37° for 4 hr. Leakage of protein through the dialysis tubing was tested with 40% w/v aqueous tri-chloroacetic acid. Radioactivity in the inside and outside phases was determined and the binding was calculated.

**Molecular Sieve Method.** Since 5-AC is not very stable in solutions at 37°, the binding of the drug to human serum albumin (5% w/v in Sorenson buffer, pH 7.4) at 37° was also measured by the molecular sieve method (which consumes much less time). The procedure used was essentially that described by Mu et al. (22).

**Stability Studies**

The stability of 5-AC in various media at several temperatures was studied by 2 methods: (a) TLC using 5-\([^{14} \text{C}]\)AC, and (b) NMR using 5-AC. For Method a, the compound was dissolved in water or buffer at various pH's and stored at appropriate temperatures (0–37°) for specific times. Aliquots were analyzed by quantitative TLC (silica gel plates, System 1) as described above. Radioactivity in the zone corresponding to 5-\([^{14} \text{C}]\)AC was determined. These values (as percentages) were plotted versus time and half-lives (\(t_{1/2}\) s) were calculated.

For Method b, 5-AC was dissolved in the appropriate medium and NMR studies were carried out at 9–37° on a Bruker Scientific HFX-90 spectrometer (Bruker Scientific, Inc., Elmsford, N. Y.). The spectra were taken at a width of 120 Hz and were time averaged over multiple scans (Nicolet 1074 computer). Tetramethylsilane and benzaldehyde in a 5-mm coaxial capillary served as external references. The rate of decrease (relative to the reference peak) in the height of the C-6 ring proton resonance of 5-AC was taken to be the rate of decomposition. The relative height of resonance peak for C-6 proton was calculated (sample peak height/reference peak height). Multiple regression analysis of the data was performed using 1st-order rate kinetics, and \(t_{1/2}\)'s at a 95% confidence level were determined.

NMR spectra at 40° were taken on a Varian A-60A spectrometer (Varian Associates, Palo Alto, Calif.). The rate of decrease of the area under the C-6 ring proton resonance of 5-AC (relative to the reference resonance) was assumed to be the rate of decomposition of 5-AC. The \(t_{1/2}\)'s were determined as described above.

**RESULTS**

**Studies in Patients**

**Single i.v. Dose.** Five patients received 5-\([^{14} \text{C}]\)AC as a single bolus dose; plasma levels of \(^{14} \text{C}\) (expressed as \(\mu\)g equivalents of 5-\([^{14} \text{C}]\)AC per ml of plasma) are shown in Table 2. The data were analyzed by computer, using a program developed by Dr. William Olson (Department of Medicine, Emory University). The plasma \(^{14} \text{C}\) levels (\(C_p\), 0 to 12 hr) fit the expression:

\[
C_p = Ae^{-\alpha t} + Be^{-\beta t}
\]

where \(A\) and \(B\) are the intercepts and \(k_\alpha\) and \(k_\beta\) are the slopes. The calculated values for \(A\) and \(B\), the \(t_{1/2}\) of \(\alpha\) and \(\beta\) phases, and the apparent volumes of distribution (\(V_j\)) are given in Table 2. The \(t_{1/2}\) of distribution phase (\(\alpha\)) ranged from 16 to 33 min, the \(t_{1/2}\) of \(\beta\) phase ranged from 3.4 to 6.2 hr, and \(V_j\) ranged from 0.58 to 1.15 liters/kg.

Preliminary studies indicate that the concentration of 5-\([^{14} \text{C}]\)AC in plasma declined much faster than total \(^{14} \text{C}\); thus in 1 patient (Patient 1), at 5, 10, 15, and 20 min after dose, the levels of 5-\([^{14} \text{C}]\)AC were 40, 37, 27, and 7%, respectively, of the total \(^{14} \text{C}\) in plasma. After 30 min the values for 5-\([^{14} \text{C}]\)AC were <2% of \(^{14} \text{C}\) in plasma. At least 2 metabolites and/or decomposition products of 5-AC were found in plasma by TLC.

The ratio of \(^{14} \text{C}\) levels in RBC to plasma was about 0.8 (RBC/plasma \(^{14} \text{C}\) concentration ratios for Patient 3 at 5, 15, and 30 min and 1 hr were 0.81, 0.82, 0.85, and 0.80, respectively). The ratio of concentration of \(^{14} \text{C}\) in leukocytes to plasma ranged from 1.1 to 2.3, while \(^{14} \text{C}\) associated with nucleic acids of WBC was 20 to 43% of \(^{14} \text{C}\) in leucocyte (Table 3).

The level of \(^{14} \text{C}\) secreted into the sputum was from 5 to 17% of plasma concentrations; the vomitus contained <0.1% of the dose (Table 2). The patients vomited about 6 times (range, 2 to 11) starting 1 hr after the dose (until 14 hr in some cases). The vomitus varied in color from light yellow...
Plasma levels of $^{14}$C in patients after administration of 5-$[^{14}]$CJAC

<table>
<thead>
<tr>
<th>Time after Plasma (µg/Leukocytes (µg/g)</th>
<th>After single i.v. dose</th>
<th>During and after continuous i.v. infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient 1</td>
<td>Patient 2</td>
</tr>
<tr>
<td>5 min</td>
<td>11.9</td>
<td>10.6</td>
</tr>
<tr>
<td>10 min</td>
<td>9.3</td>
<td>9.7</td>
</tr>
<tr>
<td>15 min</td>
<td>8.9</td>
<td>9.3</td>
</tr>
<tr>
<td>30 min</td>
<td>8.0</td>
<td>8.6</td>
</tr>
<tr>
<td>45 min</td>
<td>7.0</td>
<td>7.8</td>
</tr>
<tr>
<td>1 hr</td>
<td>6.5</td>
<td>7.2</td>
</tr>
<tr>
<td>2 hr</td>
<td>4.6</td>
<td>6.1</td>
</tr>
<tr>
<td>3 hr</td>
<td>3.7</td>
<td>5.1</td>
</tr>
<tr>
<td>4 hr</td>
<td>2.8</td>
<td>4.3</td>
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<td>12 hr</td>
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<tr>
<td>16 hr</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>20 hr</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>24 hr</td>
<td>0.44</td>
<td>0.44</td>
</tr>
<tr>
<td>32 hr</td>
<td>0.44</td>
<td>0.44</td>
</tr>
<tr>
<td>48 hr</td>
<td>0.46</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* For single i.v. dose, the time indicated is after drug administration; for continuous infusion, it is from start of infusion. For doses, see Table 1.

** Computer analysis of the 0- to 12-hr data of Patients 1 to 5 fits a 2-compartment model (r > 0.95). The t<sub>1/2</sub>'s of distribution phase (α) for these patients were: Patient 1, 33 min; Patient 2, 28 min; Patient 3, 16 min; Patient 4, 22 min; and Patient 5, 17 min. The t<sub>1/2</sub>'s for β phase were: 4.7, 5.5, 3.4, 6.2, and 3.5 hr, respectively. The corresponding apparent volumes of distribution were 0.62, 0.56, 0.60, 1.15, and 0.58 liters/kg, respectively. The vomitus contained 0.01, 0.09, 0.03, and 0.02% of dose of $^{14}$C in Patients 1, 2, 3, and 4, respectively; $^{14}$C (5-AC equivalent) in the sputum obtained at the indicated time was as follows: Patient 1, 1.5 hr, 0.27 µg/ml; Patient 2, 0.5 hr, 0.8 µg/ml, and 1 hr, 0.55 µg/ml; and Patient 3 1 hr, 1.22 µg/ml.

Continuous Infusion. Three patients received 5-$[^{14}]$CJAC to green; pH ranged from 7.5 to 7.9. Nausea was pronounced and was partially controlled by chlorpromazine or prochlorperazine.

Most of the administered $^{14}$C appeared in urine: 69 to 91% and 73 to 98% in 1 and 3 days, respectively, while less than 1% of the dose was present in the feces (Table 4). The calculated amount of $^{14}$C remaining in the body showed considerable individual variations in these patients; in 1 case about 27% of the dose was apparently present even after 3 days.

The renal clearance of $^{14}$C (drug + metabolite) for the 1st 12 hr after bolus dose varied from 74 to 210 ml/min (Table 4).

### Table 2

**Disposition of 5-AC in Humans**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>44</td>
<td>38</td>
<td>44</td>
<td>22</td>
<td>47</td>
</tr>
<tr>
<td>0-8</td>
<td>67</td>
<td>58</td>
<td>73</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>0-12</td>
<td>78</td>
<td>71</td>
<td>82</td>
<td>58</td>
<td>66</td>
</tr>
<tr>
<td>0-24</td>
<td>90</td>
<td>85</td>
<td>91</td>
<td>69</td>
<td>69</td>
</tr>
<tr>
<td>0-48</td>
<td>96</td>
<td>93</td>
<td>94</td>
<td>77</td>
<td>71</td>
</tr>
<tr>
<td>0-72</td>
<td>98</td>
<td>95</td>
<td>95</td>
<td>80</td>
<td>73</td>
</tr>
</tbody>
</table>

* Percentage of dose of $^{14}$C excreted in feces was as follows: Patient 1, 0.01 (0 to 2 days); Patient 2, 0.36 (0 to 1 day); Patient 3, 0.98 (0 to 2 days); and Patient 4, <0.01 (0 to 2 days). The renal clearance (ml/min) of $^{14}$C (5-ACJAC and metabolites) for 0-4 hr, 4-8 hr, and 8-12 hr intervals were as follows: Patient 1, 107, 128, and 210; Patient 2, 115, 118, and 160; Patient 3, 125, 178, and 127; Patient 4, 74, 175, and 93; Patient 5, 127, 77, and 79.

Urea was isolated from the urine of Patient 1 and was crystallized twice from 95% ethanol. By TLC, this fraction was shown to be contaminated with materials cocrystallized with urea. $[^{14}]$C[Urea] was quantitatively measured by converting it to $^{14}$CO<sub>2</sub> by urease. Of the excreted $^{14}$C in 0-4 hr, 4-8 hr, and 8-12 hr-urines, 0.8, 1.6, 2.9, and 5.7%, respectively, was associated with urea.

### Continuous Infusion. Three patients received 5-$[^{14}]$CJAC
by continuous infusion. Plasma levels and urinary excretion of $^1$H were presented in Tables 2 and 5. In one case (Patient 7, at 6 hr after beginning of infusion) the spinal fluid to plasma concentration ratio of $^1$H was 0.08. At 15 and 30 min and 1, 2, 4, and 6 hr after beginning of infusion, plasma 5-[^1]H/AC levels were 70, 46, 42, 34, 15, and 13%, respectively, of the total $^1$H concentration in plasma. Urinary excretion of $^1$H (0 to 24 hr) amounted to 85, 83, and 94% of the dose (Table 5) in the 3 patients, respectively.

### Partition Coefficient and Binding of 5-[^1]H/AC

The partition coefficient of 5-[^1]H/AC (concentration in the organic phase/concentration in the aqueous phase) was found to be <0.005 for all 3 systems studied.

The binding of 5-[^1]H/AC to 5% w/v human albumin in Sorensen buffer at pH 7.4, 37°, was found to be <1% both by equilibrium dialysis and molecular sieve procedures.

### Stability of 5-AC in Solutions

Freshly prepared solutions of 5-[[^1]H]AC were incubated in various media at several temperatures and analyzed by TLC. For NMR the solutions of 5-AC were allowed to remain in the probe for the duration of the experiment.

**TLC Method.** Almost all of the spotted radioactivity at time 0 was found to be in the zone corresponding to 5-AC. This value was assumed to be 100%. The radioactivity (as percentage of control) associated with zones corresponding to 5-[[^1]H]AC was measured at 1/2, 1, 2, 4, 6, 12, and 24 hr after incubations at 0°, 25°, and 37° in various media. The decomposition of 5-[[^1]H]AC followed 1st-order kinetics; the $t_{1/2}$'s of decay are given in Table 6. 5-AC was more stable in Tris buffer (10 m/M) than in other media. A summary of stability studies is given in Table 6.

### Stability studies of 5-AC

<table>
<thead>
<tr>
<th>Medium $^b$</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>NMR</td>
</tr>
<tr>
<td>EDTA (10^{-4} M)/N$_2$</td>
<td>40°</td>
</tr>
<tr>
<td>Tris buffer (10^{-5} M)</td>
<td>NMR</td>
</tr>
<tr>
<td>Ringer’s lactate</td>
<td>NMR</td>
</tr>
<tr>
<td>Ringer’s lactate-EDTA (10^{-5} M)/N$_2$</td>
<td>NMR</td>
</tr>
<tr>
<td>Human plasma (fresh)</td>
<td>NMR</td>
</tr>
<tr>
<td>Human plasma (stored)</td>
<td>TLC</td>
</tr>
<tr>
<td>Human plasma (M/15)</td>
<td>TLC</td>
</tr>
<tr>
<td>Sorensen buffer (M/15)</td>
<td>TLC</td>
</tr>
<tr>
<td>Tris buffer (10^{-2} M)</td>
<td>TLC</td>
</tr>
<tr>
<td>Ringer’s lactate</td>
<td>TLC</td>
</tr>
<tr>
<td>Human plasma (fresh)</td>
<td>TLC</td>
</tr>
<tr>
<td>Human urine</td>
<td>TLC</td>
</tr>
<tr>
<td>Ringer’s lactate</td>
<td>NMR</td>
</tr>
<tr>
<td>Ringer’s lactate</td>
<td>NMR</td>
</tr>
<tr>
<td>Tris buffer (10^{-2} M)</td>
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</tr>
<tr>
<td>Human plasma (fresh)</td>
<td>TLC</td>
</tr>
<tr>
<td>Human urine</td>
<td>TLC</td>
</tr>
</tbody>
</table>

- $^a$ Concentration of 5-AC = 30 mM (NMR) and 5-[[^1]H]AC = 3.5 mM (TLC).
- $^b$ Each $t_{1/2}$ determination represents a single experiment. In the NMR studies, experiments were carried out for 170 to 190 min (0.5 to 2.0 half-lives). For the TLC, incubations were carried out up to 48 hr.
- $^c$ Each $t_{1/2}$ is ± 95% confidence level.
- $^d$ EDTA, aqueous disodium EDTA.
- $^e$ The $R^2$ values for these 2 determinations were between 0.90 and 0.93; for others, >0.96 (NMR).
- $^f$ Fresh plasma for each experiment was obtained from different individuals. Stored plasma was stored at 0° for 1 to 10 weeks.
- $^g$ pH was not controlled.
buffer (pH 6.3, 10^{-2} \text{ M}) or Ringer's lactate at pH 6.2 and least stable in fresh heparinized plasma.

**NMR Method.** The resonance due to the proton at C-6 of 5-AC occurred at 8.7 ppm (single) in D_2O solution at 20\(^\circ\); the aldehyde proton of benzaldehyde resonated at 9.8 ppm relative to the reference, tetramethylsilane in the capillary. In Ringer's lactate at 20\(^\circ\), the resonances due to C-6 and benzaldehyde protons occurred at 9.0 and 9.9 ppm, respectively. These chemical shifts varied slightly with temperature and the composition of the medium. The decomposition of 5-AC (in D_2O) is accompanied by the progressive disappearance of the resonance at 8.7 ppm and the appearance of a new peak of 8.5 ppm (Chart 2). The addition of formic acid to the partially decomposed solution of 5-AC increased the intensity of the peak at 8.5 ppm. The resonance for C-6 protons of azacytosine or 5-azauracil occurred at 8.2 ppm (in alkaline D_2O solution).

For quantitative purposes 16 to 32 scans were taken with an accumulation time of 27 to 54 min. The molarities of the protons present were assumed to be proportional to the peak heights of the corresponding resonances. Relative peak heights were determined relative to the aldehyde proton resonance of benzaldehyde. Multiple regression analysis of the data was performed using 1st-order kinetics and the \( t_{1/2} \)'s at a 95% confidence level are shown in Table 6.

**DISCUSSION**

The disposition of 5-[\(^{14}\)C]AC was studied in a limited number of patients. The drug was administered i.v. either as a single bolus dose or as a continuous infusion; a comparison of the 2 modes of administration was 1 of the major objectives of this study.

A clinical evaluation of these 2 modes of therapy has been carried out by the Southeastern Cancer Study Group. In a Phase I study of twice-weekly rapid i.v. injections (35), it was found that all patients had severe gastrointestinal toxicity when a single dose exceeded 100 mg/sq m. When given by continuous i.v. infusion, less gastrointestinal toxicity occurred in doses less than 200 mg/sq m (36). In the present study, similar observations were made. Thus the continuous infusion is a more tolerable method of administration.

The plasma level decline of \(^{14}\)C after the bolus dose of 5-[\(^{14}\)C]AC was multiphasic; the pattern was similar to that obtained by Troetel et al. (32). The drug distributes rapidly after administration. The apparent volume of distribution of the drug \( (V_d) \) agreed with: (a) the low organic/aqueous partition coefficient and (b) the lack of binding to human albumin. In 1 patient (leukemic) higher \( V_d \) may have been due to selective uptake and incorporation of the drug (and/or metabolites) by the leukocytes. The levels of \(^{14}\)C RBC (80% of plasma levels) reflect the lack of binding of 5-AC to plasma proteins; it is also an indication of the absence of binding to the constituents of the red cells.

A small but measurable fraction of the administered radioactivity appeared in the vomitus, suggesting gastric and/or biliary secretion of the drug (and/or metabolites). The salivary \(^{14}\)C levels were about 10% of the plasma levels. The spinal fluid to the plasma concentration ratio of \(^{14}\)C was similar to that reported previously (32).

The concentration of 5-[\(^{14}\)C]AC in plasma was determined in a preliminary fashion. At least 2 metabolites and/or decomposition products of 5-AC were found in plasma. The initial plasma level pattern of 5-AC after a single bolus injection reflects at least 3 processes: (a) the distribution phase, (b) metabolism, and (c) nonenzymatic degradation. At 30 min after the administration of the drug, plasma levels of \(^{14}\)C were 70% of the concentration at 5 min. However, \(^{14}\)C associated with the parent drug was <2% of the total, indicating rapid metabolism and degradation. The maximum nonenzymatic decomposition (both in plasma and tissues) probably accounts for only 20% of the overall decrease of 5-AC in plasma at 30 min (see later).

In patients who received the dose by continuous infusion, plasma \(^{14}\)C levels indicated some accumulation. In contrast to the bolus dose, \(^{14}\)C associated with the parent drug in plasma was much higher; after 6 hr of continuous infusion about 13% of plasma \(^{14}\)C was present as the parent drug.

We sought optimum conditions for (a) preparation and storage of solutions of 5-AC for continuous infusion and (b) analysis of 5-AC in biological materials. Therefore a study of the stability of the drug was carried out in solution at various pH's, temperatures, and media.

The stability of 5-AC was measured by 2 independent methods: (a) TLC, using 5-[\(^{14}\)C]AC, and (b) NMR, using nonlabeled compound. Studies with TLC were done in a preliminary fashion and the results confirm data obtained from the NMR studies. The stability studies with NMR are more reliable, since we measured the disappearance of the resonance peak corresponding to the proton at C-6 of the pyrimidine moiety and the simultaneous appearance of a new peak. Since the addition of formic acid to this partially decomposed solution of 5-AC resulted in an increase in the intensity of the new peak, it is possible that it is due to formic acid formed from the N-formyl intermediate pro-
posed by Pithova et al. (26) and not due to decomposition products of 5-AC (5-azacytidine and/or 5-azauracil).

Although our data are not sufficient to propose a mechanism for the decomposition of 5-AC, one may write a scheme for the decomposition similar to that proposed by Pithova et al. (26). The N-formyl intermediate yields formic acid and 1-β-D-ribofuranosyl-3-guanuridylic acid. The formic acid may contribute partially to the toxic and therapeutic effects of 5-AC.

In fresh human plasma at 37°, 5-AC was fairly unstable. The stability increased by lowering of temperature. In distilled water at pH 6.5 and 40°, t₁/₂ was 4.4 hr; the addition of disodium EDTA and bubbling the solution with nitrogen doubled the t₁/₂. It is possible that disodium EDTA chelates ions, e.g., iron which may still be present at very low concentrations in distilled water, which may act as a catalyst in the decomposition of 5-AC. Bubbling with nitrogen (to remove dissolved oxygen) may also have increased the stability. However, when the same technique (EDTA and N₂) was used with Ringer’s lactate, only a small increase in t₁/₂ was observed. Apparently, the small amount of EDTA added to the solution was used up by calcium present in Ringer’s lactate.

The temperature and pH studies indicated that decomposition of 5-AC was dependent on both of these parameters. The stability increased with a decrease in pH or temperature.

The stability of 5-AC was determined in Ringer’s lactate since the latter is used as a medium for infusion of 5-AC in humans. In Ringer’s lactate, 5-AC was fairly stable at 20° (pH 6.2; t₁/₂ = 94 to 100 hr; for 10% decomposition the value was 14 to 15 hr). From these data we have concluded that, if the drug solution is prepared every 8 hr and infused at room temperature, the decomposition would not be more than 10% over the infusion period. The decomposition would be even less if the drug solution could be maintained at a still lower temperature during infusion.

Earlier studies of stability of 5-AC (4, 26) were done either under nonphysiological conditions such as high temperature (50–100°) or at acid or alkaline pH. Cihak and Sorm (4) studied the stability by determining the loss of biological activity of 5-AC (by measuring the inhibition of the growth of Escherichia coli). Pithova et al. (26) studied the hydrolysis of 5-AC by using paper chromatography and changes in molar absorbity. The reliability of the latter method is doubtful, since the absorbance (at 255 nm) of the solution of 5-AC increases initially with time and then decreases.

From our studies we conclude that 5-AC should be given to patients by continuous infusion rather than as a single bolus. This manner of administration results in lower incidence of side effects (36); plasma concentration of the parent drug stays higher for a longer period of time.

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Addendum

After submission of this manuscript, we studied the stability of 5-AC in the formulation used for i.v. injection (containing 1:1 mannitol) at room temperature by NMR. The pH of a 0.03 M solution of the injectable drug in Ringer’s lactate was 7.1 (for pure 5-AC, to pH 6.4 under the same conditions). The t₁/₂ of decomposition of the injectable drug (0.03 M) at pH 7.1 and 6.3 (adjusted with HCl) in Ringer’s lactate at 20 ± 1° were 69 ± 3 and 80 ± 4 hr, respectively.

By ['³C]NMR it was found that one of the products of the decomposition of 5-AC in water was formic acid. This is in agreement with the data obtained from the proton-NMR study.

The degradation of 5-AC has recently been studied by a UV method (Pitari, R. E., and DeYoung, J. L. Kinetics and Mechanism of Degradation of the Antileukemic Agent 5-Azacytidine in Aqueous Solutions. J. Pharm. Sci., 64: 1148–1157, 1975).

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