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FACTORS INFLUENCING THE CHROMATOGRAPHIC ANALYSIS OF 2,2'-O-CYCLOCYTIDINE

R. L. FURNER, J. D. STROBEL, S. M. EL DAREER and L. B. MELLETT
Southern Research Institute, Birmingham, Ala. 35205 (U.S.A.)
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SUMMARY

The analysis of cyclocytidine using radioactive tracer techniques coupled with instant thin-layer chromatography depends upon maintaining the stability of the compound throughout the analysis. At pH 7.0 or below cyclocytidine is stable in phosphate, citrate, and formate buffer, but the addition of heavy metal ions to these buffers may yield unstable conditions.

The data emphasize the need for carefully controlled conditions in the analysis of cyclocytidine. In general, the data are in agreement with previous reports in that cyclocytidine is hydrolyzed to arabinosylcytosine in alkaline solutions. Based upon the experiments reported herein, we suggest that the hydrolysis of cyclocytidine is non-enzymatic, and that a number of factors related to sample handling and chromatographic conditions will influence the rate of hydrolysis.

INTRODUCTION

2,2'-O-Cyclocytidine is an antitumor agent which is structurally related to arabinosylcytosine (ara-C) and arabinosyluridine (ara-U). The relationship has both chemical and biological significance since the following sequence of reactions transforms cyclocytidine to ara-U.

However, the reactions are essentially irreversible under biological conditions. Cyclocytidine offers some advantages over ara-C in that it appears to be resistant to deamination by cytidine aminohydrolase[1]. Furthermore, Neil[2], Venditti *et al.*[3], and others have observed better single-dose antitumor activity with cyclocytidine than with ara-C.

It has been suggested that cyclocytidine produces its antitumor effects through the release of ara-C by hydrolysis. But little is known of the mechanism for that hydrolysis, or indeed of the biological disposition of cyclocytidine, in different animal species and in man. This report details some of the analytical problems which have complicated the assay of cyclocytidine.

DEVELOPMENT OF METHODOLOGY

Two methods of analysis were sought for the biological measurement of cyclocytidine and its metabolites. One involved the use of UV spectrophotometry and will be described in detail elsewhere. The other method depends upon the use of instant thin-layer chromatography (ITLC). It is the latter technique which we shall discuss in detail here.

The development of a reliable analytical technique involved a detailed analysis of the mobility of cyclocytidine and its metabolites in ITLC and an attempt to determine the conditions under which cyclocytidine is reasonably stable. Mobility and stability are discussed in the following sections.

Mobility

Protein precipitation. The use of alcohol to precipitate tissue proteins is a common and convenient technique. The supernatant of such a precipitated system can be condensed in volume with considerable increase in sensitivity. It was shown, however, by the following experiment, that cyclocytidine is altered by some component in the alcoholic supernatant.

^{14}C-Cyclocytidine, 2 mg/ml (0.09 μCi/mg), was added to monkey serum at a final concentration of 200 μg/ml. The serum proteins were precipitated with four volumes of ethanol. The supernatant (100 ml) was spotted directly upon Gelman SA20 ITLC media at the time periods indicated. "Zero time" was the time at which the first sample was spotted after the alcoholic precipitation of proteins was complete. Chromatograms were developed in chloroform–methanol (50:50). The dried chromatograms were cut into channels 2.54 cm wide and each channel was further divided into 0.5-cm strips. Each strip was placed in a counting vial and eluted with 1 ml of water. Scintillation solution (10 ml) was added and the radioactivity measured for each vial. The data are expressed as observed counts per strip *versus* vial number. R_F values are given for highest levels of radioactivity only. An aqueous standard of [^{14}C]cyclocytidine yielded a single peak at R_F 0.14. Each spot (100 μl alcoholic supernatant) contained about 1200–1300 cpm.

As can be seen in Fig. 1, cyclocytidine moved from R_F 0.14 when spotted from the stock solution to R_F 0.37 after 5 min in the alcoholic supernatant. After 4 h, cyclocytidine had hydrolyzed to ara-C (25%). Confirmation of the identity of spots at R_F 0.37 and R_F 0.50 was by UV analysis. When serum proteins were precipitated with tungstate or $ZnSO_4$, no conversion to ara-C was observed and the mobility of cyclocytidine was restricted to R_F 0.16. A more convenient method of precipitation was to use a mixture of formalin and ethanol (10:90), which allowed easier volume reduction than did the heavy metal salt solutions.

Salt concentrations. It was determined that various concentrations of NaCl would alter the mobility of cyclocytidine on Gelman Type SA ITLC media. Table I describes a few such alterations.

For the standard to be representative of the samples, it must be run in a solution of the same ionic strength and character. It is interesting that the character of the spots was different for different salts.

pH. If the salt concentration was kept constant, the mobility of cyclocytidine on the chromatogram was influenced by the pH of the medium from which it was

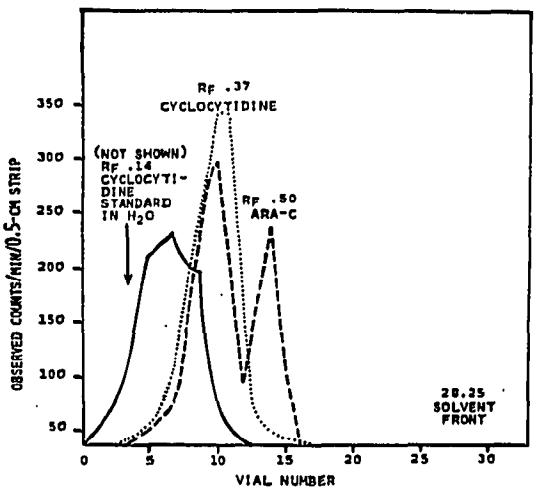


Fig. 1. Chromatographic profile of cyclocytidine after incubation in monkey serum. ——, Zero time; . . . ., 5 min; – – –, 4 h.

TABLE I

SALT CONCENTRATION AND CYCLOCYTIDINE MOBILITY

Sodium chloride was added to solutions containing cyclocytidine, 5 mg/ml. Of each solution 5 μl was spotted on Gelman SA chromatography medium and developed in chloroform–methanol (50:50). After drying, the spots containing cyclocytidine (25 μg) were visualized under UV light.

	Concentration of NaCl				
	0.308 M	*0.015 M*	*0.008 M*	*0.0015 M*	*0.0008 M*
R_F	0.17	0.15	0.13	0.06	0.06

spotted. Table II shows that cyclocytidine moved further from the origin when spotted from a solution at pH 2 than when spotted from a solution at pH 5.5. A mitigating factor is also the solvent system in which the chromatogram is developed, since only a small pH effect upon mobility was noted in the system utilizing butanol and water.

Solvent systems. In many cases the character of the material spotted on ITLC media may influence the character of the solvent system *per se*. It is tempting to discount the influence of so small an amount of material, but as the following tables and figures will show, the influence is significant.

Table III shows a few of the different solvent systems which will alter the mobility of cyclocytidine, ara-C and ara-U on ITLC. Notice that cyclocytidine is very susceptible to changes in acid concentration but not so much to organic solvent composition, whereas ara-C and ara-U are influenced by solvent composition but not by its acidity. Fig. 2 shows the mobility of cyclocytidine increasing as the con-

TABLE II

INFLUENCE OF pH OF THE MIXTURE SPOTTED ON THE MOBILITY OF CYCLOCYTIDINE

Cyclocytidine was dissolved in 0.1 *M* solutions of citrate or formate at the pH indicated. The final concentration of cyclocytidine was 5 μg/ml in all cases. The solutions were spotted on Gelman SA chromatography medium and dried before developing in chloroform–methanol (50:50) (S_1) or butanol–water (86:14) (S_2). The chromatograms were dried and the spots visualized under UV light.

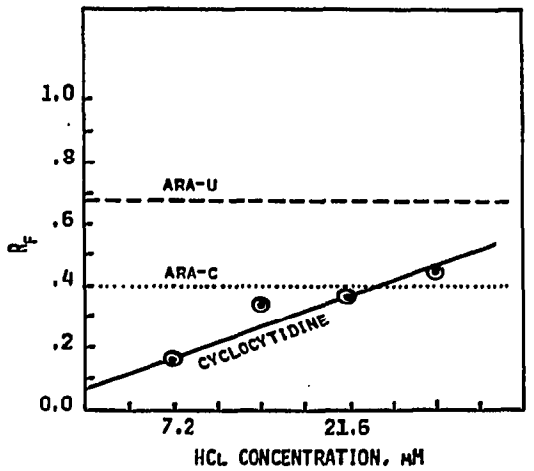
Solution before spotting	*pH*	R_F *value*	
		S_1	S_2
0.1 *M* citrate	2.0	0.21	0.08
	3.0	0.21	0.02
	3.5	0.17	0.02
	4.0	0.17	0.01
	4.5	0.17	0.01
	5.0	0.10	0.01
	5.5	0.01	0.00
0.1 *M* formate	2.3	0.10	0.07
	3.0	0.10	0.07
	3.5	0.10	0.07
	4.0	0.09	0.11
	4.5	0.07	0.11
	5.0	0.07	0.11
	5.5	0.04	0.11

TABLE III

INFLUENCE OF ACIDIFIED SOLVENT SYSTEMS UPON THE MOBILITY OF CYCLOCYTIDINE, ARA-C AND ARA-U

Solvent system	R_F *value*		
	Cyclocytidine	*Ara-C*	*Ara-U*
Butanol–0.001 *N* HCl (86:14)	0.07	0.45	0.72
Butanol–0.1 *N* HCl (86:14)	0.18	0.47	0.67
Methanol–0.1 *N* HCl (99:1)	0.18	0.74	0.80
Butanol–0.1 *N* HCl (75:25)	0.18	0.52	0.76
Chloroform–methanol–0.1 *N* HCl (90:9:1)	0.14	0.75	0.82
Chloroform–methanol–0.1 *N* HCl (80:19:1)	0.14	0.72	0.80
Methanol–0.1*N* HCl (99.9:0.1)	0.09	0.76	0.82
Chloroform–methanol–0.1 *N* formic acid (80:19:1)	0.16	0.70	0.82
Methanol–butanol–0.1 *N* HCl (80:19:1)	0.16	0.75	0.82
Chloroform–methanol–acetic acid (50:50:0.06)	0.19	0.60	0.77
Butanol–acetic acid (100:0.06)	0.05	0.17	0.53
Chloroform–methanol–acetic acid (75:25:0.06)	0.05	0.20	0.56
Chloroform–methanol–acetic acid (60:40:0.06)	0.09	0.43	0.73
Chloroform–methanol–conc. HCl (60:40:0.06)	0.38	0.46	0.74
Chloroform–methanol–conc. HCl (60:40:0.02)	0.19	0.45	0.75

centration of HCl increases from 7.2 m*M* to 21.6 m*M*. Fig. 3 demonstrates that altering the ratio of chloroform to methanol+chloroform does not alter cyclocytidine mobility



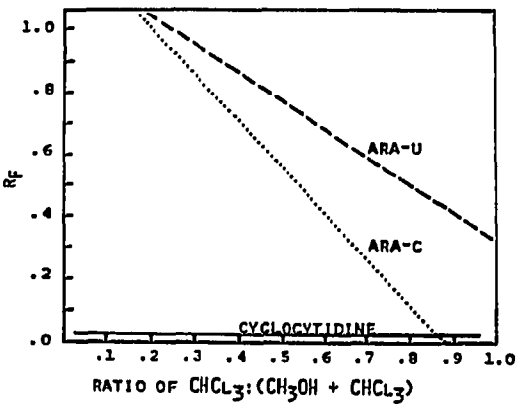


Fig. 2. Influence of the concentration of HCl on the mobility of cyclocytidine in chloroform–methanol (60:40).

Fig. 3. Influence of the ratio of chloroform to methanol+chloroform on the mobility of cyclocytidine, Ara-C, and Ara-U. The acid content was maintained at zero. The R_F values were determined by visual inspection under UV light.

From the foregoing it is obvious that great care must be taken in establishing the analytical conditions under which cyclocytidine and its metabolites are to be measured. The pH, salt concentration, and methods of protein precipitation are very important. To have a positive identification of the materials to be identified, standards must be run in the tissue system from which samples will be obtained.

Stability

Establishing a reliable analytical method was difficult since cyclocytidine is unstable in alkaline solutions. Ara-C is quickly formed hydrolytically at pH 8 or above. The critical question is, "How low must the pH be in order to stabilize cyclocytidine?"

pH and certain ions. Hydrolysis depends upon more than pH. Table IV gives a survey of conditions causing hydrolysis. For example, cyclocytidine is stable in phosphate buffer pH 7.4, but in Tris buffer pH 7.4 hydrolysis proceeds at about 15%/h.

At pH 7.0 in bicarbonate buffer no hydrolysis was revealed after 2 h if the solution was analyzed by UV spectrophotometry where the solution was scanned from 400 to 200 nm by a dual-beam instrument. Had hydrolysis been observed, a shift in the absorption spectra would have been immediately apparent. But when the solution was spotted and heat-dried on ITLC, nearly 75% of the cyclocytidine

TABLE IV

FACTORS INFLUENCING THE HYDROLYSIS OF CYCLOCYTIDINE TO ARA-C

Condition	*Rate of hydrolysis*
Phosphate buffer, pH 6.0	0
pH 6.5	0
pH 7.0	0
pH 7.5	< 1%/h
pH 8.0	> 50%/h *
Tris buffer, pH 7.4	15%/h
$ZnSO_4$–$Ba(OH)_2$, pH 6.9	> 10%/h *
20% $ZnSO_4$–10% formalin	> 50%/day *
Water, 100°	40%/h
37°	1%/day
22°	1%/week

* Estimated from visualization under UV light or from changes in the character of the UV spectrum. Quantification was not attempted. The data are from experiments designed to establish conditions under which cyclocytidine is stable. No attempt has been made to rationalize the different factors which lead to the hydrolysis of cyclocytidine.

was converted to ara-C. This suggested that as the bicarbonate solution dried, the following reaction took place:

$$NaHCO_3 \xrightarrow{+\triangle} NaOH + CO_2\uparrow$$

yielding a very alkaline medium, if for only a short time.

Hydrolysis was observed at pH 6.9 in a solution of $ZnSO_4$ and $Ba(OH)_2$ and also in a solution containing 20% $ZnSO_4$ and 10% formalin. Although cyclocytidine does hydrolyze to ara-C in heated solutions, the rate is only about 40% per hour at 100°. At 37° the rate is about 1% per day, and at room temperature the rate of hydrolysis is about 1% per week.

Mechanism of hydrolysis. To determine if cyclocytidine might be hydrolyzed by an enzyme in the serum as suggested by Hayashikawa and Nagyvary[4], we boiled the serum for 30 min. Proteins were visibly denatured, but hydrolysis of cyclocytidine was observed after addition of the drug to the supernatant. The experiment was carried out as follows. Two vessels were prepared, each containing 1 ml of monkey serum. Vessel A was placed in a boiling water-bath for 30 min while vessel B remained at room temperature. To both A and B were added 4 ml absolute ethanol and the vessels were shaken to mix the alcohol and serum. (In vessel A the coagulated protein was broken up into fragments using a glass stirring rod.) The solutions were centrifuged at $500 \times g$ at 5° for 30 min and the supernatants transferred to clean vials containing 50 μl [^{14}C]cyclocytidine (100 μg) with a specific activity of 0.18 μCi/mg. The mixture was incubated at room temperature. At zero time and at 4 h, 100 μl of the mixture were spotted on Gelman SA ITLC medium, and the chromatogram was developed in chloroform–methanol (50:50). The dried chromatogram was segmented and placed into scintillation vials for quantification. The results can be found in Fig. 4.

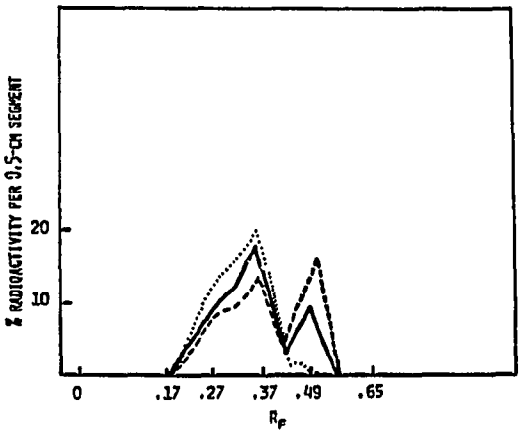


Fig. 4. Hydrolysis of cyclocytidine by an alcoholic extract of boiled serum. . . . ., Zero time, boiling and no boiling; ——, 4-h incubation after boiling; – – –, 4-h incubation, no boiling.

Alcohol was added to the supernatant to eliminate any enzymes left after heat denaturation. Hydrolysis of cyclocytidine persisted. This experiment suggests very strongly that all hydrolysis of cyclocytidine is non-enzymatic. Such a possibility is supported by direct evidence that hydrolysis of cyclocytidine proceeds in a non-biological environment at a pH similar to that found in serum[5,6].

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