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A USEFUL THIN-LAYER CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF CYTIDINE AMINOHYDROLASE ACTIVITY IN SERUM

L. T. MULLIGAN, JR. AND L. B. MELLETT
Kettering-Meyer Laboratories, Southern Research Institute, Birmingham, Ala. 35205 (U.S.A.)

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SUMMARY

A thin-layer chromatography method for the determination of serum cytidine aminohydrolase activity is presented. The method utilizes a tungstic acid deproteinization reagent and the use of new precoated thin-layer sheets to facilitate separation of substrates from their metabolite products. The method has been found to be useful in the separation of numerous pyrimidines and related analogs from both serum and aqueous solutions.

INTRODUCTION

1-β-D-Arabinofuranosyl cytosine (arabinosyl cytosine, ara-C) is an important antitumor and antiviral agent. It inhibits growth of a variety of transplantable tumors in mice and rats, mammalian cells in culture, bacteria, and DNA-containing viruses in cell culture and whole organisms. In humans, the agent has been studied for the treatment of neoplasms and of virus infection of the eye. To date, the major method for the separation in serum of ara-C from its metabolic product, 1-(β-D-arabinofuranosyl) uracil (arabinosyl uracil, ara-U), is by paper chromatography after trichloroacetic acid (TCA) deproteinization[1]. The metabolism is catalyzed by a deaminase enzyme, cytidine aminohydrolase, which has been partially purified from mouse kidney[2,3] and sheep liver[4]. The difficulties involved with the above separation method are the loss of ara-C and ara-U with the TCA precipitated proteins and the time-consuming paper chromatography. To alleviate these problems, work was initiated to find a separation method that was quicker and more accurate. This work resulted in a method which replaced the deproteinization reagent, TCA, by tungstic acid (TDR)[5], and the paper, by glass-fiber sheet and thin-layer chromatography. Coupled with the use of tritium-labeled ara-C, the method has been useful in our laboratory as an assay method for the comparative *in vivo* and *in vitro* metabolism of ara-C by various animal species and man. The method could be employed as a screening assay in human cancer patients to determine relative deaminase activity prior to treatment with ara-C. In addition, it would be useful in the rapid screening of potential inhibitors of this enzyme.

This communication will describe the method and its use in a preliminary determination of the effect of enzyme and substrate concentration on the rate of deamination of ara-C in monkey serum. The serum of this species is a reliable and easily accessible source of the enzyme.

EXPERIMENTAL

Arabinosyl cytosine was supplied by Cancer Chemotherapy National Service Center. Arabinosyl uracil was purchased from Calbiochem, Los Angeles, Calif. ^{3}H-labeled arabinosyl cytosine was obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y. The specific activity of the ring-labeled compound was 1.4 C/mmole. The radiopurity of the compound was stated as 99% by the manufacturer and was confirmed in our laboratory by the described glass-fiber sheet and thin-layer system and by paper chromatography[1]. To produce a weighable stock, 1.0 ml of the labeled compound containing 1.0 mC of radioactivity was mixed with 25 mg of unlabeled drug in 2.0 ml of H_2O. The resulting solution was dried *in vacuo* to yield a fine, white powder. The ^{3}H-labeled arabinosyl uracil, specific activity of 6.65 C/mmole, was obtained from New England Nuclear Corp., Boston, Mass., and prepared in a fashion similar to ara-C.

Fresh blood was drawn from a rhesus monkey *(Macaca mulatta)*, allowed to clot, centrifuged, and the serum immediately removed and placed on ice. This serum was then utilized as the deaminase enzyme source. To determine the effects of enzyme concentration on the rate of reactions, serum (1.00, 0.75, 0.50, 0.25 ml) is placed into a 25-ml erlenmeyer flask. Each flask is brought up to a total volume of 1.00 ml with ROBINSON's medium[6] and preincubated in a metabolic incubator at 37° for 20 min. At the end of the preincubation period 0.1 ml of substrate (1.0 ml unlabeled ara-C, 8.3×10^{-3} *M*, +1.0 ml [^{3}H]ara-C, 1×10^7 c.p.m./ml) is added to each flask.

At time intervals, 0.2-ml aliquots of the incubation solution are removed and added to a 12-ml centrifuge tube containing 2 ml of TDR. The tube is quickly placed on a Vortex mixer for rapid mixing and equilibration. TDR not only precipitates the serum proteins but also stops the enzymatic reaction. After sitting at room temperature for 10 min, the mixture is centrifuged and the supernate removed and quickly frozen.

The TDR supernates (50 μl) are spotted on Gelman's Chromatography Media (ITLC-Type SA) premarked into channels 2.25 cm wide and developed for 45 min in a methanol–chloroform–1.0 *M* phosphate buffer, pH 7.5, (30:70:4) solvent system. The ITLC-Type SA Chromatography Media are glass microfiber sheets impregnated with silica gel adsorbents.

To obtain reproducible R_F values, the TLC sheets must be preheated at 110° for 15 min and brought to room temperature in a desiccator. The spotted chromatography sheet must also be pre-equilibrated with the solvent system by suspending the sheet in the chromatography chamber for 30 min before development. Unlabeled solutions of ara-C and ara-U, as well as solutions of the ^{3}H-labeled compounds treated in the same fashion as the samples incubated in monkey serum, were utilized as reference compounds for chromatographic controls.

After development, the sheets are dried at room temperature and each channel cut from the sheet. The individual channels are then marked crosswise every 0.6 cm.

The strips are cut with a razor blade and placed into 20-ml scintillation counting vials without further cutting. One channel usually contains from 24–30 strips. To each vial is added 1 ml of water and 10 ml of a special aqueous phosphor solution (80 g naphthalene, 8 g PPO, 200 mg POPOP, 110 ml toluene, 110 ml ethylene glycol monoethyl ether, diluted to 1 l with *p*-dioxane). After capping, the solution in the vials is mixed well by shaking and radioactivity determined in a Packard Tri-Carb Liquid Scintillation Spectrometer. To determine if the labeled compounds on the ITLC strips had been completely solubilized, the strips were removed from the vials after counting, and the vials were recounted. The comparison of the two counts showed 98–100% of the radioactivity to be in the scintillation solution. Radioactive counting data, punched on tapes, is processed through an IBM No. 1130 Computer to obtain concentration data, R_F values, percent of radioactive components, and a plot of strip number *versus* percent of total radioactivity per strip.

For qualitative identification of sample components, Mallinckrodt ChromAR sheets can be substituted for the Gelman Chromatography Media. These sheets, containing a silicic acid sorbent, have the same solvent characteristics as the Gelman sheets, with the added advantage of an inorganic phosphor for UV visualization of low concentrations. These sheets also require only 20 min for development with the above solvent system.

RESULTS AND DISCUSSION

Fig. 1 shows a plot of the chromatographic data obtained on incubating ara-C

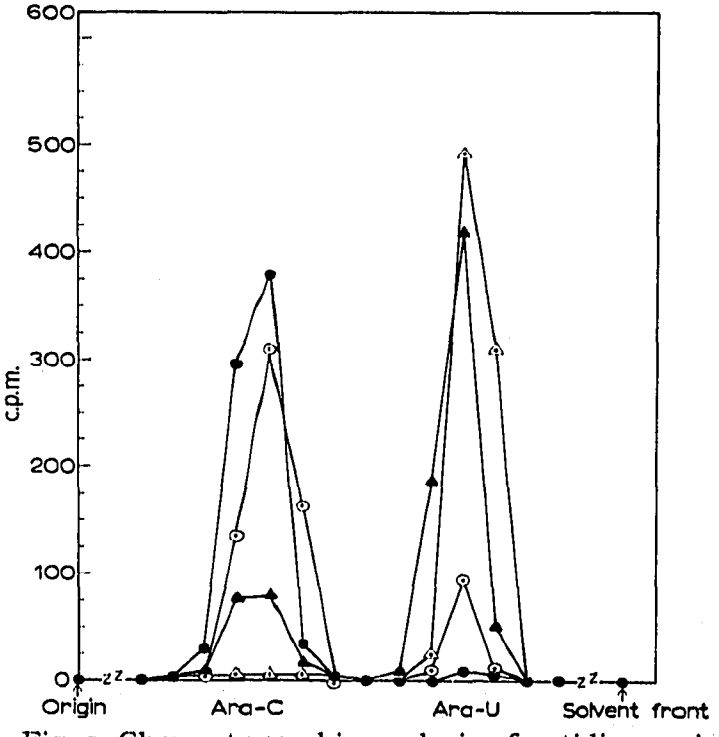

Fig. 1. Chromatographic analysis of cytidine aminohydrolase activity in monkey serum. 1 ml of serum was incubated in 8.3×10^{-3} *M* [^{3}H]ara-C substrate (5×10^6 c.p.m./ml of serum, c.p.m. = observed counts per minute minus background) for various time periods; ● = 0 min, ⊙ = 5 min, ▲ = 30 min, and △ = 360 min. The thin-layer chromatography system, described in the text, was used for the separation of ara-C from ara-U

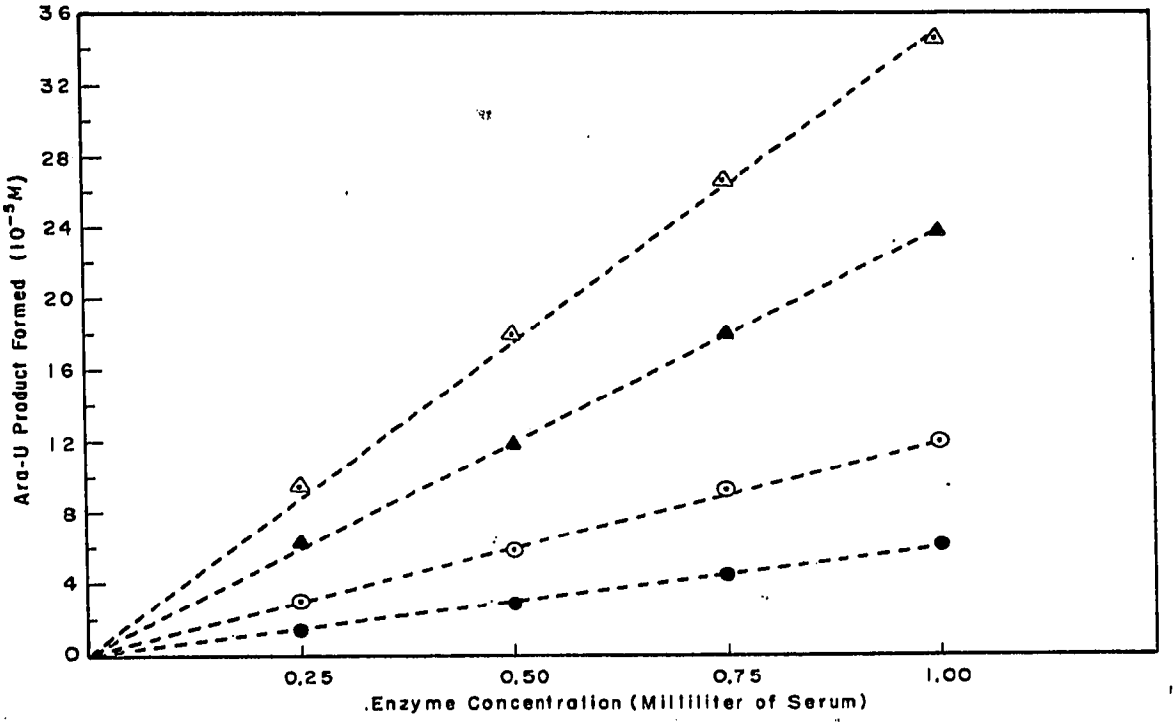

Fig. 2. Effect of enzyme concentration on the rate of deamination of ara-C in monkey serum. Various quantities of serum were mixed with 6.33×10^{-4} *M* [^{3}H]ara-C and incubated at 37° for various time periods. ● = 15 min, ⊙ = 30 min, ▲ = 60 min and △ = 90 min.

with monkey serum for various time periods. The method was also utilized to determine the effect of enzyme concentration on the deamination of ara-C (Fig. 2). The results show the substrate (6.33×10^{-4} *M*) to be in excess and the enzyme concentration to be the rate-limiting factor. The reaction rate is shown to be linear with time.

The method was also used to determine the effect of substrate concentration on the reaction rate (Fig. 3). For the reactions involving small changes in substrate concentration, the K_m values were found to be 1.3 and 1.5×10^{-4} *M*. These values agree with the K_m range of $1.2–1.6 \times 10^{-4}$ *M* found for this enzyme by CAMIENER[7] in human liver.

Monkey serum was found to be a good source for the cytidine aminohydrolase enzyme. The enzyme activity is fairly stable when the serum is kept frozen, showing a modest loss of 6% over a four-month period. On sitting at room temperature (23°) for 18 h, the enzymatic activity of the same serum decreased by only 4%.

The TDR deproteinization has several advantages over TCA: (1) excellent recovery of ara-C in the supernate (97–103% as compared with 79–85%); (2) the chromatographic characteristics of ara-C and ara-U were not affected by TDR, whereas TCA deproteinized supernates gave less effective separation of the two compounds; (3) TDR was most effective in stopping the enzymatic reaction, while TCA, freezing, or boiling were not completely effective.

The TLC method also took only 20–45 min for development, as opposed to 18 h for the paper chromatography method. In our hands, complete multiple sample

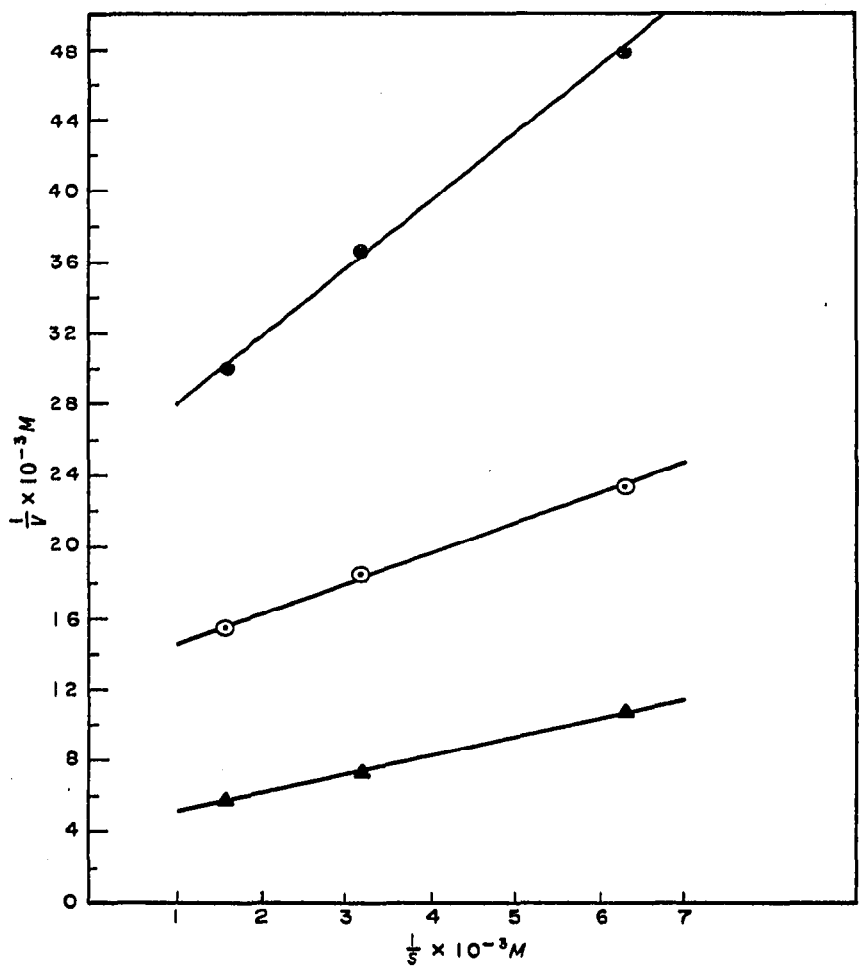

Fig. 3. Effect of substrate concentration on the rate of deamination of ara-C in monkey serum. 1 ml of serum was mixed with various concentrations of ara-C (range: 0.32–6.33 × 10^{-4} M) and incubated at 37° for various time periods. ● = 5 min, ⊙ = 10 min and ▲ = 20 min.

analysis can be accomplished in 8 h. The TLC also gave sharper peaks and better separation of ara-C and ara-U. Recovery of total radioactivity on the thin-layer strip was 95–101% and could be accounted for as either ara-C or ara-U.

R_F values in this system for cytidine, ara-C, cytosine, uridine, ara-U, and uracil were 0.28, 0.38, 0.40, 0.55, 0.60, and 0.72, respectively. R_F values for other chemotherapeutic agents were: 9-(β-D-arabinofuranosyl)adenine, 0.52; 1-(5-phosphate-β-D-arabinofuranosyl)cytosine, 0.00; 1-(β-D-arabinofuranosyl)5-fluoro-cytosine, 0.43; 1-(2,3,5-tri-O-acetyl-β-D-arabinofuranosyl)cytosine, 0.82.

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REFERENCES

1 G. W. CAMIENER AND C. G. SMITH, *Biochem. Pharmacol.*, 14 (1965) 1405.
2 W. A. CREASEY, *J. Biol. Chem.*, 238 (1963) 1772.
3 R. TOMCHICK, L. D. SASLAW AND V. S. WARAVDEKAR, *J. Biol. Chem.*, 243 (1968) 2534.
4 G. B. WISDOM AND B. A. ORSI, *Biochem. J.*, 104 (1967) 7P.
5 W. T. CARAWAY, *Chemist-Analyst*, 47 (1958) 44.
6 J. R. ROBINSON, *Biochem. J.*, 45 (1949) 68.
7 G. W. CAMIENER, *Biochem. Pharmacol.*, 16 (1967) 1681.