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BLOOD LEVELS OF 5-BROMO-2'-DEOXYURIDINE IN INTRAPERITONEAL INFUSION FOR DETERMINATION OF SISTER CHROMATID EXCHANGE INDUCTION USING THE HPLC

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ABSTRACT

A new technique was developed to measure the amount of 5-bromo-2 '-deoxyuridine (BrdUrd), a thymidine analogue, rapidly and conveniently in blood, and it was applied to measure the concentration of BrdUrd during the initiation of a paradigm for the labeling of DNA to measure the induction of sister chromatid exchanges (SCE) by genotoxic agents. Radiolabelled BrdUrd was used and blood was drawn from the abdominal aorta in pregnant Sprague-Dawley derived D rats. Using the hplc, the BrdUrd peak was easily identifiable and separable from its metabolites. The BrdUrd level in the blood stabilized in 30 minutes, and a level of about .4 mg% was enough to label the bone marrow and fetal tissue for SCE. Also, the time course of the BrdUrd metabolites suggest a constant BrdUrd metabolism by the organism. Using absorbance alone, blood levels of BrdUrd during paradigms of biological significance can be measured.

INTRODUCTION

The thymidine analogue 5-bromo-2'-deoxyuridine (BrdUrd), which labels DNA permitting the measurement of the induction of sister chromatid exhanges (SCE), is an important biological tool for estimation of genotoxicity *in vivo* (Turturro et al., 1982; Kram et al., 1980) and *in vitro* (Perry, 1979; Takehisa and Wolff, 1977). It also has various biological effects including: mutation (Fishbein et al., 1970), sensitization of DNA to radiation (Djordjevic and Szybalski, 1960), inhibition of differentiation in developing systems (Stockdale et al., 1964; Clark, 1971) and the production of terata in mice (Skalko et al., 1971) and hamsters (Ruffolo and Ferm, 1965). Quantitative interpretation of results dependent upon the concentration of BrdUrd is limited, however, because the compound is unstable *in vivo*, due to debromination as well as deribosylation (Kriss and Revesz, 1962), both as a result of metabolism and non-specific, chemical changes (Prusoff and Goz, 1975). Present methods for the quantitation of BrdUrd *in vivo* are tedious (Kriss and Revesz, 1962; Fink and Adams, 1966; King et al., 1982) or insensitive (Matz, 1980). To quantitate the levels of BrdUrd during measurement of SCE induction *in vivo* using a newly developed infusion technique, a simple hplc technique was developed which permits the separation of the parent halogenated pyrimidine from its various products.

MATERIAL AND METHODS

Animal Preparation: Pregnant Sprague-Dawley derived CD rats of 110-130 days of age at Day 20 of gestation were the test animals since these animals are routinely used for *in utero* measurement of SCE induction by intraperitoneal perfusion (Turturro et al., 1982).

After light etherization the abdomens of the animals were opened with a midline incision. The abdominal aorta was cannulated with 2 cm of a heparinized saline filled PE-50 polyethylene tubing (Clay-Adams, Parsippany, N.J.) at a level just anterior to the bifurcation of the iliac arteries. A second catheter for the BrdUrd infusion, .03 inch o.d. soft Tygon tubing (A. Daigger, Chicago, Ill.), was placed in the abdominal cavity. The animal was then placed in a Bollman restrainer cage (Bollman, 1948) and infused with a concentration of BrdUrd which delivers a total dose of one gram/kg body weight of BrdUrd over a 24 hour period in a 5% glucose solution at the rate of .78 ml/hr with a perfusion pump (Harvard Perfusion Company, Mills, Mass.). This is the dose used for measurement of SCE induction (Turturro et al., 1982). As a marker, a tracer amount of radioactive BrdUrd, 10 Cis of 5-(2-^{14}C)-bromodeoxyuridine (44 mCi/mmol Sp. Act., New England Nuclear, Boston, Mass.), was added to the infusion solution.

At timed intervals, ten drops of blood were collected from the aortic catheter. The cannula was cleared with a small amount of heparinized saline after every sampling. The results from three animals at twenty days gestation were averaged.

Analytical Technique: Ninety μls of blood, obtained as described above, were added to 10 μls of a 50% PCA solution, mixed and immediately centrifuged in a Beckman Microfuge B for one minute. The PCA-soluble fraction was recentrifuged, adjusted to approximately pH 5 with 10N NaOH and injected into a 20 μl sample chamber of a Beckman Model 334 Density Gradient hplc. The column was eluted at 1.5 ml/min for ten minutes with a 13% methanol (Fisher, Pittsburg, Pa., hplc grade) in water solution on an Altex 4.6 mm X 25 cm ODS-Ultrasphere column. Fractions were collected in scintillation vials.

The material eluted between one and 6.5 minutes was pooled together, and the BrdUrd peak was collected from 7 to 8.5 minutes of elution. Analytical grade chemicals were purchased from Sigma Co. (St. Louis, USA) except where noted.

Radioactivity Measurement: The fractions containing the BrdUrd peak from the hplc were dried in a 50 °C oven overnight, reconstituted to one ml with distilled water and mixed. The PCA-insoluble residue was redissolved in 0.5 ml Soluene (Packard, Downer's Grove, Ill.) and heated at 50 °C overnight. The samples were then reconstituted to one ml. Blood serum was separated from the remainder of the timed sample not used for PCA solubilization (approximately 60 μl) by centrifugation on a Beckman Microfuge B for one minute and diluted to one ml with distilled water. Ten mls of Biofluor (New England Nuclear) were added to each, and the samples were counted on a Searle Analytic 92 Liquid Scintillation counter. A quench curve was constructed for the PCA-insoluble fraction by adding radioactivity to samples with different levels of quenching as defined by the external standard ratio (ERS). An aliquot of infusion solution was also counted with every experiment as a control for variability in dilution of the radioactive BrdUrd.

Absorbance Measurement: Peak areas were determined either by the Altex Model C-R1A integrator of the Model 334 hplc (at .2 absorbance units full scale at 279 nm) or by weighing of peaks cut from the integrator profile. Repeated measures of a 1 nanomole standard of Brdurd were reproducible to within 2%. Peaks areas were quantitated by injection of known standard amounts of BrdUrd and also by quantitation of the radioactivity of the collected peak when tracer amounts of the radioactive species was used. Values as low as 50 picomoles could be estimated from absorbance alone.

RESULTS

At 1.5 ml/min using a solvent of 13% methanol:water, BrdUrd chromatographed as a single peak with a retention time of 7.6 minutes. The material in this peak co-chromatographed with pure BrdUrd for a number of different retention times (varied by both modifying the flow rate of the solvent and varying the percent of methanol from 6 to 13%) and was identical in ultraviolet spectrum with pure BrdUrd dissolved in 13% methanol:water solution (Figure 1). The BrdUrd peak was stable in infusion solution for at least two hours when kept in a plastic microtube (Figure 2). The products associated with BrdUrd infusion chromatograph with different retention times than the parent material. This is demonstrated for the major known metabolites of BrdUrd, uracil and 5-bromouracil (Kriss and Revesz, 1962) in Figure 3, as well as for 5-bromouridine. Thus, the material that eluted from one minute (when the material from the injected sample first passes through the column) to 6.5 minutes, just before the BrdUrd peak, was pooled into one fraction and denoted BrdUrd metabolites. Also, injected radioactivity could be quantitatively accounted for if the two fractions were added together. Comparison of hplc profiles of the PCA-soluble fraction of blood in infused animals (shown after 30 minutes in Figure 4A) and control profiles (Figure 4B), shows the BrdUrd peak clearly separated from the normal constituents of the fraction unlike some of BrdUrd metabolites (such as uracil) which are difficult to determine accurately from absorbance alone since they are submerged in a number of peaks at early retention times.

Total radioactivity as a function of time in the PCA-soluble fraction of blood, given as the amount of BrdUrd to which the radioactivity is equivalent, and serum are shown in Figure 5. The time course of the radioactivity contained in the PCA-insoluble fraction is also included. Taken together, the whole blood level of BrdUrd and its metabolites can be determined. These data demonstrate an increase in radioactivity with time, with relatively less of an increase in serum than in the PCA-insoluble fraction. The smallest increase is observed in the PCA-soluble blood fraction. In the last, the blood levels of BrdUrd (as defined by both absorbance at 279 nm and radioactivity of the BrdUrd peak) during infusion are given at Figure 6. The BrdUrd level reaches equilibrium after approximately 30 minutes and remains relatively constant, while the level of BrdUrd metabolites in blood increases linearly as a function of time.

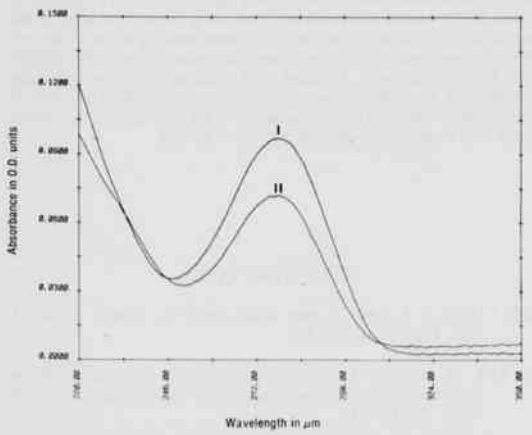

Figure 1. Ultraviolet spectrum of material from the BrdUrd peak and BrdUrd in 13% methanol:water. Peak I = BrdUrd in 13% methanol:water; peak II = directly from column.

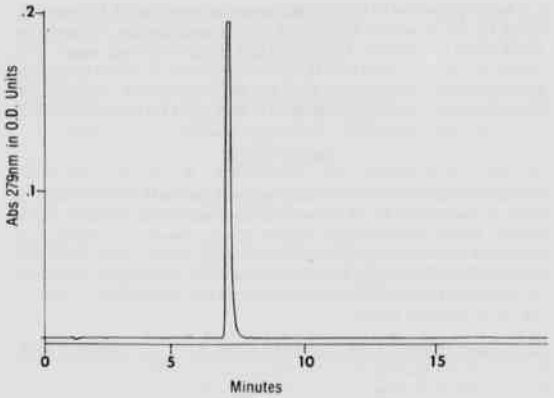

Figure 2. BrdUrd profile after two hours.

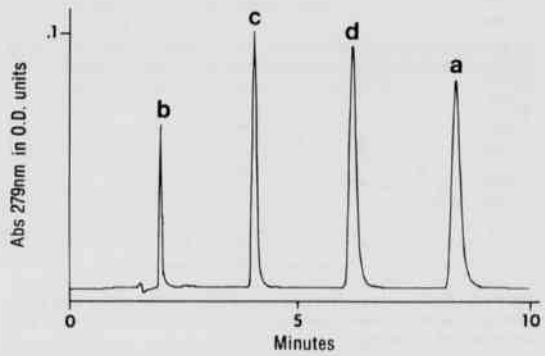

Figure 3. HPLC profile of BrdUrd (a) and uracil (b), 5-bromouracil (c) and 5-bromouridine (d).

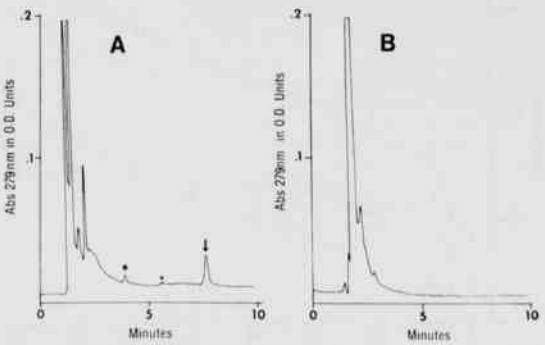

Figure 4. HPLC profile of the PCA-soluble fraction of a catherterized rat a) 30 minutes after the start of infusion and b) control (before onset of infusion). Arrow denotes BrdUrd peak, asterisk and dot denote metabolite peaks.

The concentration of BrdUrd plateaus at approximately 140 ngms/20 μls of the PCA-soluble fraction. This is approximately .35 μgms per 90 μls which converts to .39 mg% of free BrdUrd in whole blood. This level is sufficient to label clearly the chromosomes of cells in metaphase in maternal bone marrow and to provide enough material for placental transfer to label metaphases in fetal cells (Turturro et al., 1982).

DISCUSSION

This technique is able to rapidly and conveniently separate BrdUrd from its metabolites in the blood with minimum treatment of the blood sample. Other methodologies require either extensive separation from the blood and tedious procedures (Kriss and Revesz, 1962; Fink and Adams, 1966; King et al., 1982) or require more than 20 gms of material for detection (Matz, 1980), which is clearly too insensitive for measuring blood BrdUrd levels.

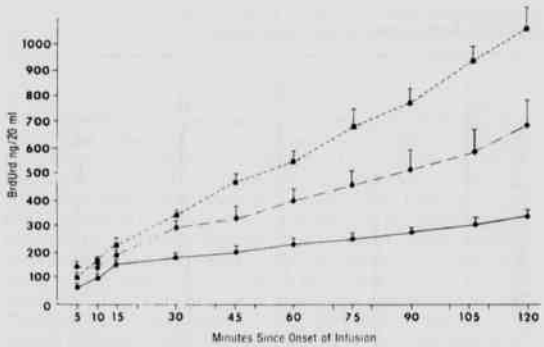

Figure 5. Radioactivity levels in the PCA-soluble (dots) and PCA-insoluble (squares) blood fractions and blood serum (diamonds). Bars are standard deviations.

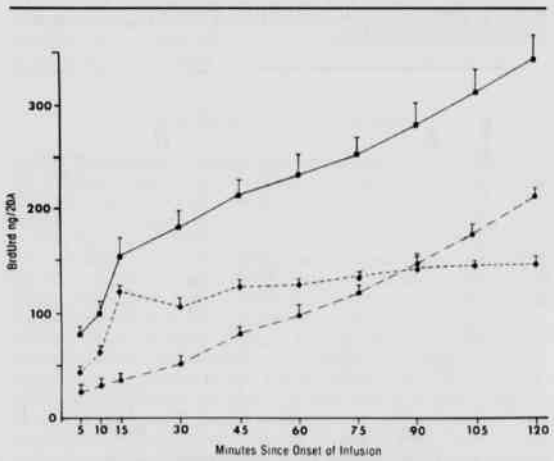

Figure 6. Total radioactivity (squares), BrdUrd (absorbance and radioactivity of BrdUrd peak [diamonds]) and products of BrdUrd metabolism (dots) of PCA-soluble fraction. Bars are standard deviations.

The data in this paper are the first *in vivo* measurement of BrdUrd blood levels after initiating a continuous infusion paradigm successful for measurement of SCE. The blood levels of BrdUrd using pellet implantation of BrdUrd (King et al., 1982) initially rise precipitously for up to 7 hours to high values and then fall to very low levels, with a mean close to the BrdUrd concentration measured here. The data presented here demonstrate that the intraperitoneal application of BrdUrd for SCE visualization results in a fairly stable level of BrdUrd in less than thirty minutes. Also, intraperitoneal administration seems to provide a steady rise in BrdUrd level up to a plateau. BrdUrd concentration can influence the number of SCE (Wolff and Perry, 1974) so, in avoiding these violent fluctuations in levels, it is a reasonable assumption that this approach will enhance accurate quantitation of SCE *in vivo*.

A blood level of .39 mg% is about a tenth of that used to determine SCE induction *in vitro* (Perry, 1979; Takehisa and Wolff, 1977). *In vitro* determinations usually exhibit a higher frequency of "spontaneous" SCE, i.e., control SCE values, than *in vivo*, which now can be seen as a direct result of the higher BrdUrd level *in vitro*.

The PCA-soluble fraction, serum and the PCA-insoluble fraction all accumulate radioactivity with time in a fairly linear fashion from thirty minutes onward. The rate of accumulation increases with the proportion of BrdUrd derivatives not in solution, suggesting that the accumulations result from a constant BrdUrd level, i.e., the free BrdUrd, which is metabolized and bound to protein and other blood components. The amount of radioactivity in the PCA-soluble fraction is derived from two components, the level of BrdUrd, which is fairly stable, and the level of other products associated with BrdUrd infusion, which are linearly increasing. Since the liver quickly metabolizes BrdUrd (Kriss and Revesz, 1962) most of the products associated with the infused material are probably the results of metabolic rather than non-specific breakdown. The linear increase of products with time suggests that either infused degradation products are accumulating at a constant rate or BrdUrd metabolism is going on at a constant rate with residue of metabolism increasing in the blood. The undetectable levels of breakdown products in the infusion solution, even after two hours, weigh against the first explanation. Therefore, the level of the metabolites seem to be some overall measure of the accumulated metabolism of BrdUrd. BrdUrd metabolism, and its correlate, thymidine metabolism, may therefore be evaluated cumulatively in the organism, an important factor in studies of DNA and DNA metabolism.

A benefit of the technique presented is that it is sensitive enough to permit the measurement of biologically significant levels of BrdUrd in blood without using radioactivity. Measuring the BrdUrd level by absorbance allows determinations to be made quickly without the cost and inconvenience of using radiolabeled species if metabolite estimation is not important. Coupled with radioactivity, the technique permits the easy determination of the levels of BrdUrd and its metabolites, important in the evaluation of the biological effects of the agent and in understanding the results of its use.

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