Journal of Chromatography, 148 (1978) 500–503

CHROM 10,534

Note

Thin-layer chromatographic separation of thymine nucleo-derivatives using a one-step, constant-concentration elution technique

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(First received February 7th, 1977; revised manuscript received July 5th, 1977)

The effects of estrogens on the incorporation of radioactive precursors into uterine macromolecules have been often described[1,2]. It has become widely appreciated, however, that rates of incorporation of radioactive tracers into large macromolecules are as much a function of the physiology of the precursor pool as they are of rates of macromolecular synthesis[3,4]. In our laboratory, the study of the effects of estrogens on uterine DNA synthesis using [^{3}H]thymidine incorporation required the measurement of thymine nucleo-derivative pools. The use of poly(ethylene)imine (PEI) cellulose anion exchangers has been shown to be particularly well suited for nucleotide analysis[5]. However, separation of mixtures containing the nucleic acid base, nucleoside, nucleotides and nucleotide sugar have required the use of two-dimensional[6,7], step-wise elution[4,5,7] or gradient elution[8] anion-exchange thin-layer chromatographic (TLC) techniques. This paper reports a simple and rapid TLC method for the separation of thymine and its major biological derivatives.

MATERIALS AND METHODS

Thymine (T), thymidine (TdR), thymidine diphosphoglucose (TDPG) and thymidine mono-, di- and triphosphate (TMP, TDP and TTP, respectively) were obtained from Sigma (St. Louis, Mo., U.S.A.). [^{3}H]Methylthymidine (20 Ci/mmole) was obtained from New England Nuclear (Boston, Mass., U.S.A.). All other chemicals were reagent grade (Fisher Scientific, St. Louis, Mo., U.S.A.). For determination of R_F values, 1 μl of a standard solution (10 nmoles each of the nucleo-derivatives) was spotted on Brinkman 20 × 20 cm PEI cellulose F plastic sheets (EM Labs., Elmsford, N.Y., U.S.A.). When indicated ^{3}H-labeled tissue extracts (2–10 μl of a NaOH neutralized 0.2 *N* $HClO_4$ homogenation extract obtained from uteri previously incubated *in vitro* with [^{3}H]thymidine) were cospotted with the standard solution above. When appropriate, the plates were preconditioned for 60 min with sulfuric acid solutions to create different humidities. Several solvent systems and humidities were tested at 23° using the Camag Vario-KS-Chamber TLC equipment (Camag, New Berlin, Wisc., U.S.A.). This system allows for testing various solvents and/or humidities simultaneously on one chromatogram. All chromatograms were run to a line *ca.* 13 cm above the origin with a running time of 45–50 min. After air drying, the compounds were located by examining the plates under UV light (254 nm) and/or by determining the distribution of radioactivity over the chromatogram.

RESULTS AND DISCUSSION

The R_F values for the thymine derivatives obtained using 4 solvent systems are given in Table I. Of the many systems tested, those listed in Table I were found to afford the best separations, giving separation distances of at least 3 mm between the inner edges of the compounds. Chromatograms run with 0.7 *M* LiCl or 0.08 *M* $MgCl_2$ showed a tendency to form more elongated and diffuse spots, especially those of TDP and TTP. This pattern, however, was less evident with those solvent mixtures containing 0.04 *M* or 0.06 *M* $(NH_4)_2SO_4$. Although the $(NH_4)_2SO_4$–LiCl mixtures were found to afford excellent separations, $(NH_4)_2SO_4$ alone failed to adequately separate all the compounds. In addition, the utilization of solvents containing various concentrations of ammonium acetate or acetic or formic acid, alone, or in mixtures resulted in incomplete separations.

TABLE I

R_F VALUES AND THEIR STANDARD DEVIATIONS OBTAINED FOR THYMINE NUCLEO-DERIVATIVES USING VARIOUS SOLVENT SYSTEMS

The nucleo-derivatives (10 nmoles each) were spotted together on Brinkman PEI cellulose F sheets and run as described in Materials and methods. Detection was by UV light (254 nm). Solvent systems: (A) 0.7 *M* LiCl; (B) 0.08 *M* $MgCl_2$; (C) 0.06 *M* $(NH_4)_2SO_4$ ± 0.1 *M* LiCl; (D) 0.04 *M* $(NH_4)_2SO_4$ + 0.3 *M* LiCl.

Nucleo-derivative	*Solvent system*			
	A	*B*	*C*	*D*
TDPG	0.97 ± 0.01	0.62 ± 0.01	0.61 ± 0.01	0.97 ± 0.01
TdR	0.87 ± 0.01	0.88 ± 0.01	0.88 ± 0.01	0.87 ± 0.01
T	0.72 ± 0.01	0.73 ± 0.01	0.74 ± 0.01	0.72 ± 0.01
TMP	0.80 ± 0.01	0.44 ± 0.02	0.53 ± 0.02	0.62 ± 0.01
TDP	0.50 ± 0.02	0.22 ± 0.02	0.36 ± 0.03	0.38 ± 0.04
TTP	0.17 ± 0.03	0.05 ± 0.01	0.11 ± 0.03	0.11 ± 0.04

The effect of humidity ranging between 9% and 72% on the ability of the solvents in Table I to completely separate the nucleo-derivatives was also tested (data not shown). The effect of humidity on chromatograms run with 0.08 *M* $MgCl_2$ was negligible. However, the R_F values of the nucleotides, especially TDP and TTP, were increased at 32% and 42% humidity when chromatograms were run with those solvents containing LiCl. Although adequate separation was maintained, the separation distance between some compounds decreased as a result of the increased migration of the nucleotides.

Although at very high solvent salt concentrations (> 2 *M* NaCl) a decrease of the R_F values of some bases and nucleosides may be observed, presumably a salting-out phenomenon[5], the R_F values of nucleic acid bases and nucleosides generally do not depend on the salt concentration of the solvent[5,8]. In contrast, the migration rate of nucleotides and nucleotide sugars depends on the electrolyte concentration of the solvent in a way characteristic for each compound. For this reason, an excess of salts or buffers in the samples to be analyzed can interfere seriously with the separation of polyphosphates[5,9]. Table II demonstrates the sensitivity of the solvent

TABLE II

THE EFFECT OF UTERINE EXTRACTS ON THE R_F VALUES OBTAINED FOR THYMINE NUCLEO-DERIVATIVES USING VARIOUS SOLVENT SYSTEMS

1 μl of a standard solution of thymine nucleo-derivatives (10 nmoles each) was co-spotted together with 2 μl of a ^{3}H-labeled tissue extract (neutralized perchloric acid uterine extract) on Brinkman PEI cellulose F sheets and run as described in Materials and methods. Detection was by UV light (254 nm). Solvent systems are the same as described in Table I.

Nucleo-derivative	*Solvent system*			
	A	*B*	*C*	*D*
TDPG	0.97 ± 0.01	0.97 ± 0.01	0.97 ± 0.01	0.97 ± 0.01
TdR	0.84 ± 0.02	0.88 ± 0.01	0.88 ± 0.01	0.88 ± 0.01
T	0.71 ± 0.01	0.73 ± 0.01	0.77 ± 0.01	0.74 ± 0.01
TMP	0.80 ± 0.01	—*	0.55 ± 0.02	0.60 ± 0.3
TDP	0.58 ± 0.01	—	0.38 ± 0.04	0.43 ± 0.4
TTP	0.35 ± 0.01	—	0.19 ± 0.02	0.17 ± 0.4

* TMP, TDP and TTP formed one elongated spot over the first half of the chromatogram.

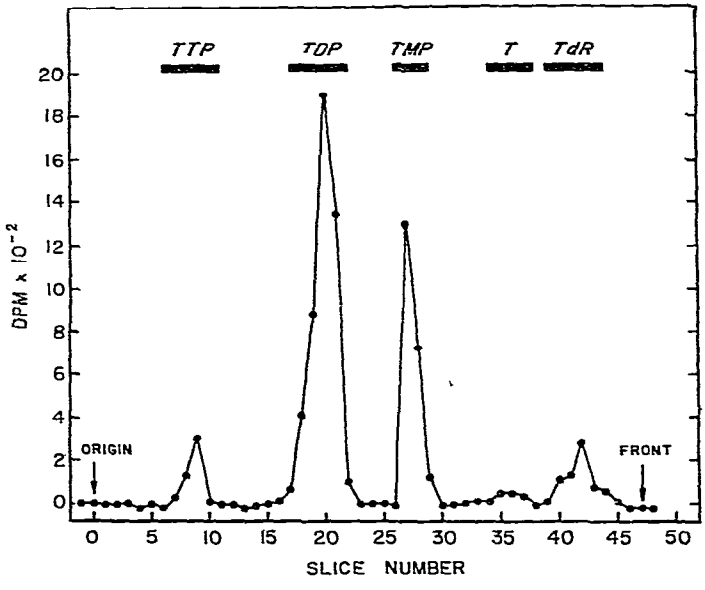

Fig. 1. Chromatographic analysis of the acid-soluble fraction obtained from uteri labelled *in vitro* with [^{3}H]thymidine. Uteri (3 per group) excised from immature rats 21–25 days old were incubated in 3 ml of Eagle's Hela Medium containing 2 μCi/ml of [^{3}H]thymidine for 1 h at 37° in sealed flasks under an atmosphere of O_2–CO_2 (95:5). The uteri were homogenized in 1 ml of iced distilled water and, following the addition of perchloric acid to a final concentration of 0.2 *N*, the homogenate was centrifuged at 1200 *g* for 10 min. The acid-soluble fraction (supernatant) was neutralized, made to 1 ml and was cospotted (10 μl) with 1 μl of a standard solution of thymine nucleo-derivatives (10 nmoles each) on a Brinkman PEI cellulose F sheet. Chromatography was performed as previously described using 0.06 *M* $(NH_4)_2SO_4$ + 0.1 *M* LiCl as the solvent system. The dried chromatogram was cut into 3 × 15 mm sections and the compounds quantitatively extracted by incubating the sections in scintillation vials containing 1 ml of 0.7 *M* $MgCl_2$–0.02 *M* Tris–HCl (pH 7.4) overnight at room temperature[11]. After thorough mixing with 10 ml of scintillation fluid (25% v/v Triton X-114 and 0.4% w/v Omnifluor in xylene)[12] the samples were counted with 34% efficiency. Running positions of the carrier compounds as detected with UV light are indicated.

systems in Table I to the presence of high concentrations of electrolytes in samples to be chromatographed. When compared to the other systems tested, solvent C (0.06 *M* $(NH_4)_2SO_4$ + 0.1 *M* LiCl) gave the best and most consistent separations as well as sharper and less elongated substance spots. In Fig. 1 an example is given showing the chromatographic distribution of radioactivity of a labeled uterine extract. The solvent system utilized is 0.06 *M* $(NH_4)_2SO_4$ + 0.1 *M* LiCl. It is clear that this system affords excellent separation between the nucleotide mono-, di- and triphosphates as well as between the nucleoside and the base. Similar performance is demonstrated even when considerably larger volumes of sample are spotted. Thus, the tedious and often impractical desalting of samples[5,9,10] can be avoided. The report of this rapid and simple one-step constant-concentration elution technique for the separation of the major thymine nucleo-derivatives allows investigators in the area of DNA synthesis to quickly measure cellular radiolabeled precursor pool size.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. M. Ruh and Miss K. Landis for assistance in the preparation of this manuscript. This work was supported by NIH Program Project (Washington University): Hormonal Control of the Reproductive Tract NIH HD 08235.

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