ION-EXCHANGE THIN-LAYER CHROMATOGRAPHY

XV. PREPARATION, PROPERTIES AND APPLICATIONS OF PAPER-LIKE PEI-CELLULOSE SHEETS*

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Until recently thin layers have been prepared in the laboratory almost exclusively on glass plates[1]. A few authors reported the preparation of thin layers on different materials, *e.g.* aluminum plates[2], aluminum foil[3], and plastic sheets[4,5].

In our experience with ion-exchange and cellulose thin layers, plastic sheets have been found to offer many advantages when compared with glass plates. The present communication describes the preparation of PEI-cellulose** anion-exchange thin layers on plastic sheets and their application in qualitative and quantitative nucleotide analysis.

EXPERIMENTAL

Materials

Bakelite® Rigid Vinyl Sheets (21 × 50 in.), type VSA 3310 Clear 31 Matte 06, 0.010 in., manufactured by Union Carbide Corporation, Cincinnati, Ohio, U.S.A., were obtained from Commercial Plastics and Supply Corporation, 630 Broadway, New York 12, N.Y., U.S.A. MN 300 cellulose powder, manufactured by Macherey und Nagel, Düren, West-Germany, and a STAHL type applicator[1] with adjustable slit width were supplied by Brinkmann Instruments, Westbury, N.Y., U.S.A. Poly(ethyleneimine) (Polymin P "BASF") was obtained from Chemirad Corporation, East Brunswick, N.J., U.S.A. Nucleotides were supplied by Sigma Chemical Company, St. Louis, Missouri, U.S.A., and by Calbiochem, Los Angeles, California, U.S.A.

Preparation of sheets

The conventional procedure is modified in the following way: Instead of a series

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** A cellulose anion-exchange material obtained by impregnating chromatography cellulose with poly(ethyleneimine)[6]. Abbreviations for nucleotides: dAMP = deoxyadenosine-5'-monophosphate; GMP = guanosine-5'-monophosphate; GDP = guanosine diphosphate; TDP = thymidine diphosphate; ATP = adenosine triphosphate; GDP-glucose = guanosine diphosphate glucose; GDP-mannose = guanosine diphosphate mannose; UDP-glucose = uridine diphosphate glucose.

of glass plates one long sheet of the plastic material (21.0 cm × full length*) is coated at a time, and a glass plate (127 × 21.5 × 0.6 cm) is used as a mounting board instead of a plastic template**.

The glass template is laid on the bench with one long side protruding a few mm over the edge. The bench top should be horizontal, otherwise the template must be leveled. This may be done by attaching thick adhesive tape to the bottom of the template.

Prior to coating the plastic sheet is degreased on one side using a commercial cleanser and is rinsed on both sides under tap water. After excess water has been allowed to drain, the wet sheet is placed on the glass template in such a way that the front edge protrudes about 1 mm over the corresponding edge of the template***. The sheet is being attached firmly to the template due to adhesion forces. Subsequently, the surface of the sheet is dried by wiping with a towel.

After the slit width has been adjusted to 0.5 mm, the applicator is placed on the right hand side of the sheet so that its guide bar is in contact with the template edge protruding over the bench. To ensure an even surface the empty spreader is moved twice over the sheet. It is then filled with a PEI-cellulose suspension which is prepared as follows:

20 g commercial 50 % poly(ethyleneimine) solution is diluted with distilled water (700 ml), adjusted to pH 6 with concentrated HCl, and made up to 1 l with water (final concentration: 1 %).

A suspension of 22 g cellulose MN 300 in 145 ml of the 1 % PEI hydrochloride solution is homogenized for 15–20 sec in an electric mixer. In order to remove air bubbles the suspension is transferred to a beaker and is stirred about 1 min with a glass rod. Subsequently, it is poured into the applicator and is stirred a few seconds in the applicator chamber. The sheet is then coated immediately. (A narrow band along one side of the sheet will be left uncoated due to the fact that the sheet is 1 cm wider than the slit of the spreader.)

The coated sheets are allowed to dry overnight at room temperature. This will result in a layer with optimal ion-exchange properties. Under no circumstances should drying of the layer be accelerated by heating.

After drying, the sheet is cut into shorter pieces; 7–10 cm wide strips from both ends of the long sheet are discarded. For cutting the coated sheets, a cutting board is recommended.

After cutting, the layers are washed to remove impurities which would interfere with separations and assays. The following procedures may be used:

1. Sheets are washed by ascending irrigation with distilled water in the same way as PEI-cellulose layers on glass plates[7,§]. The water is allowed to rise in a direction perpendicular to the coating direction. Impurities are removed most effectively if a wick of thick absorbent paper (*e.g.*, Whatman No. 3 MM) is attached to the top region by stapling.

* The commercial sheets may be cut with scissors. Alternatively, they can be obtained cut-to-size from local dealers.

** A glass template is not required if a hard and completely level bench is available; in this case the sheet may be laid directly on the bench top for coating.

*** This will prevent the suspension from entering the space between glass template and sheet which would alter the layer composition close to this edge of the sheet.

§ It was found unnecessary to "scratch" the bottom part of the layer as described for glass plates[7,8], because PEI-cellulose layers are bound more firmly to plastic material than to glass.

2. Sheets are washed using the ascending NaCl/water procedure described for PEI-cellulose on glass plates[8,9]. Attaching a wick of absorbent paper to the top part of the layer (see under 1) is again recommended.

3. Each sheet is soaked for 1 min in 10% NaCl solution (800–1000 ml)*. Immersion is started from one end of the sheet and continued in slow and steady motion. After excess solution has been allowed to drain, the sheet is dried in the air for several hours. (It may be suspended using a clip attached to a cord.) It is then soaked for 5 min in 800–1000 ml distilled water and is again dried. Subsequently, the layer is washed by ascending irrigation with distilled water. A paper wick may be attached to the top region, see under 1.

Dialysis of the poly(ethyleneimine) solution[7,8] is not required if impurities are transferred to the paper wick (procedures 1 and 2) or if the more thorough procedure 3 is used. Procedure 1 is sufficient for one-dimensional separations; for two-dimensional separations and for quantitative techniques (see below) procedures 2 and 3 are preferable.

After washing and drying at room temperature, five to ten sheets are placed on top of each other, wrapped in foil and are stored in a freezer. They are still suited for qualitative and quantitative work after several months at —10 to —20°.

Qualitative procedures

Ascending chromatography is carried out essentially as described for PEI-cellulose on glass plates[7,8]. Nucleotides are applied 2 cm from the lower edge.

For *descending chromatography*, compounds are applied about 4.5 cm from the upper end and the sheet is folded along a line 3.5 cm from this end so that the layer forms an angle of about 45 degrees. The antisiphon rod of commercial paper chromatography equipment is attached with adhesive tape to the uncoated side of the sheet between 3.0 and 3.4 cm from the upper end. This arrangement allows the sheet to hang vertically without touching the wall of the solvent trough. The solvent assembly may be supported by two glass rods (7 cm long) attached to the walls of a rectangular tank (about 28 × 25 × 7.5 cm) by rubber tubing.

For *continuous-flow chromatography*, a paper wick is attached to the distant part of the sheet (see above). In ascending chromatography, it may be folded back behind the uncoated side of the sheet. In descending continuous-flow chromatography for longer periods of time, a long paper wick (> 20 cm) is attached, and the separation is carried out using equipment for descending paper chromatography. To facilitate draining of the solvent, the distant edge of the paper may be serrated.

Two-dimensional chromatography is carried out essentially as described for glass plates[9,10], and the same procedures are used to remove electrolytes prior to development in the second direction. The front area of the first dimension is cut off and discarded. If desired the chromatogram may be cut perpendicularly to the first dimension into several strips containing different groups of compounds which can then be chromatographed in the second dimension with different solvents.

Rechromatography of fractions separated on a PEI-cellulose sheet may be carried out in the following way: the area of the compound(s) to be rechromatographed is cut out using a cutting board. The cut-out is desalted by soaking in anhydrous

* The NaCl solution may be used repeatedly, *e.g.*, for all pieces obtained from one long sheet.

methanol (see refs. 7 and 9) and is dried. Its layer is then brought into contact with the starting area of a fresh sheet and is held in place by attaching two flat magnetic bars or a magnetic bar and a metal sheet to the uncoated sides. Subsequently, chromatography is carried out in the usual way. The compound(s) is (are) transferred quantitatively to the fresh sheet during development.

*Quantitative procedures**

Transfer procedure. This technique, which comprises direct transfer of the nucleotide from the layer to a paper wick, subsequent elution from the paper and spectrophotometry[8], may be used on plastic sheets. A triangle-shaped area containing the substance spot is cut out with scissors rather than isolated *in situ*[8]. To hold the triangle its uncoated side is attached to an uncoated sheet of the same plastic material using a small drop of acetone. About 3 mm substance-free layer between the triangle base and the spot is scraped off with a razor blade, and the paper wick is brought into contact with the straight-cut edge thus obtained. Eluant is applied to the opposite corner with a micropipet as described[8].

Direct procedure. This technique is made possible by the paper-like properties of the ion-exchange sheets. The area of the compound to be assayed is located under short-wave ultraviolet light. A rectangle around the spot is marked with a pencil and is cut out using a cutting board or sharp scissors. Care must be taken that the uncoated side is clean. The rectangle is transferred, layer side up, to the bottom of a tube. Eluant (1.0 ml) is added slowly from a capillary pipet, *e.g.*, a 1000-μl Carlsberg pipet. We found 0.7 *M* $MgCl_2$–2.0 *M* Tris hydrochloride, pH 7.4 (100:1, v/v) to be capable of quantitatively eluting all common mononucleotides, *e.g.*, nucleoside diphosphate sugars, nucleoside mono-, di-, and triphosphates. After an extraction time of 1 h, which is sufficient for these compounds, the eluate is transferred to a centrifuge tube with a capillary pipet and is centrifuged at 2000 r.p.m. for 5 min. Subsequently, extinction at 260 mμ is measured against a blank value from an adjacent area of the sheet processed in the same manner as the compound area. A Zeiss spectrophotometer (model PMQ II) or a Cary spectrophotometer (model 14) equipped with a sensitive slide wire may be used. It is possible to scale down the quantitative procedure to the 1 mμmole range by reducing the eluant volume and using microcuvettes.

As special examples, quantitative determinations of dAMP, UDP-glucose, TDP and ATP were carried out on sheets prewashed according to procedure 3 (see above). For the elution experiments, 5 μl of solutions containing 1.4–2.0 mμmoles nucleotide/μl each were spotted in replicate. Compounds were chromatographed using the following solvents: 0.5 *M* LiCl (dAMP), 0.3 *M* LiCl (UDP-glucose), and 1.0 *M* LiCl (TDP). Development distance was 10 cm. Stepwise elution[7] was used for ATP: 0.5 *M* $(NH_4)_2SO_4$ solution was allowed to ascend to 3 cm from the origin, followed, without intermediate drying, by 0.7 *M* $(NH_4)_2SO_4$ up to 10 cm. After drying, direct extraction was carried out as described above.

Radioactive nucleotides (^{32}P) may be assayed prior to elution: rectangles are cut out as described, transferred to planchets for counting and are subsequently eluted for spectrophotometry. For semiquantitative assays of labeled compounds, sheets are scanned in standard equipment for scanning paper chromatograms.

* Since assays of very small quantities (a few mμmoles) are intended, precautions have to be taken to ensure a low and even background, see ref. 8.

RESULTS

Qualitative results

Properties of the ion-exchange sheets. The ion-exchange cellulose adheres very well to the VSA 3310 plastic material, and layers obtained are homogeneous. Mechanically, they are considerably more stable than plain cellulose layers on the same material[5,11] due to a binding effect of poly(ethyleneimine). Bending or folding of the sheets (see above) does not disrupt the continuity of the ion-exchange coating. Sheets are sufficiently rigid to stand upright when leaned against the wall of a chromatography chamber. The plastic material is stable against all aqueous solvents used in ion-exchange thin-layer chromatography[7,8,10] and against alcohols, aliphatic hydrocarbons, and ethers.

Chromatographic results. Nucleotide mobilities were found not to depend on the support of the PEI-cellulose layer. R_F values given previously for PEI-cellulose on glass[7] are therefore correct for sheets. Spot patterns on two-dimensional chromatograms[9,10] are the same in both cases.

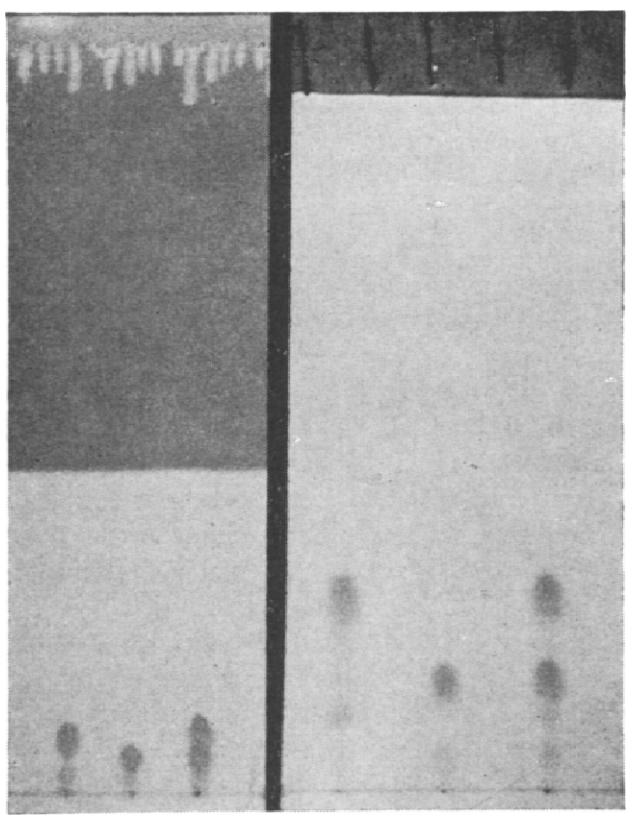

Fig. 1. Comparison between ascending and continuous-flow descending chromatography of closely related nucleotides. 0.5 mm (wet thickness) PEI-cellulose layers on VSA 3310 plastic sheets. About 20 mμmoles of each compound. From left to right: GDP-glucose; GDP-mannose; GDP-glucose plus GDP-mannose. Solvent: a solution of 6 g $Na_2B_4O_7 \cdot 10\ H_2O$, 3 g H_3BO_3, and 25 ml ethylene glycol in 70 ml water. Left: Ascending development for about 3 h (13 cm development distance; R_F values: GDP-mannose 0.05 and GDP-glucose 0.08). Right: Descending continuous-flow development for 20 h; a 25 cm long sheet of Whatman No. 3 MM paper was attached to the ion-exchange sheet at a distance of 12 cm from the origin. Impurities (close to the origin) are GMP and GDP. Photographed by short-wave ultraviolet light. Background fluoresces behind a secondary front.

The sheets may be cut to any size desired. Microscopic slide size sheets were found to be useful for preliminary experiments and for development of new solvent systems. The borate system for separation of nucleotide sugars (see Fig. 1) was developed using a series of such sheets. On the other hand, descending separations of complex mixtures may be carried out on 30–40 cm long sheets.

For many separations, ascending chromatography is sufficient, and resolution of complex mixtures[9,10] can be achieved by two-dimensional ascending chromatography on ion-exchange layers. Some closely related mononucleotides, however, are difficult to separate by simple ascending development, *e.g.*, the GDP-glucose/GDP-mannose pair. These nucleotides can be partially resolved by ascending development with a borate solvent (Fig. 1); descending continuous-flow development, using the same solvent, results, however, in a much more distinct resolution (see Fig. 1)*. Separation of these compounds on paper requires development times of 70 hours and 7 days, respectively, depending on the solvent[12]. As a rule, solvents of low elution power (R_F values of the compounds to be separated < 0.2, preferably 0.05–0.15) are best suited for continuous-flow chromatography on ion-exchange layers.

In the case of two-dimensional separations, cutting the sheet after chromatography in the first dimension (see above) and individually developing each section obtained makes possible a resolution of practically all common mononucleotides on one plate**.

The novel rechromatography procedure described under Experimental facilitates further analysis and purification without time-consuming desalting steps.

Although chromatography paper impregnated with poly(ethyleneimine)[14,15] resembles PEI-cellulose layers in many respects, a comparison[13] clearly shows the latter to be superior with regard to sensitivity and resolution power. For a comparison between PEI-paper and other anion-exchange papers, see ref. 15.

Quantitative results

In Table I data are listed which illustrate the reliability and effectiveness of the direct elution procedure described. The results closely resemble those obtained with the transfer procedure previously described for glass plates[8]: accurate and precise spectrophotometric determinations of small quantities of individual nucleotides can be performed. The direct technique, however, is less time-consuming: quantitative analysis of a compound can be completed within 75–90 min after separation. U.V. spectra may also be recorded.

DISCUSSION

The results presented show PEI-cellulose thin-layer sheets to combine advantages of both paper and thin-layer chromatography. Due to the thinness of the plastic material (0.25 mm) used as a support for the anion-exchanger and to the stability of the layer the sheets can be cut with scissors or a cutting board. They may be folded without disrupting the layer continuity, and a paper wick may be attached by stapling. These three operations make possible the following analytical procedures:

* The solvent used is also capable of separating other nucleoside diphosphate sugars according to the hexose moiety[13].

** Unpublished experiments.

TABLE I

QUANTITATIVE ELUTION OF MONONUCLEOTIDES (7–10 mμMOLES EACH) FROM PEI-CELLULOSE SHEETS (DIRECT PROCEDURE)

Eluant: 1.0 ml 0.7 *M* $MgCl_2$–2.0 *M* Tris hydrochloride, pH 7.4 (100:1, v/v).

Nucleotides	*N**	*Applied* ($E_{260\,m\mu}$, 1 cm)	*Recovered in replicate (mean)*	σ**	σ_{rel}*** (%)	*Recovery* (%)
dAMP	10	0.107	0.1077	0.0025	2.3	100.5
dAMP	10	0.109	0.1113	0.0029	2.6	102.1
ATP	8	0.149	0.1468	0.0033	2.5	98.5
UDP-glucose	8	0.087	0.0867	0.0017	2.0	99.7
TDP	8	0.067	0.0650	0.0015	2.3	97.1

* Number of determinations
** Standard deviation = (variance)$^{1}/_{2}$.
*** Relative standard deviation = $\sigma \cdot 100/\text{mean}$ %.

1. Direct quantitative evaluation (spectrophotometry or counting) of a cut-out.
2. Direct rechromatography.
3. Chromatography with different solvents in the second dimension.
4. Descending chromatography.
5. Continuous-flow development.

Descending and continuous-flow chromatography would be more difficult to perform on glass plates; direct quantitative assays and rechromatography could not possibly be carried out on glass plates.

As an additional advantage, homogeneous layers are more readily obtained on the plastic material than on conventional small glass plates, because one long sheet is coated in one operation. As a consequence, layer preparation and analytical techniques can more easily be standardized than on glass plates.

The plastic sheets should also be useful in micropreparative work: 1–10 mg of a nucleotide may be chromatographed as a band on a 10 cm wide sheet. After cutting out the band, the compound may be eluted by a transfer procedure[8] or directly with electrolyte solutions in the way described for quantitative assays. If the nucleotide is sufficiently stable against acid or alkali, dilute hydrochloric acid or dilute ammonia may be used for elution; otherwise, salt or buffer solutions (preferably volatile) are suitable. In our experience with the direct procedure, 15 min are sufficient for 90 % extraction if the eluant is capable of quantitative extraction in 1 h. Since temperature has little effect on the rate of the ion-exchange process, elution may be carried out at low temperature if required. For removal of non-volatile salts, the procedure reported by DANECK *et al.*[16] should be considered.

Due to their properties the thin-layer sheets are well suited for qualitative and quantitative routine analyses, *e.g.*, of biological extracts and of reaction mixtures. They are useful tools in both biological and preparative chemistry of nucleotides.

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SUMMARY

The present communication describes preparation and properties of anion-exchange (PEI-cellulose) thin layers on plastic sheets. The ion-exchange sheets are shown to combine advantages of paper and ion-exchange thin-layer chromatography. Procedures are reported which are difficult to perform or cannot be performed on the conventional glass plates. These include techniques for quantitative assays, rechromatography, descending and continuous-flow chromatography.

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