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Note

Separation of adenosine and its derivatives by thin-layer chromatography on silica gel

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Adenosine, non-cyclic adenine nucleotides (AMP, ADP, ATP) and cyclic nucleotides have recently been proposed as neurotransmitters and/or neuromodulators[1–3]. It therefore seems of interest to develop a simple method to identify these compounds. Separations by electrophoresis[4] and high-performance liquid chromatography[5] have been described. Thin-layer chromatography (TLC) has also frequently been used to identify purines, pyrimidines and related compounds[6–8]. However, time consuming two-dimensional techniques have been employed[9–12]. In a recent communication a rapid one-dimensional TLC method for separation of the degradation products of adenine nucleotides was described[13], but two developments were employed.

In the present paper a simple and rapid one-dimensional one-development TLC method, associated with scanning radiochromatography or liquid scintillation, capable of separating adenine, adenosine, inosine, hypoxanthine, 2′-AMP, 5′-AMP, ADP, ATP, cyclic AMP and dibutyryl cyclic AMP (dbcAMP), is described.

MATERIALS AND METHODS

Reagents

All solvents were analytical reagent grade (BDH, U.K.) or pro-analysis (E. Merck, Darmstadt, F.R.G.). Thin-layer plates were prepared from silica gel $60F_{254}$ (Merck, art. 7730), or were precoated plates of silica gel $60F_{254}$, 20 × 20 cm, layer thickness 0.25 mm (Merck, art. 5715).

[^{3}H]Adenosine with a specific activity of 500 mCi/m*M* was obtained from the Radiochemical Centre (Amersham, U.K.). Adenine, adenosine, adenosine 2′-monophosphate (2′-AMP), adenosine 5′-monophosphate (5′-AMP), adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate (ATP), adenosine 3′:5′-cyclic monophosphate (cAMP), N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate (dbcAMP), inosine and hypoxanthine were purchased from Sigma (St. Louis, MO, U.S.A.).

Thin-layer chromatography

Solvent mixtures with the following compositions were used:

System 1. *n*-Butanol–acetone–acetic acid–5% ammonia solution–water (9:3:2:2:4)

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System 2. *n*-Butanol–acetone–acetic acid–5% ammonia solution–water (9.5:3:1:1:5.5)

System 3. *n*-Butanol–acetone–acetic acid–5% ammonia solution–water (9:5:2:2:3)

System 4. *n*-Butanol–acetone–acetic acid–5% ammonia solution–water (9:5:3:3:1)

System 5. *n*-Butanol–acetone–acetic acid–5% ammonia solution (9:6:3:3)

System 6. *n*-Butanol–acetone–acetic acid–5% ammonia solution (10:5:3:3)

System 7. *n*-Butanol–acetic acid–5% ammonia solution–water (11:3:3:3) + 0.5 ml of acetone per 100 ml

System 8. *n*-Butanol–acetone–acetic acid–water (11:3:3:3)

System 9. *n*-Butanol–acetone–5% ammonia solution–water (11:3:3:3)

System 10. *n*-Butanol–acetic acid–5% ammonia solution (14:3:3)

System 11. *n*-Butanol–water (14:6) + 2.5 ml of acetone per 100 ml.

All systems were prepared 24 h before use in order to allow equilibrium. The solvent front was 17 cm above the starting point, which was placed 1.5 cm from the bottom of the plates. Thin-layer plates 0.25 mm thick were prepared in 20 × 10 or 20 × 20 cm glass plates, by mixing for 2 min silica gel $60F_{254}$ (Art. 7730, 50 g) with 95 ml of water and then spreading using a Quickfit spreader. The layers were dried at room temperature and activated for 1 h at 105°C. All the layers were previously washed with ethanol. The spots on the developed chromatograms were rendered visible under UV light at 254 nm.

All the standards were dissolved in sterile water at concentrations of 1 m*M*, and aliquots corresponding to 5 nmol of each were chromatographed.

Dilutions of [^{3}H]adenosine were also made in sterile water and corresponded to 25000 cpm when chromatography was associated with liquid scintillation or to 100000 cpm when TLC plates were analysed by scanning radiochromatography.

Scintillation counting by zonal scan

After development and evaporation of solvent, the TLC plates prepared in our laboratory were scraped in 2-mm sections with a zonal scraper (Analabs, North Haven, CT, U.S.A.) directly into vials. The radioactivity of the samples was measured in a Beckman LS 8100 liquid scintillation system. To each vial, 5 ml of scintillation fluid were added (100 g of naphthalene, 7 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene per litre of dioxane.

Quenching correction of the samples was made by use of graphs of counting efficiency previously obtained with tritium-quenched standards by the H-number technique (an external standardization technique for Beckman LS 800 liquid scintillation systems).

Thin-layer radiochromatography

After development, precoated plates (Merck, art. 5715) were placed under a hot air flow to evaporate the solvent. Then the radiochromatograms were obtained in a thin-layer scanner system RTLS-1A (Panax Nucleonics, Redhill, U.K.).

The tritium efficiency was determined by applying different amounts of [^{3}H]adenosine (measured by liquid scintillation) to a precoated TLC plate previously developed with solvent 9 under the same conditions as for the chromatograms. These

quantities were applied at different intervals as small circles with diameters not greater than 0.5 cm. From the relative intensity of the peaks (cps) corresponding to different quantities (cpm) of [^{3}H]adenosine it was possible to calculate the efficiency. Argon–propane (98:2) was used as the carrier gas for the detector.

RESULTS AND DISCUSSION

In the present work, different mixtures of solvents have been used according to previous descriptions[13–17]. The solvent mixture using *n*-butanol were the most efficient to identify adenosine and its derivatives; these derivatives could be detected in small amounts, *i.e.*, as little as a few nmoles.

The hR_F values in the eleven solvent mixtures, containing *n*-butanol, tested on precoated plates and developed either in a glass jar (Camag) with saturation for 2 h or in a sandwich chamber (Camag), were determined. Each value was the mean of two determinations, with one development for each system. In all systems the relative positions of adenosine and its derivatives were maintained, except in system 10, which did not contain acetone and, therefore, inverted the polarity of dbcAMP. The hR_F values were about the same for the two types of developments. However, the sandwich development was quicker than in the glass jar. As the saturation in the sandwich chamber was too rapid sometimes disequilibrium occurred, and this caused demixing of the mobile phases in the layers. The development in the glass jar increased the separation of the standards in the layers previously treated with ethanol. The best separation was obtained when system 9 was used. The development time for this system was 3 h for precoated plates and 4 h for the thin layers prepared in our laboratory.

In Table I are shown the mean hR_F values for adenosine and its derivatives ± S.E., as well as the relationship between the hR_F values of the standards and that of adenosine when using solvent 9 as developer. It is seen that with only one development it was possible to separate adenine, adenosine, 2'-AMP, 5'-AMP, ADP, ATP,

TABLE I

hR_F VALUES OF ADENOSINE AND ADENOSINE DERIVATIVES IN SOLVENT 9

Precoated plates (20 × 20 cm) of silica gel (Merck, Art. 5715) spotted with 5 nmol of each standard were visualized under UV light at 254 nm. For the composition of solvent 9 see Materials and methods. S.E. = Standard error of the mean.

Compound	hR_F	*n*	*S.E.*	hR_F *relative to adenosine*
Adenosine	44	18	0.5	1
Adenine	57	18	1.0	1.3
5'-AMP	25	16	0.5	0.7
ADP	16	16	0.4	0.6
ATP	12	16	0.4	0.4
2'-AMP	29	7	1.0	0.3
cAMP	39	16	0.6	0.9
dbcAMP	61	16	1.1	1.4
Inosine	38	8	1.4	0.9
Hypoxanthine	44	8	1.5	1

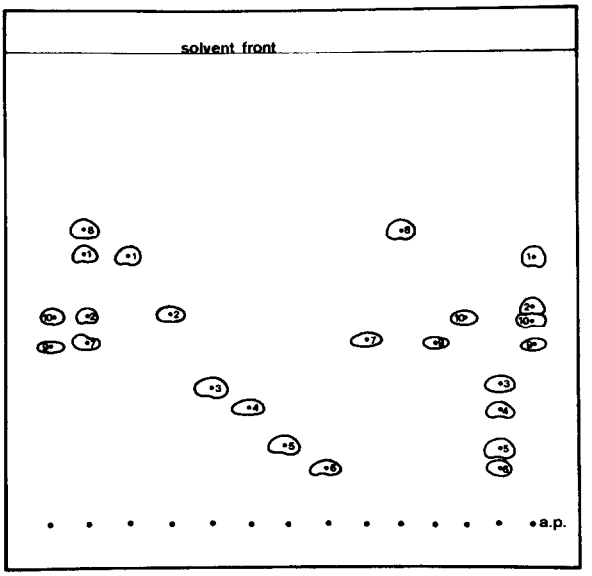

Fig. 1. TLC of 5 nmol of each standard on a precoated plate (20 × 20 cm) of silica gel (Merck, Art. 5715) using one development with system 9. Visualized under UV light at 254 nm. Spots: 1 = adenine; 2 = adenosine; 3 = 2'-AMP; 4 = 5'-AMP; 5 = ADP; 6 = ATP; 7 = cAMP; 8 = dbcAMP; 9 = inosine; 10 = hypoxanthine. a.p. = Application point.

cAMP and dbcAMP. Inosine and hypoxanthine were also tested; it was possible to separate inosine from all compounds except cAMP and hypoxanthine from all but adenosine.

Fig. 1 shows a typical chromatogram visualized by UV light at 254 nm; all compounds are well separated and it was possible to detect amounts as little as 1 nmol (5 nmol for dbcAMP).

[^{3}H]Adenosine was analyzed on silica gel plates (20 × 10 cm) prepared in our laboratory, and on precoated plates using solvent 9 as developer, concomitantly with standards of cold drugs. The purity of the sample and the relative percentages of adenosine contaminants determined by TLC–zonal scan/liquid scintillation and thin-layer radiochromatography are shown in Figs. 2 and 3 respectively.

Fig. 2. also shows the graph obtained from the radioactivity expressed in counts per minute (cpm) for each scraped fraction, corresponding to 2.1 mm of the layer; each plate had 75 fractions. After quenching correction, the radioactivity was converted into a percentage of the total recovered from the plates; the values presented are the mean values obtained by analysis on two plates. The total radioactivity recovered from the plates was 99.1% of that applied.

The percentage recoveries shown in Fig. 3 were determined in relation to the sum of the values for adenosine and adenine which was the main contaminant. The values in counts per second (cps) were converted into cpm and quantified in relation to the value of the efficiency previously determined for tritium using the same technique; they represent the average of analyses made on two plates, corresponding to 89.3% of the total radioactivity initially applied.

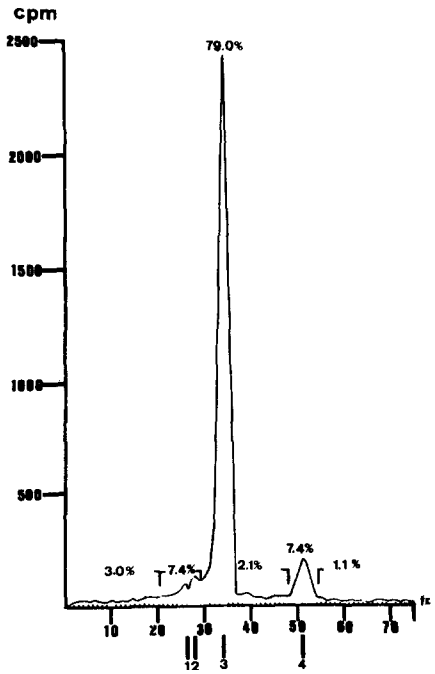

Fig. 2. Zonal scan (2.1 mm) of [^{3}H]adenosine (25000 cmp) on 20 × 10 cm plates of silica gel GF_{254} (Type 60) (Merck), prepared in our laboratory using one development with system 9. Peaks: 1 = cAMP; 2 = inosine; 3 = adenosine; 4 = adenine. fr = Fraction.

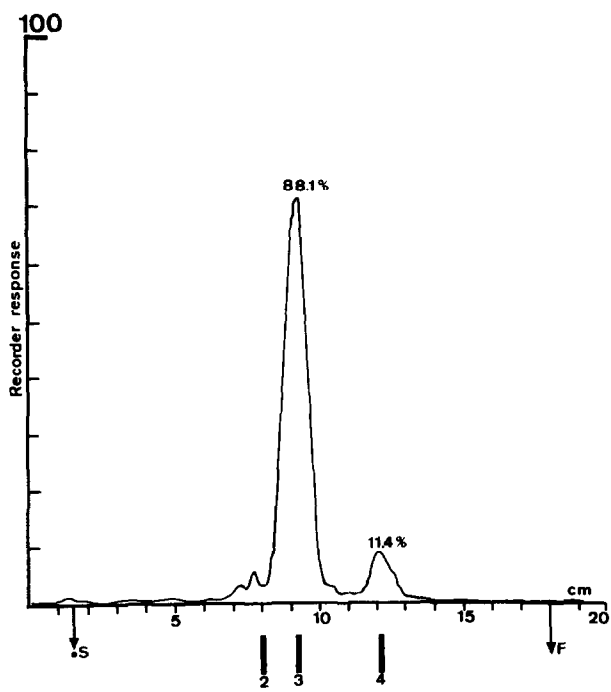

Fig. 3. Thin-layer radiochromatography of [^{3}H]adenosine (125000 cpm) on a precoated plate of silica gel (Merck, art. 5715) using one development with system 9. Scanning conditions: range, 10 cps; time constant, 30 sec; chart speed, 600 cm/h; tritium efficiency, 39.8%. Peaks: 2 = inosine; 3 = adenosine; 4 = adenine. S = Start; F = front.

TABLE II

PERCENTAGE RECOVERIES FOR ADENOSINE AND ADENINE

100% represents adenosine plus adenine.

Compound	*TLC-zonal scan/liquid scintillation*		*Thin-layer radiochromatography*	
Adenosine	91.5	91.4	86.7	89.6
Adenine	8.5	8.6	12.3	10.4

The results of the present work show that it is possible to detect small amounts of adenosine and its derivatives by using either TLC on silica gel with one development, and/or associated with scanning radiochromatography or liquid scintillation. TLC alone detected as little as 1 nmol of all compounds except dbcAMP (5 nmol). TLC associated with radiochromatography quantified as little as 74 pmol of adenosine and TLC associated with liquid scintillation measured 410 pmol of adenosine. Nevertheless, the percentage recoveries obtained for adenosine and adenine by either TLC-zonal scan/liquid scintillation or thin-layer radiochromatography (see Table II) suggest that both methods can give accurate information.

In conclusion, a good separation of adenosine and its derivatives has been achieved by using this one-dimensional one-development TLC technique. The radioactivity of the relevant spots can still be determined easily, and the method is rapid and enables more samples to be separated on a single TLC plate.

REFERENCES

1 J. A. Ribeiro, *Life Sci.*, 22 (1978) 1373.
2 J. W. Phillis and P. H. Wu, *Progr. Neurobiol.*, 16 (1981) 187.
3 T. W. Stone, *Neuroscience*, 6 (1981) 523.
4 H. Kizaki and T. Sakurada, *J. Chromatogr.*, 211 (1981) 409.
5 M. R. Preston, *J. Chromatogr.*, 275 (1983) 178.
6 K. Randerath, *Thin-Layer Chromatography*, Academic Press, London, 1966, p. 220.
7 G. Pataky, *Advan. Chromatogr.*, 7 (1968) 47.
8 N. C. Bols and D. D. Mosser, *J. Chromatogr.*, 229 (1982) 460.
9 G. W. Crabtree and F. Henderson, *Cancer Res.*, 31 (1971) 985.
10 V. Zhivkov and E. Gavazova, *Int. J. Biochem.*, 7 (1976) 497.
11 C. M. Smith, L. M. Rovano, M. P. Kekomäki and K. O. Raivio, *Can. J. Biochem.*, 55 (1977) 1134.
12 R. J. Sharma, *Biochem. Soc. Trans.*, 9 (1981) 581.
13 R. J. Sharma and J. R. Griffiths, *Biochem. Soc. Trans.*, 11 (1983) 291.
14 P. Grippo, M. Iaccarino, M. Rossi and E. Scarano, *Biochim. Biophys. Acta*, 95 (1965) 1.
15 K.-H. Scheit, *Biochim. Biophys. Acta*, 134 (1967) 217.
16 G. Pataki, *J. Chromatogr.*, 29 (1967) 126.
17 J. Cadet and R. Téoule, *J. Chromatogr.*, 76 (1973) 407.