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Note

Separation of prostaglandins and thromboxane by two-dimensional thin-layer chromatography

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Prostaglandins (PGs) and thromboxane (Tx), which are produced mainly from arachidonic acid through the cyclooxygenase pathway, play a role in several important cellular functions. Prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) have become of major interest, since these compounds are known to be involved in haemostasis and oncogenesis [1,2]. In studying PGs and Tx [3–5], only a few investigators have used two-dimensional thin-layer chromatography (TLC), which is widely used in the other fields of biochemistry.

This paper describes a two-dimensional TLC procedure for the separation of PGs and Tx with excellent resolution; the method can be applied to the analysis of PGI_2 and TxA_2.

EXPERIMENTAL

Chemicals

[1-^{14}C]Arachidonic acid (specific activity 52.0 mCi/mmol), [5,8,9,11,12,14,15-^{3}H(N)]-6-ketoprostaglandin $F_{1\alpha}$ (specific activity 150 Ci/mmol) and [5,6,8,9,12,14,15-^{3}H(N)]prostaglandin D_2 (specific activity 100 Ci/mmol) were purchased from New England Nuclear Research Products (Boston, MA, U.S.A.). Arachidonic acid (AA) was purchased from Sigma (St. Louis, MO, U.S.A.). Authentic PG and Tx standards ($PGF_{2\alpha}$, PGE_2, PGD_2, 6-keto-$PGF_{1\alpha}$ and TxB_2) were the generous gifts of Ono Pharmaceutical (Osaka, Japan). The solvents used were all analytical-grade reagents from Nakarai Chemicals (Kyoto, Japan).

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The radioactive compounds were purified by one-dimensional TLC with the solvent system ethyl acetate–iso-octane–acetic acid–water, (11:5:2:10, v/v) [6] prior to each experiment.

Chromatographic separation of radioactive compounds

One-dimensional TLC. A 20-μg sample of each of the authentic compounds and 10^4 dpm of each of the radioactive compounds (PGD_2, 6-keto-$PGF_{1\alpha}$, AA) were applied to a silica gel 60 plate (0.25 mm thickness, 20×20 cm, E. Merck, Darmstadt, F.R.G.), which was pre-washed with acetone. The plates were developed with three different solvent systems: (I) ethyl acetate–iso-octane–acetic acid–water (11:5:2:10, v/v) [6], (II) chloroform–methanol–acetic acid (90:8:6, v/v) [7] and (III) diethyl ether–methanol–acetic acid (90:1:2, v/v) [8].

Authentic standards were visualized by spraying with 10% phosphomolybdic acid in ethanol, and each spot was scraped off into separate vials. PGs and Tx were extracted with methanol, to which 5 ml of aqueous scintillant (ACS II, Amersham, U.K.) were added. The radioactivity was determined by a liquid scintillation counter and the recovery rates were determined for each of the labelled compounds.

Two-dimensional TLC. A 20-μg sample of each of the authentic compounds and 10^4 dpm of each of the labelled compounds were applied to the right bottom corner of the TLC plates and developed first to the left and secondly towards the top. The solvent systems used were I and II (I/II) or II and III (II/III) described by Srivastava and Awasthi [4]. Each spot of PGs and Tx was visualized and scraped off, and the radioactivity was determined as described above.

Two-dimensional TLC in the analysis of PGs and Tx production by human neuroblastoma cells in culture

Cultured cells of a human neuroblastoma cell line, NB-1 [9], were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% carbon dioxide. For the study, $5 \cdot 10^5$ cells were seeded in a culture flask (25 cm^3, Corning), and the medium was changed every 48 h. After eight days, cells were treated with a fresh medium containing 1 μCi of [1-^{14}C]AA and cultured for another 15 h. The medium was then collected and analysed for PGs and Tx produced from [1-^{14}C]AA by the cells.

After acidification of the medium to pH 4.0 with hydrochloric acid, PGs and Tx were extracted three times with three volumes of ethyl acetate. The extracts were evaporated and the residues were reconstituted in a small volume of ethanol. An aliquot ($3 \cdot 10^5$ dpm) of the ethanol extract was applied with 20 μg each of the authentic standards on a TLC plate, and two-dimensional TLC was carried out with the solvent systems I and II (I/II) or II and III (II/III). Each spot of PGs and Tx was visualized and scraped off, and the radioactivity was determined as described above.

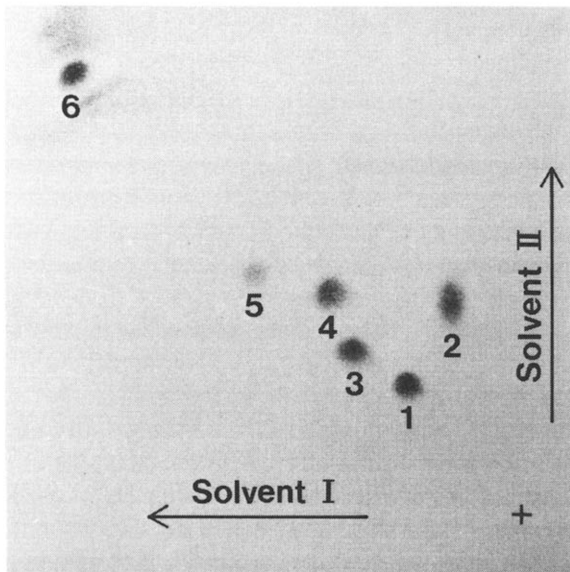

Fig. 1. Separation of prostaglandins, thromboxane and arachidonic acid by two-dimensional TLC with the solvent system I/II. Spots: 1 = $PGF_{2\alpha}$; 2 = 6-keto-$PGF_{1\alpha}$; 3 = TxB_2; 4 = PGE_2; 5 = PGD_2; 6 = AA.

RESULTS

Chromatographic separation of radioactive compounds

In one-dimensional TLC with the solvent system I, resolution was poor between TxB_2 and PGE_2. In solvent system II PGE_2 and 6-keto-$PGF_{1\alpha}$ were not well resolved, and in solvent system III TxB_2 and PGE_2, and $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were not clearly separated. Two-dimensional TLC with the solvent system I/II

TABLE I

RECOVERY OF RADIOACTIVE COMPOUNDS IN ONE- AND TWO-DIMENSIONAL TLC

10^4 dpm of each labelled compound were applied with authentic standards (20 μg) on a plate and developed. Each spot of PGs and Tx was visualized and scraped off, and the radioactivity was determined. Results are mean ± S.D. of four to eight separate experiments.

Solvent system	Radioactivity (dpm)		
	6-Keto-$PGF_{1\alpha}$	PGD_2	AA
I	4677 ± 880	4591 ± 389	6779 ± 617
II	3797 ± 438	3976 ± 951	5724 ± 1038
III	5226 ± 901	4229 ± 204	6253 ± 755
I/II	2800 ± 298	4243 ± 372	6789 ± 485
II/III	3033 ± 445	3917 ± 174	6618 ± 1473

TABLE II

PGs AND Tx PRODUCTION BY NB-1 CELLS FROM [1-^{14}C]AA ANALYSED BY TWO-DIMENSIONAL TLC WITH THE TWO SETS OF SOLVENT SYSTEMS

$3 \cdot 10^5$ dpm of sample extract were applied with authentic standards (20 μg) on a plate, and two-dimensional TLC was carried out. Each spot of PGs and Tx was visualized and scraped off, and the radioactivity was determined. Results are the mean ± S.D. of six separate experiments.

Solvent system	Radioactivity (dpm)					
	$PGF_{2\alpha}$	6-Keto-$PGF_{1\alpha}$	TxB_2	PGE_2	PGD_2	AA
I/II	1604±192	2238±116	1354±201	1448±308	850±269	58 500±14 400
II/III	1909±355	2422±431	1455±240	1364±205	2137±620	69 000±19 300

(Fig. 1) led to clearer separation of all major PGs and Tx than one-dimensional TLC. However, two-dimensional TLC with the solvent system II/III did not afford consistent separation of PGE_2 and 6-keto-$PGF_{1\alpha}$. As shown in Table I, the recoveries of 6-keto-$PGF_{1\alpha}$ and PGD_2 by one-dimensional TLC were 37–52% and that of AA was 57–67%. In contrast, the recoveries of PGD_2 and AA by two-dimensional TLC were almost the same as those by one-dimensional TLC. However, the recovery of 6-keto-$PGF_{1\alpha}$ was only 28–30%.

Assay of PGs and Tx synthesis by NB-1 cells

PGs and Tx production from [1-^{14}C]AA by NB-1 cells was analysed by using two sets of two-dimensional TLC solvent systems. As shown in Table II, the synthetic radioactive $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, TxB_2, PGE_2 and PGD_2 were identified. When the solvent system II/III was used, the radioactivities of these compounds were higher than those determined by the solvent I/II, except for PGE_2. With the solvent system I/II, PGD_2 was recovered particularly in significantly lower amounts.

DISCUSSION

Gas chromatography–mass spectrometry is now considered to be the most sensitive and reliable method for the measurement of very small amounts of PGs in biological materials [10]. However, since this method is expensive and time-consuming, it is accessible only to a limited number of institutes. In the majority of research centres, investigators still use radiolabelled AA and study the production of PGs and Tx in biological materials with TLC.

One-dimensional TLC has most often been used for this purpose [11–13]. Since it was difficult to obtain complete separation of all PGs and Tx by a single one-dimensional TLC procedure, the most adequate solvent system was selected for specific types of PGs and Tx with the aim of separating them from the others [6]. In such a system, the possibility of cross-channel contamination between different PGs exists during the TLC separation [14]. Our attempts at using one-

dimensional TLC with any one of the three solvent systems were unsatisfactory.

To date, there have been only a few reports about two-dimensional TLC methods that give complete resolution of major PGs and Tx. In Granström's three solvent systems [15], one gave incomplete separation between PGE_2 and TxB_2, and the other two lacked information about the location of 6-keto-$PGF_{1\alpha}$. The report by Bailey et al. [5] did not provide the information about 6-keto-$PGF_{1\alpha}$, probably because of the relatively late discovery of PGI_2.

The solvent system II/III reported by Srivastava and Awasthi [4] was more effective, but the separation between PGE_2 and 6-keto-$PGF_{1\alpha}$ was not consistent. By contrast, our solvent system I/II consistently gave a complete separation of all major PGs and Tx. In terms of the recovery from TLC, our results were comparable with those in other reports [6,16]. AA, a less polar substance, was recovered in a higher percentage (68%), but 6-keto-$PGF_{1\alpha}$, a polar substance, had a low recovery (38%) in one-dimensional TLC. This phenomenon became more apparent in two-dimensional TLC (Table I).

Using two-dimensional TLC (Table II), we showed that cultured human neuroblastoma cells (NB-1) synthesize $PGF_{2\alpha}$, PGE_2, PGD_2, 6-keto-$PGF_{1\alpha}$ and TxB_2 from [1-^{14}C]AA. In our solvent system I/II, the radioactivities of each PG and Tx, especially of PGD_2, were lower than those determined by the solvent system II/III.

Recent increased interest in PGI_2 (6-keto-$PGF_{1\alpha}$) and TxA_2 (TxB_2) means that a reliable method is required for the extraction and separation of these two compounds. Two-dimensional TLC, as described here, is a simple, rapid and reliable method for the separation of these two compounds from other PGs.

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REFERENCES

1 C. Ingerman-Wojenski, M.J. Silver and J.B. Smith, J. Clin. Invest., 67 (1981) 1292.
2 C. Chiabrando, M. Broggini, M.N. Castagnoli, M.G. Donelli, A. Noseda, M. Visinitainer, S. Garattini and R. Fanelli, Cancer Res., 45 (1985) 3605.
3 J. Männistö, Prostaglandins, 26 (1983) 681.
4 K.C. Srivastava and K.K. Awasthi, J. Chromatogr., 275 (1983) 61.
5 J.M. Bailey, R.W. Bryant, S.J. Feinmark and A.N. Makheja, Prostaglandins, 13 (1977) 479.
6 F.F. Sun, J.P. Chapman and J.C. McGuire, Prostaglandins, 14 (1977) 1055.
7 K.C. Srivastava, K.P. Tiwari and K.K. Awasthi, Microchem. J., 27 (1982) 246.
8 D.H. Nugteren and E. Hazelhof, Biochim. Biophys. Acta, 326 (1973) 448.
9 S. Imashuku, S. Todo, N. Esumi, T. Hashida, K. Tsunamoto and F. Nakajima, in A.E. Evans, G.J. D'Angio and R.C. Seeger (Editors), Advances in Neuroblastoma Research, Alan R. Liss, New York, 1985, p. 89.
10 I.A. Blair, S.E. Barrow, K.A. Waddell, P.J. Lewis and C.T. Dollery, Prostaglandins, 23 (1982) 579.
11 J.A. Salmon and R.J. Flower, Methods Enzymol., 86 (1982) 477.
12 A. Riutta, E. Seppälä and H. Vapaatalo, J. Chromatogr., 307 (1984) 185.

13 B.J. Smith, R.M. Ross, C.R. Ayers, M.R. Wills and J. Savory, J. Liq. Chromatogr., 6 (1983) 1265.
14 A.E. Barden, K.D. Croft, E.L. Mathews, J.S. Soltys, R. Vandongen and L.J. Beilin, J. Chromatogr., 275 (1983) 229.
15 E. Granström, Methods Enzymol., 86 (1982) 493.
16 H.G. Morris, N.A. Sherman and F.T. Shepperdson, Prostaglandins, 21 (1981) 771.