Journal of Chromatography, 161 (1978) 406–409

CHROM. 11,277

Note

Separation of myocardial purine nucleosides and nucleotides by one-dimensional thin-layer chromatography

DIANE K. REIBEL* and MICHAEL J. ROVETTO

Department of Physiology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pa. 19107 (U.S.A.)

(Received May 30th, 1978)

A number of reports have appeared concerning separation of purine and pyrimidine nucleosides and nucleotides by thin-layer chromatography (TLC) on polyethyleneimine (PEI) cellulose[1–6]. These represent modifications of the basic techniques developed by Randerath and Randerath[7–9] who first employed this resin for such separations. Nucleosides and nucleotides in biological fluids have been separated on PEI[2,4], but with few exceptions[3,5] these techniques were not applied to separation and quantification of nucleotides and nucleosides in tissue extracts. Moreover, methods for simultaneous separation of adenine nucleotides and nucleosides have previously relied on two-dimensional chromatography. Development of the TLC plates in a second dimension greatly restricts the number of samples per plate and significantly prolongs the time required for analysis.

We have employed one-dimensional development with a solvent system which enables simultaneous separation of adenine nucleotides and nucleosides and their quantification in myocardial tissue extracts.

METHODS

Isolated rat hearts perfused as Langendorff or working hearts[10,11] were quickly frozen between metal clamps cooled in liquid nitrogen. The frozen tissue was pulverized in a liquid nitrogen cooled stainless-steel mortar, and extracted in ice-cold 6% perchlorate (PCA). The PCA extract was centrifuged at 10^4 *g* for 10 min, the supernatant added to cold 0.1 ml of 1 *M* morpholinopropane sulfonic acid (MOPS) and the solution neutralized with 30% KOH. Potassium perchlorate was removed by centrifugation. PCA was also removed with a 4:1 mixture of Freon TF® (Miller-Stephenson Chemical Co., Danbury, Conn., U.S.A.) and Alamine® 339 (McKerson Corp., Minneapolis, Minn., U.S.A.)[12]. Four volumes of Freon–Alamine were added per volume PCA extract. After 1 min of vigorous mixing, the samples were centrifuged at 10^4 *g* for 5 min and the upper phase carefully removed and added to 10 μl 1 *M* MOPS, pH 7.2. Extracts prepared in this manner contained much less salt than

* Present address: Department of Physiology, Hershey Medical Center, Hershey, Pa. 17033, U.S.A.

those prepared by KOH neutralization which allowed larger sample application to TLC plates.

Ten to 150 μl of the neutralized extract were spotted 2 cm from the bottom of 20 × 20 cm commercially prepared plastic-backed PEI cellulose plates with UV flourescent background (Schleicher and Schüll F1440 PEI/LS254; Schleicher and Schüll, Keene, N.H., U.S.A.). Effective separation was acheived on similar plates supplied by Brinkmann Instruments (Westbury, N.Y., U.S.A.) but with higher sample volumes, these became overloaded with salt.

Nucleotide and nucleoside standards were made singly or in combination to 1 m*M* final concentration in aqueous media. Standards were spotted on the plates to give 5–20 nmoles of each compound.

Separation was achieved by one-dimensional development using the following solvents, and solvent migration distances (the plates were placed directly in the succeeding solvent without drying); butanol–methanol–water (1:1:8), 5 cm; deionized distilled water, 3 cm; 1.4 *M* LiCl, 12 cm. At this point, the plates were examined without drying under UV (254 nm) light. Generally additional development ($\frac{1}{2}$–1 h) in LiCl was necessary to completely separate adenosine 5′-monophosphate (AMP) and inosine 5′-monophosphate (IMP) and to achieve a greater separation of adenosine and hypoxanthine.

After complete separation, the plates were dried, the spots observed under UV light, circled with a soft pencil, scraped off, and eluted with 1 ml of 2 *M* LiCl. UV absorption of the eluate was determined spectrophotometrically and the concentration of each compound calculated using the appropriate molar extinction coefficient. Blanks were prepared in a similar manner by taking areas of cellulose adjacent to the spots. Blank values were independent of the quantity of PEI cellulose taken from the plate and averaged about 0.005 and 0.015 O.D. units for nucleotides and nucleosides, respectively. Adenine nucleotides in tissue extracts were determined by standard enzymatic techniques[13] for comparison to values obtained by chromatography.

RESULTS AND DISCUSSION

Fig. 1 represents a PEI cellulose plate on which standards, sample, and sample plus standards were separated. The relatively large spots which appeared in tissue samples between IMP and hypoxanthine corresponded to the migration of standard nicotinamide-adenine dinucleotide (NAD). Standard nicotinamide-adenine dinucleotide phosphate (NADP) was located between AMP and IMP and was not completely separated from these compounds in this development. Contamination of tissue AMP or IMP by NADP or its acid breakdown products was considered negligible as NADP amounts to only about 5% of the AMP and IMP concentrations in myocardial tissue[13]. The nucleosides adenosine, inosine and hypoxanthine are in very low concentrations in myocardial tissue and were not visualized on the plates and could not be quantified in the amounts of tissue sample spotted. However, radiolabeled nucleosides from tissue extracts could be detected. Because we were interested in obtaining a method for determining radioactive content of tissue nucleosides, no problem was posed by our inability to determine concentrations of these compounds.

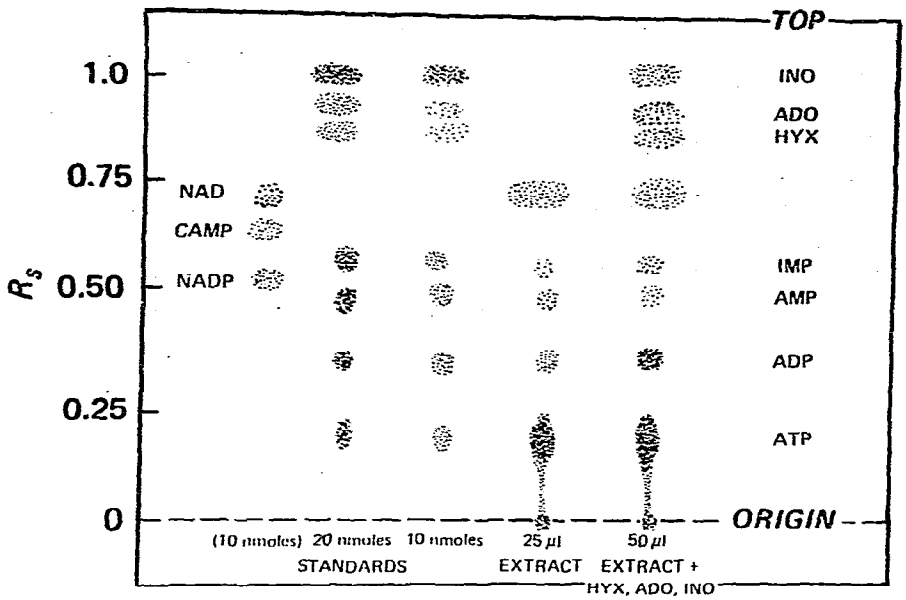

Fig. 1. Thin-layer chromatogram of purine nucleosides and nucleotides. Standards and tissue extracts were spotted on polyethyleneimine (PEI) cellulose plates and developed as described. The spots were traced from a UV illuminated chromatogram. The density of dots in each spot approximates the quench intensity of the fluorescent background of the PEI plates. R_s corresponds to the migration distance from the origin where inosine (INO) standard has a value of 1.0. Abbreviations: adenosine (ADO), adenosine 3',5'-cyclic monophosphate (CAMP), adenosine 5'-monophosphate (AMP) adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), hypoxanthine (HYX), inosine 5'-monophosphate (IMP), nicotinamide-adenine dinucleotide (NAD), nicotinamide-adenine dinucleotide phosphate (NADP).

Recovery data for standards and tissue nucleotides are shown in Table I. Although not shown, subtraction of values obtained with standards alone from those obtained with standards plus tissue extracts gave values comparable to tissue extracts alone. Also, ^{14}C-labeled adenosine standard was eluted quantitatively. Enzymatic analysis of tissue extracts gave values for ATP, ADP and AMP comparable to those obtained by TLC.

The techniques described represent modifications of TLC techniques employing PEI cellulose for separation of adenine nucleotides and nucleosides. However, to our knowledge, separation of the nucleosides from nucleotides and from each other by one-dimensional TLC has not been reported. Generally two-dimensional systems have been employed which is both time consuming and expensive when large numbers of samples are involved. In addition, reports of TLC systems for these separations have employed standard nucleosides and nucleotides and with few exceptions[3,5] have not applied the techniques to separation and quantification of tissue constituents.

The solvent system we presently employ allows separation and quantification of adenine nucleotides and nucleosides within about 3½ h. The technique is directly applicable to studies of radiolabel incorporation into nucleotides and nucleosides using tissue perchlorate extracts.

TABLE I

RECOVERY OF STANDARD AND TISSUE EXTRACT NUCLEOSIDES AND NUCLEOTIDES

Ten and 20 nmoles of each standard were applied to and developed on PEI cellulose plates. Each compound was subsequently eluted in 1 ml of 2 *M* LiCl and quantified spectrophotometrically using the appropriate UV extinction coefficient. These values were used to calculate percentage recovery which was found to be the same for 10- and 20-nmole samples. The amount of standards applied to the TLC plates was determined spectrophotometrically by UV absorption and enzymatically (ATP, ADP, AMP). Tissue ATP, ADP and AMP concentrations were determined by enzymatic analysis of tissue extracts and by TLC separation of the same extracts with subsequent quantification by UV absorbance. Each value represents the mean ± SEM for 8–16 standards or hearts.

AN	*Standards*		*Tissue extracts*	
	Recovery (%)		*μmoles/g dry tissue*	
	Extinction coeff.	*Enzymatic analysis*	*Enzymatic analysis*	*TLC*
ATP	99.8 ± 1.6	99.3 ± 1.9	20.9 ± 0.60	21.1 ± 0.70
ADP	99.8 ± 3.0	105.9 ± 8.7	3.7 ± 0.50	3.3 ± 0.40
AMP	100.5 ± 3.2	97.1 ± 7.0	0.35 ± 0.04	0.37 ± 0.04
IMP	100.6 ± 3.5	—	—	—
ADO	95.0 ± 2.1	—	—	—
HYX	99.0 ± 4.3	—	—	—
INO	101.0 ± 2.7	—	—	—

ACKNOWLEDGEMENTS

This research is supported in part by Research Grant HL-17895 and Career Development Award HL-00289 from the National Heart, Lung and Blood Institute of the National Institutes of Health.

REFERENCES

1 R. A. Barton, A. Schulman, E. L. Jacobson and M. K. Jacobson, *J. Chromatogr.*, 130 (1977) 145.
2 J. J. E. Chivot, M. J. Leibrandt, H. L. Rouvray, J. Simeon and J. P. Caen, *Clin. Chim. Acta*, 53 (1974) 69.
3 U. E. Honegger, S. S. Bogdanov and P. R. Bally, *Anal. Biochem.*, 82 (1977) 268.
4 C. E. Jones, in J. C. Touchstone (Editor), *Quantitative Thin Layer Chromatography*, Wiley-Interscience, New York, 1973, p. 155.
5 J. Lust and M. A. Sahud, *J. Chromatogr.*, 71 (1972) 127.
6 R. M. Trifilo and J. G. Dobson, *J. Chromatogr.*, 116 (1976) 465.
7 K. Randerath and E. Randerath, *J. Chromatogr.*, 16 (1964) 111.
8. E. Randerath and K. Randerath, *J. Chromatogr.*, 16 (1964) 126.
9 E. Randerath and K. Randerath, *Anal. Bichem.*, 12 (1965) 83.
10 J. R. Neely, H. Liebermeister, E. J. Battersby and H. E. Morgan, *Amer. J. Physiol.*, 212 (1967) 804.
11 J. R. Neely and M. J. Rovetto, *Methods Enzymol.*, 39D (1975) 43.
12 J. K. Khym, *Clin. Chem.*, 21 (1975) 1245.
13 H. U. Bergmeyer, *Methods in Enzymatic Analysis*, Vol. 4, Academic Press, New York, 1974.