Journal of Chromatography, 214 (1981) 111–117
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,953

PAPER CHROMATOGRAPHIC SEPARATION OF PHOSPHATE ESTERS, TRICARBOXYLIC CYCLE ACIDS AND AMINO ACIDS IN EXTRACTS FROM MALARIA PARASITES

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(First received March 28th, 1980; revised manuscript received April 28th, 1981)

SUMMARY

A paper chromatographic method for the separation and identification of complex mixtures of phosphate esters, tricarboxylic cycle acids and amino acids in biological extracts is described. The method has been applied to the investigation of carbohydrate metabolism pathways in the intraerythrocytic, simian malaria parasite *Plasmodium knowlesi*; on treatment of parasitized erythrocytes with [U-C^{14}]glucose, a perchloric acid extract was prepared and separated by chromatography on Whatman 31 ET filter-paper. A simple procedure for the measurement of the specific activities of radioactive compounds without prior elution from the chromatographic support is also described.

INTRODUCTION

We have reported earlier that the simian malaria parasite *Plasmodium knowlesi* possesses a novel pathway for metabolizing carbohydrates[1,2]. Several procedures have been described for the separation and the identification of the products of metabolic pathways[3–5]. These cannot easily be applied for the identification of unknown compounds such as mixtures of phosphate esters, tricarboxylic acid cycle acids and amino acids, which are the major products of carbohydrate metabolism in *Plasmodium knowlesi*.

We have now developed a paper chromatographic method that is more useful for the separation and identification of complex mixtures of the above three classes of compounds. The previous techniques were not adequate for the identification of such compounds and also lacked information on R_F values. In this study, this shortcoming has been overcome by determining the R_F values of all three classes of compounds in various solvent systems.

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0021-9673/81/0000–0000/$02.50

MATERIALS AND METHODS

Whatman 31 ET filter paper and other grades of chromatographic papers were purchased from Reeve Angel (London, Great Britain). Phosphate esters, tricarboxylic acid cycle acids, sugars, enzymes and nucleotides were obtained from Boehringer (London, Great Britain) and Sigma (St. Louis, MO, U.S.A.). Powdered cellulose and N-2-hydroxypiperazine-N-2-ethanesulphonic acid (HEPES) were supplied by Calbiochem (London, Great Britain), carbon-14 labelled compounds by The Radiochemical Centre (Amersham, Great Britain) and 2,5-diphenyloxazole and *p*-bis-(*o*-methylstyryl)benzene by New England Nuclear (Boston, MA, U.S.A.). All other chemicals were of the highest grade obtainable from BDH (Poole, Great Britain).

Chromatographic methods

Four solvent systems were employed. Solvent 1, for descending development, was ethanol–ethyl formate–1 *M* ammonium formate (pH 4) (6:1:3); solvent 2, for ascending development, was ethanol–1 *M* ammonium formate (pH 8) (3:2); solvent 3, for descending development, was propanol-1–ammonia–water (50:4:9); and solvent 4 was butanol-1–acetic acid–water (12:3:5), corresponding to the upper layer of Patridge's solvent[6]. The 1 *M* ammonium formate solution in solvent 1 contained 1.7 mmol/l of EDTA (free acid), the pH being adjusted to 4 with formic acid. The pH of the 1 *M* ammonium formate solution in solvent 2 was adjusted to 8 with ammonia solution. All solvents were prepared when required, except solvent 4, which was aged for 48 h before use. The chromatograms were developed at laboratory temperature (22 ± 2°C).

Carboxylic acids were detected with aniline–xylose reagent[7,8], citrate with *p*-dimethylaminobenzaldehyde reagent[9], pyruvate and oxaloacetate with 2,4-dinitrophenylhydrazine reagent[8] and reducing sugars with aniline–xylose reagent[10]. For the detection of phosphate esters, the paper was sprayed with a solution of potato acid phosphatase (orthophosphoric acid monoester phosphohydrolase, E.C. 3.1.3.2). The reaction mixture (50 mg of acid phosphatase, 5 ml of 0.1 *M* magnesium sulphate solution and 100 ml of 0.1 *M* sodium acetate solution adjusted to pH 5 with sodium hydroxide) was sprayed uniformly on to the paper, which was covered with a thin film of polythene and left at ambient temperature for 30 min for reaction to proceed. Phosphate ion was revealed by spraying the paper with ascorbic acid–ammonium molybdate reagent[11].

Biological methods

The Nuri strain of *Plasmodium knowlesi*, a simian malaria parasite, was maintained by serial blood passage in rhesus monkeys (*Macaca mulatta*) at the Liverpool School of Tropical Medicine. Heavily infected blood samples containing 40 % or more of infected erythrocytes were withdrawn from the saphenous vein under general anaesthesia in heparinized syringes. Approximately 10 ml of blood were passed through a 5 × 1 cm I.D. glass column packed lightly with powdered cellulose (Cellex-N; Bio-Rad Labs., Richmond, CA, U.S.A.), to remove leucocytes. The leucocyte-poor blood was centrifuged and the plasma discarded. The sedimented cells were washed twice with HEPES saline and reconstituted in the same medium to give a suspension containing 4 × 10^9 cells/ml. One litre of HEPES saline contained 139

mmol of sodium chloride, 1.73 mmol of potassium chloride, 2 of potassium dihydrogen orthophosphate, 1.07 of magnesium sulphate, 1.00 of calcium chloride and 20 mmol of HEPES, the pH being adjusted to 7.4 with sodium hydroxide. For metabolic studies, 10 μCi of [U-C^{14}]glucose was added and the cells were incubated at 37°C for 15 min on a metabolic shaker incubator. The reaction was terminated by adding 4 ml of ice-cold 8% (w/v) perchloric acid. The resulting mixture was centrifuged at 500 *g* for 15 min, the clear supernatant was filtered to remove floating debris and neutralized with solid potassium carbonate to a methyl orange end-point and precipitated potassium perchlorate was discarded. Following the termination of incubation, all operations were carried out at 4°C. Approximately 10–20 μl of neutralized extract were spotted on chromatographic paper by repeated application and drying in a stream of nitrogen.

Detection of ^{14}C-labelled compounds

The developed chromatogram, dried in a stream of nitrogen, was exposed to Kodak Royal Blue X-ray film, placed inside lead-lined cassettes and stored at −20°C for 6–8 weeks. After processing the X-ray film, profiles of radioactive spots were traced on chromatograms and R_F values were recorded. The radioactive spots were cut out from the chromatograms, immersed in vials containing a scintillation mixture containing 4 g of 2,5-diphenyloxazole and 0.08 g of *p*-bis(*o*-methylstyryl)benzene per litre of toluene and counted in a Packard Tricarb liquid scintillation spectrometer.

Measurement of the specific activity of glucose 6-phosphate was carried out without eluting the compound from chromatographic support. The strips of paper containing glucose 6-phosphate recovered from the counting vial were washed twice with diethyl ether to remove trace amounts of scintillation solution and air dried. Glucose 6-phosphate was assayed enzymatically at 340 nm utilizing glucose 6-phosphate dehydrogenase and NADP[12]. The assay mixture was pipetted into the cuvette and the reaction was started by inserting the paper strip containing glucose 6-phosphate directly into the cuvette. The total change in absorbance was recorded and the concentration of glucose 6-phosphate was calculated from the value of the molar absorptivity of NADP. To check the reliability of the method, a known amount of pure glucose 6-phosphate was dried on a paper strip and assayed similarly. The error of the determination was around 5%.

RESULTS AND DISCUSSION

R_F values of reference compounds in solvents 1–4 are presented in Table I. Solvent 1 was found to be useful for the separation of phosphate esters and could be used for one-dimensional chromatography or, in combination with solvent 2, for two-dimensional chromatography. Some amino acids and organic acids could also be resolved with solvent 1.

Solvent 1 was evolved through several modifications of a parent solvent containing methanol, formic acid, ammonia and water. Trials indicated that the mobilities and overall resolution of phosphate esters were influenced by changes in the pH and the polarities of the components. To simplify the formulation of the solvent, the combination of ammonia and formic acid was replaced with a solution of ammonium formate whose pH was adjusted to a desired value.

TABLE I

R_F VALUES OF REFERENCE COMPOUNDS IN SOLVENTS 1–4

Solvent 1: ethanol–ethyl formate–1 *M* ammonium formate (pH 4) (6:1:3). Descending development for 6 h in machine direction on Whatman 31 ET paper. Solvent 2: ethanol–1 *M* ammonium formate (pH 8) (7:5). Whatman 31 ET paper was washed in solvent 1 and dried prior to spotting of compounds. Development was carried out for 4 h by ascending flow in the machine direction of paper. Solvent 3: propanol-1–ammonia–water (50:4:9). Descending development for 6 h on Whatman 31 ET paper at right-angles to the machine direction. Solvent 4: butanol-1–acetic acid–water (12:3:5). Whatman 31 ET paper was washed in solvent 3 and dried prior to spotting of compounds. Development by ascending chromatography for 5 h in machine direction.

Compound	R_F *values*			
	Solvent 1	*Solvent 2*	*Solvent 3*	*Solvent 4*
Adenosine 5-triphosphate	10	23	0	9
Adenosine 5-diphosphate	20	27		11
2,3-Diphosphoglycerate	20	23	0	25
Cystine	20			
6-Phosphogluconate	25	35		20
Fructose 1,6-diphosphate	25	25	0	
Leucine			6	69
Isoleucine	35		62	68
Aspartate	35	54	6	32
Homocysteine	35		10	
Adenosine 5-monophosphate	37	37	3	22
3-Phosphoglycerate	40	43	5	42
Glucose 6-phosphate	41	52	1	17
Histidine	44		31	21
Glucose 1-phosphate	45	50		18
Ornithine	45		15	18
Coenzyme A	45			19
Phosphoenol pyruvate	46	46	3	37
Alanine	47			42
Acetyl coenzyme A	50		1	21
Glutamate	50	54	7	33
Glycine	50		24	
Fructose 6-phosphate	52			33
Arginine	52		15	22
Serine	52		24	30
Citrulline	52		21	30
Lysine	52		19	15
Ribose 5-phosphate	52	54	2	25
Orthophosphate	53		3	32
Hydroxyproline	57		22	34
Taurine	58		33	28
Threonine	59		38	37
Tryptophan	59		49	56
Cysteine	60		5	13
α-Glycerophosphate	61	59	5	24
β-Glycerophosphate			5	27
Citrate	65	47	2	52
Proline	66			48
Glucose	67	80	45	37
α-Ketoglutarate	74			

TABLE I *(continued)*

Compound	R_F *values*			
	Solvent 1	*Solvent 2*	*Solvent 3*	*Solvent 4*
Tyrosine	75		41	56
Pyruvate	76		62	60
Malate	77		10	66
Methionine	77		64	52
Phenylalanine	78		72	60
Oxaloacetate	78		11	64
α-Aminoisobutyrate			47	50
β-Aminoisobutyrate	79			88
γ-Aminoisobutyrate			37	
Glyoxalate	80			60
Fumarate	80		16	85
Valine	81		57	56
Succinate	82		17	80
α-Ketobutyrate			71	87
α-Ketoglutarate	83		18	66
Lactate	84	77	48	81
Oxalate			0	
Asparagine			7	32
Glutamine			8	
Maleic acid			11	72

Table II shows the effects of variation of pH, ionic strength and polarity of the solvents by altering either the composition or proportion of ammonium formate solution. An increased pH resulted in improved resolution but at the same time a drastic reduction in the mobilities of diphosphates tended to crowd these compounds close to the origin. In general, a decrease in pH increased the R_F values of all com-

TABLE II

INFLUENCE OF pH, IONIC STRENGTH AND RELATIVE PROPORTION OF AMMONIUM FORMATE SOLUTION INCORPORATED INTO SOLVENTS

Compounds were chromatographed on Whatman grade 54 paper and separated by descending chromatography in the machine direction. The time of separation varied between 8 and 40 h. Solvent composition: ethanol–propyl acetate–ammonium formate, 60:10:30 for data in columns 1–6 and 60:10:20 for data in column 7. Data given are 100 × R_{G6P} values.

Compound	*Ionic strength and pH of ammonium formate*						
	1 M, pH 4	*1 M pH 5*	*1 M, pH 6*	*1 M, pH 8*	*2 M, pH 5*	*3 M, pH 5*	*1 M, pH 5*
Fructose 6-phosphate	120	125	135	126	137	130	166
Fructose 1,6-diphosphate	60	47	31	17	32	29	14
Glycerate 3-phosphate	97	85	63	–	70	75	70
2,3-Diphosphoglycerate	49	30	29	30	26	20	5
6-Phosphogluconate	60	45	27	67	25	22	12
Orthophosphate	129	141	146	–	160	170	197

pounds. However, fructose 1,6-diphosphate and 6-phosphogluconate failed to separate at acidic pH.

A change in the ionic strength of the ammonium formate solution from 1 to 3 *M* had no significant influence on the R_{G6P} values. However, an increase in ionic strength increased the viscosity of the solvent and prolonged the development time. When the polarity of the solvent was reduced by decreasing the proportion of ammonium formate solution (Table II, last column), the resolution was significantly improved. However, a reduction in polarity greatly decreased the mobilities of compounds. In order to achieve satisfactory separations of spots, development was prolonged to 18 h and the solvent was allowed to flow beyond the edge of the paper. In spite of its obvious advantage, solvent 1 was not considered satisfactory for routine use.

Solvent 2 was intended to be used in the second dimension for separating those compounds which were not resolved by solvent 1 at acidic pH. In these experiments, the chromatographic paper was washed with solvent 1 and dried prior to spotting reference compounds to simulate conditions that would be encountered during two-dimensional separation of biological extracts. Solvent 2 was found to give satisfactory resolutions of a number of nucleotides (Table I). The polarity of solvent 2 was considerably increased to allow for the rapid resolution of diphosphates and nucleotides.

The R_F values of dicarboxylic acids and monocarboxylic acids were high in solvents 1 and 2, and many of these compounds migrated with the solvent front. These compounds, in a two-dimensional separation with solvent 3 in the first dimension and solvent 4 in the second dimension, gave satisfactory resolutions. The R_F values in these solvents are given in the last two columns in Table I. It should be noted that phosphate esters stay close to the origin in solvent 3 but show significant mobilities in solvent 4.

In selecting an appropriate solvent for the separation of phosphate esters, their stabilities at different pH values had to be taken into consideration. Hydrolysis and decomposition are accelerated at both alkaline and acidic pH values. Most of the phosphate esters are stable at about pH 4, but nucleotides and some esters show optimal stability at about pH 9[13]. During the formulation of the solvents their pH values were kept close to these two values. To minimize the risk of decomposition of labile compounds, the development time was reduced to a minimum by a judicious choice of chromatographic paper and solvent composition. Using Whatman 31 ET paper and solvent 1, 2, 3 or 4 satisfactory resolution could be obtained within 6 h. Decomposition was more difficult to prevent during two-dimensional separation. During such separations, at the end of the first development, the paper was dried in a stream of nitrogen, stored overnight at −20°C and developed in the second solvent on the following day.

The solvents reported represent a compromise between rapid separation and resolution. Solvents of increased resolving power could be formulated by decreasing the polarity of solvents 1 and 2 and/or by increasing the pH of the ammonium formate solution. Such solvents are expected to require longer development times but offer possibilities of improved resolution.

In another series of experiments, the performance of different grades of chromatographic paper was examined. Whatman grades 1 and 3MM paper gave very poor resolution of phosphate esters. The resolution was considerably improved and

R_F values were increased by washing the paper with 0.04% EDTA solution. Acid-washed grades, such as Whatman grades 541, 540 and 542, were found to be satisfactory. The surface of these papers gave an acidic reaction that had little effect on R_F values because of the buffering action of ammonium formate. When Whatman 31 ET paper was tested it gave a performance comparable to that of Whatman 541 paper. Its extremely fast flow-rates and high capacity made it particularly suitable for rapid separation work and thus it was chosen for all subsequent experiments.

Separation was also attempted on thin layers prepared from Whatman CC 41 microcrystalline cellulose, Cellex-N cellulose powder and a variety of ion-exchange cellulose powders. The performances of all types of thin layers examined were found to be inferior to paper and resolution was hampered by streaking or excessive diffusion of spots.

With radioactive glucose as a substrate, 26 radioactive spots were detected on a two-dimensional chromatogram. In contrast, a simpler pattern of labelling was obtained with radioactive lactate and eight distinctly separated spots appeared on the chromatogram. The significance of these findings in relation to the metabolism of malaria parasite will be reported separately.

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