QUALITATIVE AND QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF GUANIDINE DERIVATIVES AND DIFFERENTIATION OF PHOSPHAGENS FROM OTHER PHOSPHORUS COMPOUNDS*

FERNANDO DI JESO
Department of Biochemistry, University of Pavia, Pavia (Italy)
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About fifty biological guanidines are known at present; some of these, like arginine, are involved in essential metabolic pathways, or have particular biochemical functions, such as phosphagens and their dephosphorylated bases, but the role of some of them, such as octopine, remains unknown. The distribution in the living matter of most of them is surprisingly specific, but free guanidines or their phosphagens have been found in all organisms.

We have applied thin-layer chromatography (TLC) to the separation, identification and determination of the naturally occurring guanidines and of some other synthetic analogues. The paper chromatographic analysis of the guanidines has been extensively studied by ROCHE and coworkers[1–4], and some data have been published on the paper chromatography of phosphagens[5–8], but no attempts have been made for a systematic study by TLC. In the case of the phosphagens, these derivatives must first be separated from other naturally occurring phosphorus derivatives and the products of their hydrolysis (guanidine derivative and orthophosphate) must be identified later chromatographically. We have chosen cellulose-layer chromatography (CLC) chiefly because some classical location reagents lose their specificity on supports other than cellulose and we have avoided the presence of plaster of Paris in the cellulose because it may interfere in the chromatography of the phosphate compounds in basic solvents. The solvent systems chosen are as simple as possible, because the complex ones, frequently used in chromatography, often lead to variable results due to the evaporation or chemical reaction between the solvent components.

EXPERIMENTAL

Materials

Thirty guanidine derivatives (see Tables I and II) have been studied. Most of them were synthesized according to SCHÜTTE[9] (amidination at room temperature by S-methylisothiourea of the corresponding amines dissolved in aqueous ammonia, 12 %). 1 mmole of amine was dissolved in 1 ml of ammonia and left to react overnight with 1.2 mmoles of reagent at 20°. Guanidinoethylphosphate and opheline were synthesized by a method already described[6,10] and purified by column chromato-

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graphy on Dowex 50 X2[6]. Phosphoarginine and phosphocreatine were synthesized[11,12] from the two bases and $POCl_3$, at 0° and pH > 8, and purified on Amberlite IRC 50.

Lombricine, hirudonine and octopine were extracted by 2 % acetic acid from the muscles of the earthworm (*Lumbricus terrestris* L., (1758) (Müller)), the leach (*Hirudo medicinalis* L.) and the sepia (*Sepia officinalis* L.), respectively, purified by ion-exchange chromatography on buffered 200–400 mesh Dowex 50 X4[6] or Amberlite IRC 50[13]. Agmatine was prepared by the degradation of hirudonine by mercuric acetate in a sealed tube at 125°[13].

Arginine, creatine, creatinine, glycocyamine (B.D.H.), γ-guanidobutyric acid (Light), canavanine (KEK Lab., California) and dimethyl-biguanide (Fluka) were commercially pure products.

The non-phosphagen phosphorus compounds studied for comparison (see Table II) were all pure Sigma products.

Chromatographic analysis

Five 20 × 20 cm layers, 300 μ thick, were prepared from 15 g of Cellulose MN 300 (Macherey, Nagel and Co.) with a Chemetron automatic spreader (after mixing the cellulose for 40 sec with 90 ml of distilled water, in a Waring blendor). 600 μ thick layers have been prepared in the same way for preparative purposes. The layers are dried overnight at room temperature. A front line is marked by scraping off the cellulose at 18.5 cm from the bottom edge of the plates. For one-dimensional runs, the substances are applied in a straight starting line at 1.5 cm from the bottom edge of the plate as single spots (1–2 mm^2). Individual path-runs for each spot are limited by marking vertical straight lines 1 cm apart or more. For two-dimensional runs, the mixture is spotted at a starting point 1.5 cm from the bottom and the left edge of the plate; two front lines are marked, for the first solvent at 18.5 cm from the bottom edge of the plate, and for the second solvent at 18.5 cm from the left edge; alongside the front lines two other paths of 1.5 cm each allow one-dimensional chromatography for standard substances. On the preparative layers the same sample is spotted on a starting line; no paths are marked, but the extreme lateral edges of the layer are excluded, as generally they are irregular.

The plates are developed at 23° by the ascending technique, sloping at an angle of 110°, in 22 × 10 × 22 cm tanks containing 100 ml of freshly prepared solvent. Two walls (those 22 × 22 cm) of the tanks are previously lined with filter paper soaked with solvent. The plates are removed 20 min after the solvent has reached the front line, and promptly evaporated in an air stream. "Multiple chromatograms" can be carried out by developing again a developed and dried plate in the same solvent and direction. The chromatograms of non-hydrolysed phosphagens are developed at 2°. The following solvents are proposed*: S_1 = PWEa (8:2:1); S_2 = iAWPb (4:7:8); S_3 = MWFa (20:1:4); S_4 = MFa (17:3); S_5 = iBiPWAb (20:40:39:1); S_6 = PWAb (6:1:3); S_7 = MAb (7:3); S_8 = BWEa (12:5:3); S_9 = tBWPa (20:5:1 v/v/w); S_{10} = MAb (85:15); S_{11} = WPbAb (1:6:2).

* The following abbreviations have been used: Ab = aqueous ammonia, sp.gr. 0.910; iA = 3-methyl-1-butanol; B = 1-butanol; iB = 2-methyl-1-propanol; tB = 2-methyl-2-propanol; Ea = ethanoic acid, sp.gr. 1.050; Fa = formic acid, sp.gr. 1.200; M = methanol; P = 1-propanol; Pa = trinitrophenol; Pb = pyridine; W = water. If not otherwise stated, the proportions between the concentrations are expressed by volume.

For the qualitative evaluation of the chromatographic results a multiple spraying technique is employed using some reagents already employed in paper chromatography, and combining them in the following suitable sequence: (I) Heat at 110° for an hour; (II) after spontaneous cooling, spray the molybdate reagent (aqueous solution of 12 g/l $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$, 50 g/l $HClO_4$, about 0.1 *N* HCl); (III) after a few minutes, heat at 80° for 30 min; (IV) expose to H_2S for a few minutes after having humidified the cellulose layer slightly with steam produced by boiling water; (V) spray α-naphthol reagent (8 ml of 1 % $NH_2\cdot CO\cdot NH_2$ in water + 2 ml of 4 % NaOH in water + 0.2 ml of 1 % α-naphthol in ethanol); (VI) when the plate is dry, spray hypobromite reagent (11 g/l Br in 4 % NaOH); (VII) spray diacetyl reagent (18 ml of 4 % NaOH + 2 ml of 1 % α-naphthol in ethanol + one drop of 2,3-butanedione); (VIII) spray picrate reagent (8 ml of saturated trinitrophenol aqueous solution + 2 ml of 10 % NaOH); (IX) spray silver nitrate reagent (20 ml of 2-propanone + 0.2 ml of saturated $AgNO_3$ aqueous solution + some drops of water to just redissolve the flocculent precipitate).

It is possible to eliminate steps I, II + III + IV, IV, V + VI, VII, VIII or IX from the complete multiple spraying sequence and to insert a ninhydrin spray, *e.g.*, before II or V. VIII can be used before V or VII. A modified sequence without I–IV can be suitably combined with the "complete sequence", on two different plates, for a routine screening of the guanidine derivatives in biological materials.

Only three longitudinal paths of the preparative layers, 0.5 cm wide, are kept on the plate, one at the center and the other two at the lateral edges of the plate and these are sprayed with the reagents, the remaining cellulose layer having been scraped off earlier in the form of successive transverse strips, each 0.5 cm wide. Each of these strips is placed in a numbered centrifuge tube. After location of a specific substance on the three longitudinal paths, the corresponding strips in the centrifuge tubes are eluted by distilled water and studied.

Quantitative determinations

Both guanidine groups and phosphate can be evaluated quantitatively after location. Nitrogen can be estimated only if the reagents IV–VII are employed without urea, and the other reagents have been omitted. The layer bearing a specific spot is scraped off into a centrifuge tube, a digestion flask, or Pyrex tube.

In the first case, the ROSENBERG method for estimation of guanidine groups[14] is applied with the only modification that the cellulose is centrifuged off just before reading the density of colour at 535 nm. In the second case, total phosphorus is determined in the following way: the sample is digested in 1 ml of $HClO_4$, then 3.5 ml of 5 % trichloroacetic acid, 5 ml of 2-methyl-1-propanol–benzene (1:1) and 0.5 ml of 10 % ammonium molybdate are added and the flasks shaken by a vortex mixer; 2 ml of the top layer are transferred into a tube containing 4 ml of 1 *N* H_2SO_4 in ethanol, then 0.5 ml of freshly prepared $SnCl_2$ solution (0.15 ml of 10 % $SnCl_2$ in HCl, stored in a freezer, + 20 ml of 1 *N* H_2SO_4) are added and the optical density is determined at 730 nm, after 15 min or more; the colour is stable for many hours. When "phosphagen labile phosphorus" is to be estimated, the cellulose is suspended in a small volume of 0.1 *N* HCl and immersed in a boiling water-bath for 1 min, then cooled; 4 ml of a 1:7 mixture of perchloric and 5 % trichloroacetic acids, 5 ml of the isobutanol–benzene mixture and 0.5 ml of the ammonium molybdate are quickly added and

TABLE I

R_F VALUES OF GUANIDINE DERIVATIVES ($R_1R_2N—CNH—NHR_3$) ON CLC (300 μ) AT 23°

Detection by the multiple spraying technique described in the text. Reagents: V–VIII.

Compounds	R_1	R_2,R_3	R_F × 100	
			S_1	S_2
Agmatine	$—(CH_2)_4—NH_2$		33	13
Arcaine	$—(CH_2)_4—NH—CNH—NH_2$		47	14
L-Arginic acid	$—(CH_2)_3—CHOH—COOH$		51	46
L-Arginine	$—(CH_2)_3—CHNH_2—COOH$		19	10
L-Canavanine	$—O—(CH_2)_2—CHNH_2—COOH$		17	22
Creatine	$—CH_3$	$R_2 = —CH_2—COOH$	42	42
Creatinine	$—CH_3$	$R_2–R_3 = —CH_2—CO—$	54	66
Dimethylbiguanide	$—CNH—NCH_3—CH_3$		61	60
Glycocyamine	$—CH_2—COOH$		37	42
γ-Guanidinobutyric acid	$—(CH_2)_3—COOH$		74	48
Guanidinoethanol	$—(CH_2)_2—OH$		67	56
Guanidinoethylphosphate	$—(CH_2)_2—O—PO_3H_2$		17	27
β-Guanidinopropionic acid	$—(CH_2)_2—COOH$		63	46
δ-Guanidinovaleric acid	$—(CH_2)_4—COOH$		80	52
Hirudonine	$—(CH_2)_3—NH—(CH_2)_4—NH—CNH—NH_2$		17	4
D-Lombricine	$—(CH_2)_2—O—PO_2H—O—CH_2—CHNH_2—COOH$		6	26
Monomethylguanidine	$—CH_3$		74	53
L-N-amidinoalanine	$—CHCH_3—COOH$		53	50
L-N-amidinoaspartic acid	$—CHCOOH—CH_2—COOH$		34	36
L-N-amidinoglutamic acid	$—CHCOOH—(CH_2)_2—COOH$		41	40
L-N-amidinohistidine	—CHCOOH— 		55	38
L-N-amidinomethionine	$—CHCOOH—(CH_2)_2—S—CH_3$		67	70
L-N-amidinophenylalanine	—CHCOOH— 		79	75
L-N-amidinotyrosine	—CHCOOH—  —OH		65	76
L-N-amidinovaline	$—CHCOOH—CHCH_3—CH_3$		69	63
Octopine	$—(CH_2)_3—CHCOOH—NH—CHCH_3—COOH$		17	35
Opheline	$—(CH_2)_2—O—PO_2H—O—CH_3$		39	50
Taurocyamine	$—(CH_2)_2—SO_3H$		29	59

shaken. The free phosphate is then determined as described above. Any inorganic phosphorus that may be present in the same spot is determined on another chromatogram by direct treatment of the cellulose in a mixture of the perchloric–trichloroacetic acids and the 0.1 *N* HCl. The nitrogen is estimated by the JACOBS method[15] (digestion in sealed tube, in 50 % H_2SO_4 at 470° for 30 min).

RESULTS AND DISCUSSION

The R_F values of the guanidines studied in one-dimensional runs, and in the two solvents chosen (S_1 and S_2), are reported in Table I. Table II shows the R_F values of the most common phosphagens, phosphoarginine and phosphocreatine, in some solvents, compared with arginine and creatine and the most common non-phosphagen phosphorus compounds. Solvents S_7 and S_5 are usually able to separate the phosphagens from the other biological phosphorus compounds. The solvents S_{10}, S_{11}, S_9, S_6, S_5, S_7 and S_8 separate the phosphagens from the other naturally occurring guanidines (the cyclic guanidines run more quickly than the mono- and the di-substituted ones reported in the table). Thus, it is easy to chose two successive solvents to demonstrate the presence of a phosphagen in a preparation or purify it on preparative layers. When a spot of a free guanidine base is also detected in a phosphagen preparation, it is possible with a "multiple chromatogram" to decide whether it is an impurity of the preparation or whether it derives from the hydrolysis of the phosphagen; if the hydrolysis occured during development a new free guanidine spot appears in the second run, between the two spots detectable on the first run.

TABLE II

R_F VALUES OF PHOSPHAGENS AND OTHER PHOSPHORUS COMPOUNDS ON CLC (300 μ) AT 2°
Detection by the multiple spraying technique described in the text. Reagents: I–VII.

Compounds	$R_F \times 100$								
	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}
Phosphoarginine	59	46	31	28	24	25	21	6	3
Phosphocreatine	72	74	33	29	30	27	21	6	4
L-Arginine	64	60	40	45	38	32	72	20	19
Creatine	86	81	52	50	54	45	72	43	33
Orthophosphate	76	78	0	6	0	25	65	0	0
ATP	4	3	7	2	3	4	2	0	2
ADP	8	6	7	6	6	5	6	0	4
AMP	31	33	23	29	15	18	33	0	9
Phosphoenolpyruvic acid	74	78	4	1	21	30	64	4	2
DL-Glyceraldehyde-3-phosphate	89	66	26	23	19	25	21	3	2
D-Ribose-5-phosphate	62	56	9	2	14	14	35	0	2
D-Glucose-6-phosphate	61	43	25	15	19	12	21	3	4
D-Glucosamine-6-phosphate	52	46	11	1	15	11	26	2	3
D-Glucose-1-phosphate	50	39	28	25	17	11	21	4	2

The R_F values are generally affected by a standard deviation of about ± 3 %. A reproducibility better than that usual in paper chromatography has been obtained by taking the following precautions: (a) the composition and thickness of layers, the positions of starting points and of front line, the dimension of tanks and the

TABLE III

EXAMPLES OF DIFFERENTIAL STAINING BY MULTIPLE SPRAYING METHOD

The Roman numbers indicate the staining steps described in the experimental part. After the first positive reaction the successive positive ones change the colour of the spots. In the case of negative reaction there is no spot, or the same spot appeared by the previous reaction, with the same colour.

Compound examined	*First plate*				*Second plate*				
	V + VI	*VII*	*VIII*	*IX*	*I + II + III + IV*	*V + VI*	*VII*	*VIII*	*IX*
Monosubstituted guanidines	+	+	—	—	—	+	+	—	—
Asymmetrically disubstituted guanidines	—	+	—	—	—	—	+	+	—
Cyclic guanidines	—	—	+	—	—	—	—	+	—
Phosphoamidic disubstituted guanidines	—	—	—	—	+	+	+	—	—
Phosphoamidic trisubstituted guanidines	—	—	—	—	+	—	+	+	—
Phosphoamidic cyclic guanidines	—	—	—	—	+	—	—	+	—
Sugar phosphate	—	—	—	±	+	—	—	—	+
Inorganic or organic non-phosphoamidic phosphorus	—	—	—	—	+	—	—	—	—
Simple sugar	—	—	—	+	—	—	—	—	+
Non-phosphoamidic monosubstituted guanidine + non-phosphoamidic phosphorus	+	+	—	—	+	+	+	—	—
Non-phosphoamidic asymmetric disubstituted guanidines + non-phosphoamidic phosphorus	—	+	—	—	+	—	+	+	—
Non-phosphoamidic cyclic guanidines + non-phosphoamidic phosphorus	—	—	+	—	—	—	—	+	—

quantity of solvent are constant; (b) the temperature and humidity of the chromatographic room is continuously controlled; (c) the developing solvent is freshly prepared from reagents kept at the temperature of the chromatographic room, and the run started only when the tank is saturated; (d) the division of the layers into individual paths for each sample and the previous marking of a front line keeps the distances travelled by substance and by solvent sensibly equivalent to the distances conventionally measured from the starting point; they also maintain all the samples under the same conditions, and help to eliminate edge effects; (e) stopping the run 20 min or more after the solvent has reached the whole front line, the R_F values are about 10% greater than in conventional chromatography, but their reproducibility is improved; (f) the plates are always held in the same standard position when they are uniformly and lightly sprayed; (g) the center of the developed spot for the R_F measurement is visually estimated, and the spots with a major axis of more than 1 cm are taken off.

Table III summarizes the possibility of differentiating many different structures, even before R_F estimations. The correct staining with a complete sequence of multiple spraying gives, in some cases, a better identification, but, for many purposes, the steps VIII and IX can be omitted.

The use of the solvents S_1 and S_2 in two-dimensional chromatography allows orientative screening for unknown guanidines, and the rapid and accurate identification of phosphagens. Three chromatograms are necessary to identify one of these: the complete sequence on the first, locates a single spot, which stains with both IV and VI or VII; the modified sequence does not locate this spot on the second chromatogram; on a third the hydrolysis products (100° in 0.1 N HCl) of the preparation do not give this spot at any step of the complete sequence. Two new spots appear after hydrolysis: one stains with III and has the R_F of orthophosphate (see Fig. 1); another stains with VI or VII, it has the R_F of one of the known phosphagen bases reported in Fig. 1, or of the hypotaurocyamine (2-guanidoethanesulphinic acid). This last has a behaviour

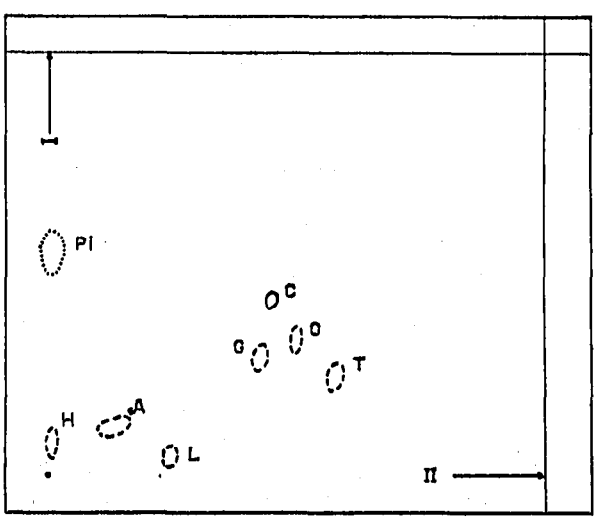

Fig. 1. Two-dimensional chromatography at 23° of a mixture of arginine (A); creatine (C); glycocyamine (G); hirudonine (H); lombricine (L); opheline (O); taurocyamine (T) and orthophosphate (Pi) in the solvent systems S_1 and S_2 respectively for the first (I) and the second (II) run. The spots are located at the following steps of the multiple spraying sequence: III (···), VI (– – –) and VII (———).

similar to that of glycocyamine (especially in S_2) and, of course, after oxidation, the same behaviour as of taurocyamine.

The quantitative evaluation of the guanidine groups, phosphorus or nitrogen in the spots is easy and practical, as their elution is not necessary. The blank of the first method on a piece of scraped-off cellulose in a guanidine-free zone, does not absorb at 535 nm even in the presence of phosphorus. A 100 % recovery may be obtained on the cellulose layer treated for the detection of phosphorus and guanidines until step VI. Also the blank of the second method is not affected by the presence of the cellulose or of guanidine compounds; a 100 % recovery is possible if the location is limited to the detection steps before VIII. All the methods give a satisfactory reproducibility of results. Besides the low limits of detection (about 0.5 and 10 nmoles respectively for the guanidines and phosphates), and other advantages quoted before, the possibility of direct quantitative evaluation on the chromatographically separated and identified spot is a further substantial advantage of the application of CLC to the study of the phosphagens and of other phosphorus or guanidine derivatives.

CONCLUSIONS

Thin-layer chromatography on cellulose and a suitable succession of spray reagents have been applied to the identification of very small amounts of guanidine derivatives. The mono-, di- and tri-substituted guanidines and the phosphagens can be differentiated rapidly. The phosphagens can be separated from other phosphorus derivatives in extracts of biological origin. All these substances can be rapidly determined in very low amounts after chromatographic separation and location, without elution. Preparative chromatography can be elaborated on the same principles, but with thicker layers. Because of the reproducibility of the R_F values it is possible to identify, without control substances, all the known phosphagens, and, tentatively a great number of guanidines.

The method permits accurate demonstration, identification and determination of phosphagens in biological material even on very low concentration of these compounds. In bacteria, *e.g.*, the absolute concentration of phosphoarginine is about 10^{-2} μmoles per gram of wet weight, and the relative concentration is about 1 % of total guanidines and 0.05 % of total phosphorus[16], while the absolute concentration of this substance in the leg muscles of orthopterans[8] and of phosphocreatine in the frog gastrocnemius[17] is about 14 μmoles per gram of wet weight, that is about 50 to 200 % of the free guanidines and 20 to 40 % of total phosphorus. Thus, the application of this technique revealed a more widespread presence of phosphagens in living matter than was expected before the availability of TLC techniques.

SUMMARY

Thin-layer chromatography on cellulose and a suitable succession of spray reagents have been applied for qualitative and quantitative analysis or for preparative purposes on very small amounts of mono-, di- and tri-substituted guanidine bases and phosphagens. These substances can be rapidly differentiated in preparations of biological origin containing different guanidine and phosphorus derivatives. R_F values of some natural and synthetic guanidines are given. A tracing of the two-dimensional

chromatographic behaviour of hydrolysis products of phosphagens is reported, facilitating the identification of all the known phosphagens.

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