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MICROELECTROPHORETIC AND CHROMATOFOCUSING TECHNIQUES FOR THE QUANTITATIVE SEPARATION AND IDENTIFICATION OF IMIDAZOLE DERIVATIVES

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SUMMARY

The separation of eleven naturally occuring imidazole compounds by low-voltage electrophoresis on cellulose acetate using a wide-range buffer covering the pH range 3.5–11.5 is described. The recoveries of urocanic acid, histamine and histidine varied from 80 to 100% for concentrations of 1–7 μg. The results are reproducible and the technique could be useful for the rapid identification and determination of histidine metabolites.

A mixture of nine imidazole derivatives was resolved on Dowex 50W-X8 (200–400 mesh) using a linear pH gradinent of the wide-range buffer. The elution pH values of the different imidazole compounds varied from +0.6 to – 0.4 pH unit above or below their isoelectric pH values. The recoveries of the standards ranged from 86 to 98%. This technique was applied successfully to the separation of histidine metabolites in *A. aerogenes* culture medium.

INTRODUCTION

Different paper and thin-layer chromatographic methods have been reported for the separation of histidine metabolites from different biological materials[1–4]. Also, different column chromatographic techniques using Dowex–50W-X8 (H^+) and Dowex 1 (Cl^-) have been described[5–8]. These methods have the disadvantage of being limited in application to a small number of imidazole derivatives.

High-voltage electrophoresis has been used for the separation and identification of amino acids and histidine degradation products in histidenaemic urine and human sweat[8–11]. Cellulose acetate has been used as a supporting medium for the separation and semiquantitative determination of amino acids and formiminoglutamic acid in biological fluids[12];

A method for the separation of histidine and histamine on Whatman No. 1 paper using low-voltage electrophoresis and ninhydrin as the locating reagent[13] was applied successfuly to the measurement of the histidine decarboxylase activity in rat hypothal-

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amus homogenate. The described electrophoretic methods for the separation of imidazole compounds have the disadvantage of using buffer systems containing pyridine, acetic acid and formic acids[1,10,13]. These reagents are usually unpleasant to work with and associated with enviromental hazards.

In this paper a simple and rapid micromethod for the separation and determination of histidine and related imidazole compounds employing cellulose acetate strips and non-hazardous buffer is reported. Also, based on the determined isoelectric pH, a method for the separation of members of this group of compounds on Dowex 50W-X8 (H^+) is described, which has been applied successfuly to the separation of histidine metabolites in *A. aerogenes* culture media.

EXPERIMENTAL

Materials

Imidazole compounds were purchased from Sigma (St. Louis, MO, U.S.A.) and 4-aminoimidazole-5-carboxamide from Koch-Light Laboratories (Colnbrook, U.K.). Super Sepraphore cellulose acetate strips (5.7 × 14.4 cm) were purchased from Gelman Instrument (Ann Arbor, MI, U.S.A.).

Buffer

Wide-range citrate–phosphate–borate–barbitone buffer was prepared as recommended by Varley *et al.*[12]. Unless otherwise stated, the molarity of all buffers used was 0.075 *M*. The final pH was checked with a pH meter (Electronic Istruments, type 1020).

Preparation of A. aerogenes histidine metabolites for chromatographic analysis

A. aerogenes were grown aerobically in shaking culture at 30°C for 18 h in 250-ml erlenmeyer flasks containing 50 ml of basal synthetic medium[14], supplemented with 0.15% of histidine. Cell-free media were concentrated to a yellow syrup under reduced pressure. Imidazole compounds were extracted with three successive portions of hot acetone and the residue after acetone extraction was re-extracted with 95% ethanol. The pooled acetone and alcohol fractions were evaporated to dryness and the extracted imidazole compounds (acetone and alcohol fractions) were dissolved in 0.075 *M* buffer (pH 3.5).

Electrophoretic procedures

For the separation of imidazole compounds, the samples were applied to the pre-soaked cellulose acetate strips as 0.5 μl of 0.2% solution. The strips were subjected to a low constant voltage (10 V/cm) with a current of 39–45 mA for 15 min using the appropriate buffer. The cellulose acetate strips were air dried (1–3 min) and the spots were revealed by spraying with sulphanilic acid spray reagent[2]. For testing the effect of electroendoosmosis, the mobility of glucose was examined at pH 3.5, 7.0, 9.0 and 10.5. Glucose bands were revealed by spraying the air-dried cellulose acetate strips with aniline-phosphoric acid reagent[2]. The mobility of glucose never exceeded 0.05 mm at pH 7.0 and 9.0 and 0.1 at pH 3.5 and 10.5 and therefore no correction was made for the recorded electrophoretic mobilities of the imidazole derivatives. For quantitative determinations, the coloured zones and equal blank areas were cut out and eluted with

2 ml of 20% acetone in 5% sodium carbonate solution. The intensity of the colour was measured at 500 nm and the concentration was determined using a previously established calibration graph.

Chromatographic procedures

Dowex 50W-X8 (200–400 mesh) was activated as recommended by Hirs *et al.*[15] and equilibrated with 0.15*M* wide-range buffer (pH 3.5) before packing into the column (10 × 0.4 cm I.D.). Sufficient volumes of the same buffer (0.075 *M*) were passed before application of the sample. A 1-ml volume of standard solution containing nine imidazole compounds was applied to the top of the column. The column was routinely washed with 20 ml of 0.075 *M* wide-range buffer (pH 3.5) after sample application to flush out any unadsorbed compounds. The adsorbed material was eluted with 300 ml of a linear gradient of buffer made from two 150-ml portions of 0.075 *M* wide-range buffer and 0.1 *M* sodium hydroxide solution. Fractions of 5 ml were collected at a flow-rate of 5 ml per 7 min. The concentration of imidazole compounds in the eluted fractions was determined by the sulphanilic acid method[16] and the concentration of each individual imidazole derivative was calculated from a previously established calibration graph.

The elution sequence was established by paper chromatography using two solvent systems: Solvent I, 1-butanol–acetic acid–water (120:30:50) and Solvent II, 1-butanol–pyridine–water (1:1:1). The spots were revealed by spraying the air-dried chromatograms with sulphanilic acid spray reagent.

RESULTS AND DISCUSSION

The problems encountered in obtaining a complete separation of a complex mixture of histidine metabolites and their derivatives by partition chromatography is well known[1,2,4]. As a starting point, a systematic investigation of the electrophoretic separation of different imidazole compounds and histidine peptides using cellulose acetate as the support medium was attempted. The changes in the electrophoretic mobilities of the tested standard imidazole compounds with pH are shown in Fig. 1.

The apparent p*I* (isoelectric point) values for imidazolelactic acid, carnosine, 4-aminoimidazole-5-carboxamide, imidazoleacetic acid, histidine, imidazoleacrylic acid and glycylhistidine were derived from the intercept of the elctrophoretic mobility profiles with the pH axis (Table II). However, no such intercepts could be recorded for imidazole, histamine and histidinol in the pH range investigated, possibly because they are devoid of carboxyl groups and because of the slight acidity of the imidazole ring.

A scheme for the separation of the tested imidazole derivatives is shown in Fig. 2. This scheme should be of particular value for the identification of these substances when present in biological fluids and mammalian tissues. At acidic pH (below pH 5.5), all the tested compounds were positively charged and completely separated and moved towards the cathode. On the other hand, at alkaline pH, imidazoleacetic acid, imidazolelactic acid, histidine, urocanic acid and glycylhistidine were negatively charged and migrated towards the anode. Ergothioneine apparently carried no significant net charge at any pH tested, as it moved slightly towards the anode. Carnosine (5), imidazolelactic acid (6), histamine (7), histidine (8) and imidazoleacrylic acid (9), which are diagnostic biochemical compounds in urine, can be resolved and clearly identified

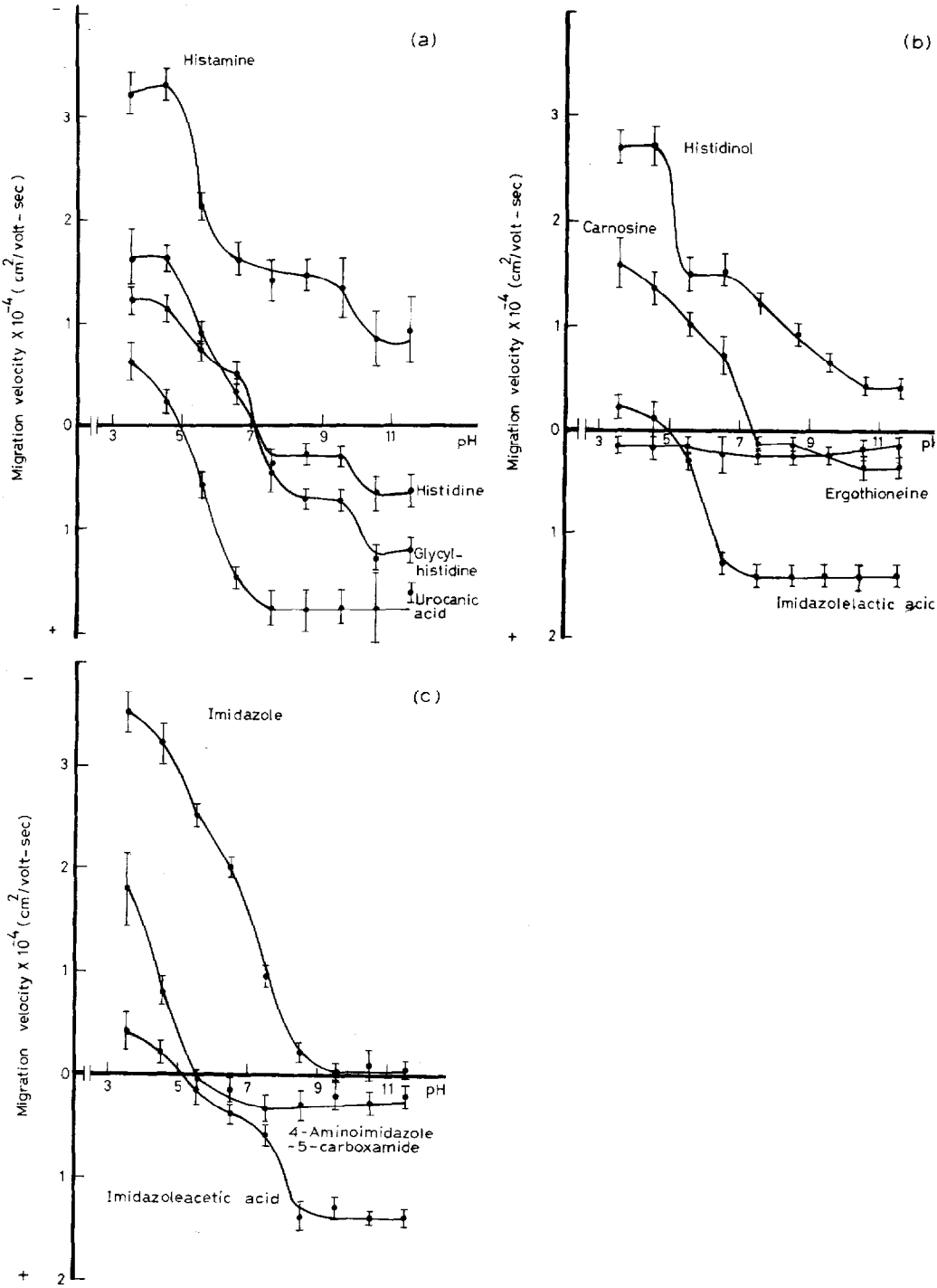

Fig. 1. Electrophoretic mobilities of imidazole compounds at different pH values.

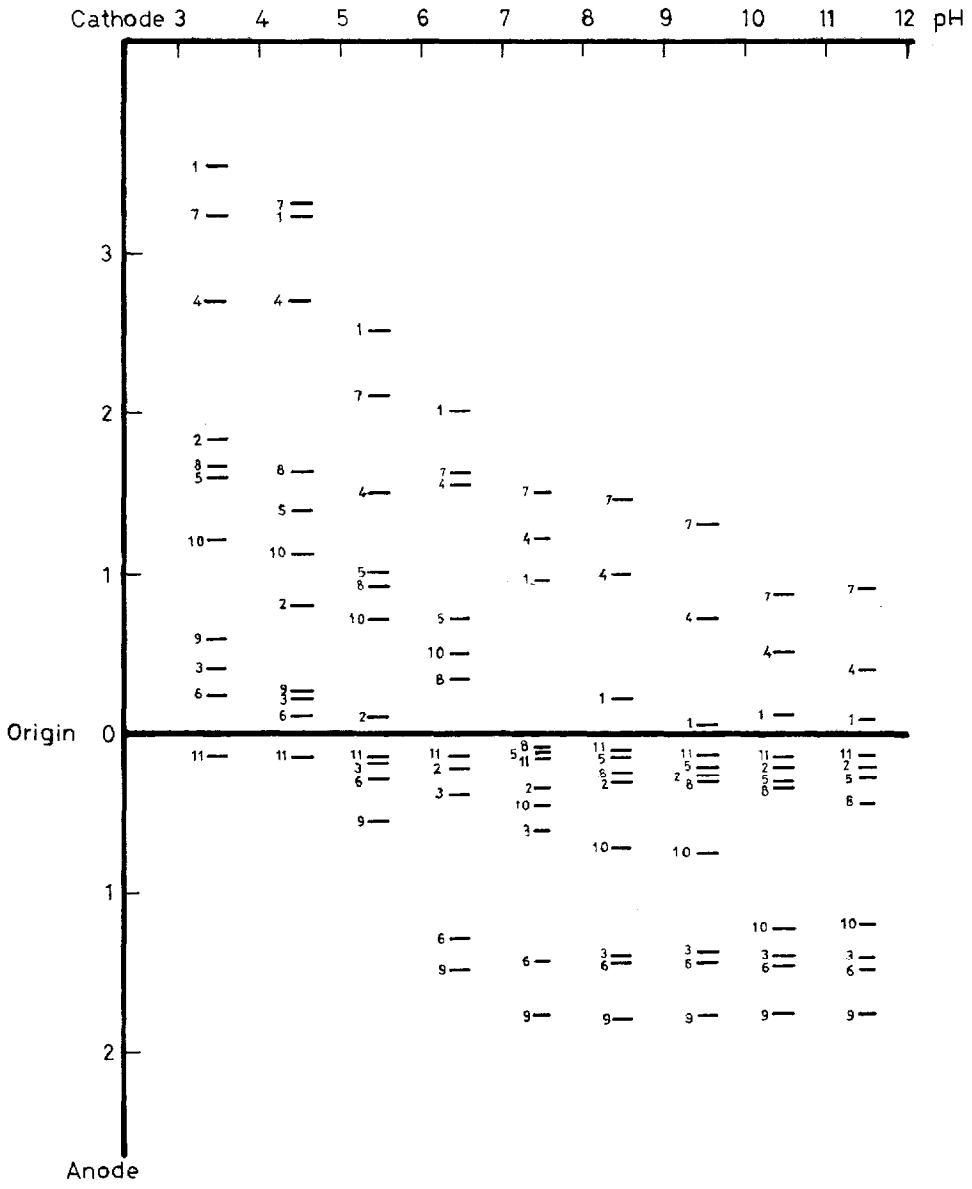

Fig. 2. Scheme for the separation of imidazole compounds on cellulose acetate strips: (1) imidazole (2) 4-aminoimidazole-5-carboxamide; (3) imidazoleacetic acid; (4) histidinol; (5) carnosine; (6) imidazolelactic acid; (7) histamine; (8) histidine; (9) imidazoleacrylic acid; (10) glycylhistidine; (11) ergothioneine.

at pH 3.5 and 6.5. A typical example of the separation of a mixture of seven imidazole derivatives and four other compounds at pH 8.5 is shown in Fig. 3.

For microscale quantitative determinations, a mixture of histidine, histamine and urocanic acid was applied to pre-soaked cellulose acetate strips and electrophoresis was carried out for 15 min at pH 7.5. The recoveries of the eluted colours are shown in Fig. 4 and Table I.

It is obvious from the recoveries that no irreversible adsorption takes place on the cellulose acetate strips. The recoveries of urocanic acid, histamine and histidine ranged from 80 to 100% for amounts ranging from 1 to 7 μg. The results are reproducible (Table I, Fig. 4), and the technique could be useful for the rapid identification and determination of histidine metabolites. The methods can be carried out with simple, compact and inexpensive apparatus currently used in many laboratories for quantitative separation of proteins.

Prins[17] established a correlation between the pH of elution of various abnormal haemoglobins from carboxymethylcellulose and their isoelectric points as determined by free electrophoresis. Later, the same phenomenon was used for the determination of the isoelectric points of different proteins by chromatography on carboxymethyl-Sephadex[18]. More recently, it has been demonstrated that a pH gradient on an ion exchanger could be established by taking advantage of the buffering action of the charged groups of the ion exchanger[19,20]. It occurred to us that the elution pH might be a func-

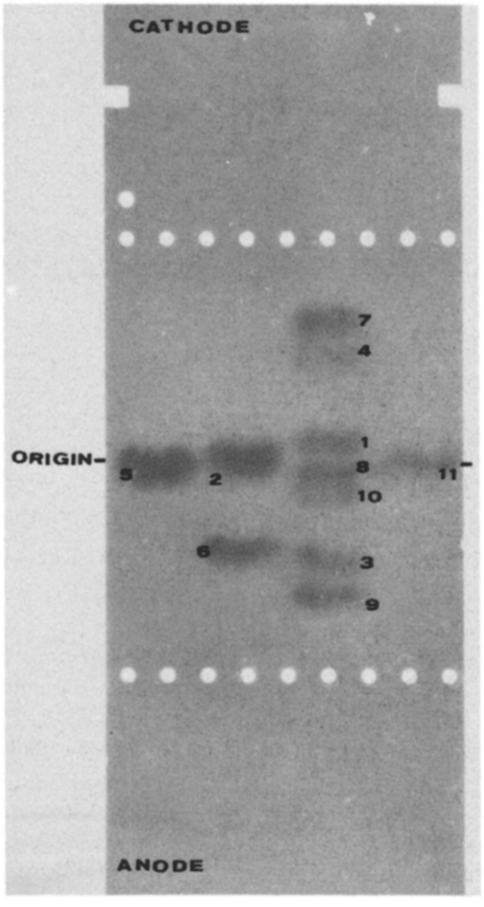

Fig. 3. Typical example of the separation of a mixture of seven imidazole compounds and four other compounds on cellulose acetate strips at pH 8.5: (1) imidazole; (2) 4-aminoimidazole-5-carboxamide; (3) imidazoleacetic acid; (4) histidinol; (5) carnosine; (6) imidazolelactic acid; (7) histamine; (8) histidine; (9) imidazoleacrylic acid; (10) glycylhistidine; (11) ergothioneine.

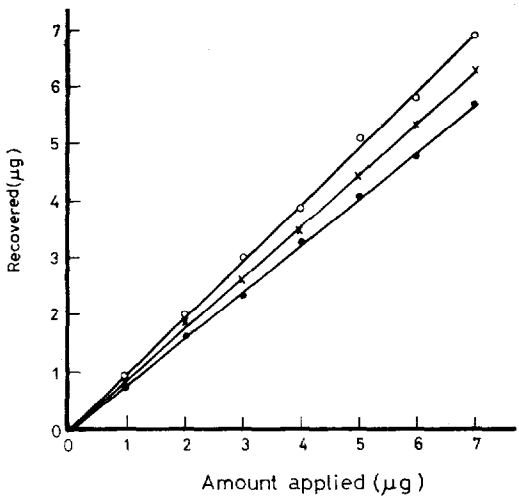

Fig. 4. Recovery of histidine (•), histamine (X) and amidazoleacryclic acid (O) as a function of the amount of sample applied to cellulose acetate strips. Each point represents the mean of at least three runs, ± S.E.

TABLE I

RECOVERY OF HISTIDINE, HISTAMINE AND UROCANIC ACID FROM CELLULOSE ACETATE STRIPS

Results are means (n = □), ranges and standard errors.

Amount (μg)	*Compound*		
	Histidine	*Histamine*	*Urocanic acid*
1	0.96	0.93	0.93
	(0.77–1.10)	(0.77–1.10)	(0.79–1.02)
	(S.E. ±0.09)	(S.E. ±0.07)	(S.E. ±0.03)
2	1.70	1.93	2.00
	(1.60–1.80)	(1.66–2.20)	(1.42–2.06)
	(S.E. ±0.06)	(S.E. ±0.21)	(S.E. ±0.04)
3	2.30	2.70	3.01
	(2.10–2.60)	(2.50–2.90)	(2.56–3.58)
	(S.E. ±0.14)	(S.E. ±0.19)	(S.E. ±0.30)
4	3.33	3.44	3.87
	(3.00–3.70)	(3.00–3.70)	(3.46–4.35)
	(S.E. ±0.16)	(S.E. ±0.19)	(S.E. ±0.21)
5	4.15	4.52	5.13
	(3.70–4.20)	(4.20–4.80)	(5.10–5.20)
	(S.E. ±0.15)	(S.E. ±0.13)	(S.E. ±0.05)
6	4.86	5.33	5.77
	(4.60–5.20)	(5.10–5.60)	(5.30–6.20)
	(S.E. ±0.18)	(S.E. ±0.06)	(S.E. ±0.16)
7	5.76	6.37	6.90
	(5.50–6.00)	(6.30–6.72)	(6.60–7.30)
	(S.E. ±0.15)	(S.E. ±0.19)	(S.E. ±0.20)

tion of the isoelectric pH of the different imidazole compounds. To investigate such a possibility, a mixture of nine imidazole derivatives was subjected to chromatographic analysis on Dowex 50W-X8 (200–400 mesh) prepared as described above. The adsorbed material was eluted with a linear continuous pH gradient.

The behaviour of the imidazole derivatives was studied and the pH gradients established are shown in Fig. 5. The identities and the elution sequence of the eluted imidazole compounds were established by paper chromatography using two solvent systems (Table II). The standards were eluted in the following order; ergothioneine, imidazolelactic acid, imidazoleacetic acid, imidazoleacrylic acid, histidine, carnosine, imidazole, histidinol and histamine. The calculated recoveries (means ± standard errors, S.E.) were 93.8 ± 1.48, 93.0 ± 0.0, 87.5 ± 2.3, 95.3 ± 2.6, 90.8 ± 4.9, 86.0 ± 1.6, 98.3 ± 1.6 and 96.3 ± 1.2%, respectively. It is obvious from the data in Table II and Fig. 5, that the imidazole derivatives were desorbed from the resin at the point where the buffer pH approached the apparent p*I* of the eluted compound. The basic compounds imidazole, histidinol and histamine did not elute from the column unless the pH of the eluent buffer reached 8.8, 9.6 and 10.2, respectively. These compounds might have been transformed into the non-protonated basic form, *i.e.*, to the non-exchangeable form at the appropiate pH. Ergothioneine appeared in the void volume of the column at pH 3.5, which indicates a net zero charge at this pH. The elution pH values of each of the imidazole compounds varied between – 0.4 and +0.6 pH unit below or above their isoelectric pH values.

The methods available for the colorimetric determination of histidine metabolites in biological fluids such as urine, serum and fermentation media lack specificity[1,2,6].

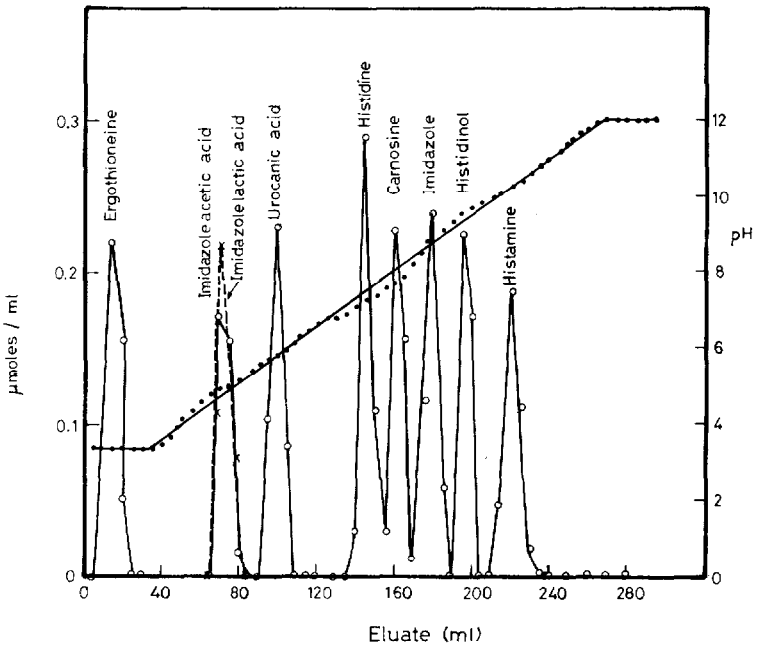

Fig. 5. Typical elution profile of a standard mixture of imidazole compounds (2 µmole each) on a Dowex 50W-X8 column (10 × 0.4 cm I.D.); imidazolelactic acid chromatographed separately.

TABLE II

ELUTION pH VALUES, ISOELECTRIC pH VALUES AND R_F VALUES OF STANDARD IMIDAZOLE COMPOUNDS

Compound	*Isoelectric pH*		R_F	
	Cellulose acetate strips	*Dowex 50W-X8 column*	*Solvent I**	*Solvent II**
Ergothioneine	–	–	0.29	0.24
Imidazoleacetic acid	5.0	4.9	0.42	0.33
Imidazolelactic acid	4.9	4.9	0.34	0.41
Imidazoleacrylic acid	5.0	5.6	0.59	0.48
Histidine	7.0	7.3	0.18	0.28
Carnosine	7.4	7.8	0.17	0.27
Imidazole	–	8.8	0.51	0.81
Histidinol	–	9.6	0.18	0.55
Histamine	–	10.2	0.41	0.57
Glycylhistidine	6.9	–	0.17	0.32
4-Aminoimidazole-5-carboxamide	5.4	–	0.49	0.67

* Solvent I = 1-butanol–acetic–water (120:30:50); solvent II = 1-butanol–pyridine–water (1:1:1).

Also, interference by phenolic compounds present in such materials with the sulphanilic acid reagent on the chromatograms of partially purified fractions is well known[2,21].

The proposed method was used for the isolation and identification of *A. aerogenes* histidine metabolites. The imidazole compounds in the culture media were extracted and chromatographed on a Dowex 50W-X8 column as described above. The elution sequence was established by paper chromatography. Three major peaks (I–III) were eluted from the column (Fig. 6). The first peak appeared at pH 4.3 and exhibited

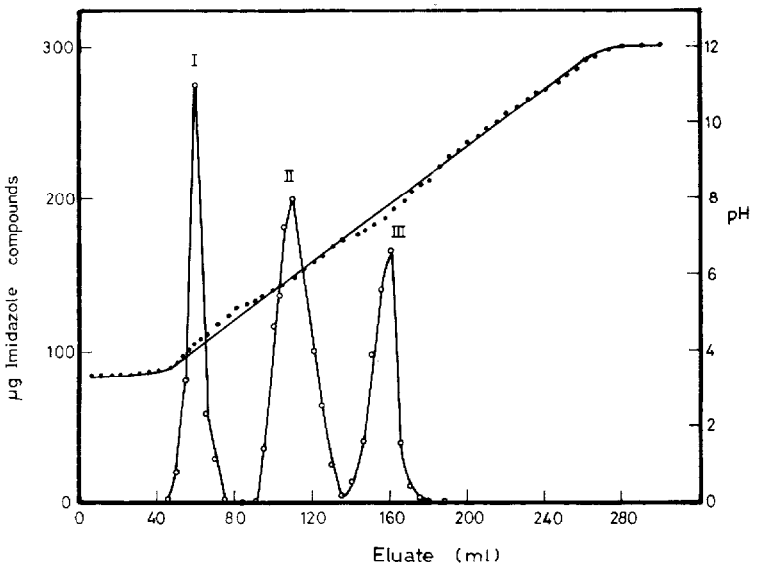

Fig. 6. Separation of histidine metabolites in the alcohol fraction from *A. aerogenes* culture media on a Dowex 50W-X8 column (10 × 0.4 cm I.D.).

R_F values of 0.34 and 0.41 in solvents I and II, respectively. This compound could be identified as imidazolelactic acid. The second peak appeared at pH 5.7, and chromatographic analysis revealed the presence of two imidazole compounds with R_F values of 0.38 and 0.60 in solvent I and 0.37 and 0.49 in solvent II. One of these compounds could be identified as urocanic acid and the other as imidazolepropionic acid. Unfortunately, we were unable to investigate the behaviour of imidazolepropionic acid on cellulose acetate strips and a Dowex 50W-X8 column because of the unavailability of an authentic sample. However, this compound exhibited the characteristic cherry red colour of imidazolepropionic acid with sulphanilic acid spray reagent. Form the calculated pI of imidazolepropionic acid [pK_1 = 3.5 (COOH) and pK_2 = 5.8 (imidazole)][22], one would predict its elution from the column at around pH 4.9. Paper chromatographic analysis of the third pak eluted at pH 7.1 showed one spot with R_F values of 0.17 and 0.28 in solvents I and II, respectively, and was identified ad histidine.

Compared with reported electrophoretic and chromatographic methods[1,2,9], the method proposed here has the advantages of the sharp resolution of mixtures of different imidazole derivatives and of expanding the column size for preparatory purposes. Also, commercially available ion exchangers and inexpensive buffer components are utilized.

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