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COMBINED APPLICATION OF ION-EXCHANGE CHROMATOGRAPHIC METHODS FOR THE STUDY OF "MINOR BASIC AMINO ACIDS"

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SUMMARY

A two-dimensional ion-exchange thin-layer chromatographic method is described for the separation of all methylated basic amino acids from biological fluids or protein hydrolysates; development is carried out on Fixion 50-X8 plates, which contain a resin of the Dowex 50-X8 type. An automatic amino acid analyzer was used for quantitative analysis of the minor basic amino acids.

INTRODUCTION

Enzymic methylation of the side-chains of protein molecules after genetic translation yields, among other compounds, N^{ε}-methylated lysines, guanidino-methylated arginines and imidazole-N-methylated histidines[1,2]. These methylated basic amino acids are generally found in histones, muscle proteins, cytochrome *c* and brain proteins, but they occur in insignificant amounts; in this respect, these compounds are "minor amino acids". Various methylated basic amino acids also occur free in brain, thymus, plasma and urine[1,2]; these are probably derived from the hydrolysis of methylated proteins *in vivo*[3].

These minor basic amino acids (both in free and bound forms) have important functions in living organisms, *e.g.*, they exert tumour-promoting and retarding effects[4–6].

We have found that a combination of ion-exchange chromatographic methods offers a suitable approach to the study of these amino acids.

MATERIALS AND METHODS

Authentic amino acids

The compounds N^{ε}-monomethyl-DL-lysine hydrochloride (MML), $N^{\varepsilon},N^{\varepsilon}$-dimethyl-DL-lysine hydrochloride (DML) and $N^{\varepsilon},N^{\varepsilon},N^{\varepsilon}$-trimethyl-DL-lysine dihy-

drochloride (TML) were prepared by total synthesis[7,8]; the guanidino-methylated arginines N^G-monomethyl-L-arginine (MMA), N^G,N^G-dimethyl-L-arginine (DMA) and N^G,N'^G-dimethyl-L-arginine (DMA') were gifts and partly prepared by synthesis[9]. Other authentic amino acids and their derivatives were obtained from various foreign firms.

Chromatoplates

Most experiments were performed on Fixion 50-X8 (Na^+) containing Dowex 50-X8 type resin (Chinoin-Nagytétény, Budapest, Hungary). Identical results were obtained when Ionex 25 SA (Na^+) chromatosheets (Macherey, Nagel and Co., Düren, G.F.R.) were used. The plates were equilibrated with sodium citrate buffer (pH 3.28, 0.02 *N* in Na^+) as previously described[10], and development was carried out with the solvents listed in Table I.

TABLE I

COMPOSITION, PER LITRE, OF DEVELOPING SOLVENTS FOR TLC

Component	*Buffer solution*		
	A (pH 6.0)	*B (pH 5.28)*	*C (pH 6.0)*
Hydrated citric acid, g	100.0	24.6	105.0
Hydrochloric acid (sp. gr. 1.19), ml	14.0	6.5	—
Sodium hydroxide, g	60.0	14.0	60.0
Sodium chloride, g	—	—	58.5
Sodium ions, *N*	1.5	0.35	2.5

Ninhydrin spray reagent. To prepare this reagent, 0.5 g of ninhydrin and 0.05 g of copper sulphate were dissolved in 100 ml of acetone.

Amino acid analyzer methods

A Japan Electron Optical Ltd. automatic amino acid analyzer (type JLC-5AH) was used; the minimum material requirement of the instrument was less than 1 nmole. The single-column method was used for complete analysis, the temperature of the column being maintained at 40° for 25 min from the beginning of the analysis and then raised to 60°.

When only the methylated lysines were examined, 0.35 *N* citrate buffer was used, with a 10-cm column containing LC-R-1 resin and operated at 25° and pH 6.5; the buffer velocity was about 30 ml/h. For the separation of guanidino-methylated arginines, we used a modification of the method of Kakimoto and Akazawa[11]; the arginine and its methylated derivatives were eluted from a 55-cm column containing LC-R-1 resin (Mitsubishi Chemical Industries, Japan) with 0.51 *M* sodium chloride in 0.20 *M* sodium citrate buffer of pH 3.25 at a flow-rate of 100 ml/h at 60°.

RESULTS AND DISCUSSION

Table II shows $R_F \times 100$ values for the 30 amino acids so far identified in proteins by the proposed TLC method. The results show that these buffer systems,

TABLE II

$R_F \times 100$ VALUES OF 30 AMINO ACIDS SO FAR IDENTIFIED IN PROTEINS ON FIXION 50-X8 CHROMATOPLATE

Amino acid	*Developing buffer*		
	A	*B*	*C*
Asp	80	79	82
Thr	79	78	80
Ser	80	80	81
Glu	80	79	80
Gly	78	64	79
Ala	72	60	72
Pro	51	49	51
Val	65	61	65
Met	58	50	57
Ile	52	49	52
Leu	53	50	53
Tyr	51	41	50
Phe	54	50	55
Try	10	—	11
Asn	69	—	70
Gln	61	—	60
Cys	77	—	79
$(Cys)_2$	60	—	62
HyPro	75	—	78
His	47	16	48
1-MeHis	37	15	36
3-MeHis	26	12	24
Lys	59	25	58
MML	38	19	39
DML	23	16	21
TML	14	12	13
Arg	29	8	28
MMA	21	8	20
DMA	14	9	13
DMA′	15	8	16

chiefly used for the separation of aromatic and basic amino acids, are very suitable for our purpose.

On the basis of the one-dimensional experiments with buffers A, B and C on the Fixion 50-X8 chromatoplates, a two-dimensional ion-exchange method was devised for the separation of all the methylated basic amino acids; the results are shown in Fig. 1. This method offers possibilities for studying minor basic amino acids from several biological fluids and protein hydrolysates. In Fig. 2 is shown the "layer-fingerprinting" of normal human urine with this method. Further, this thin-layer chromatographic method can conveniently be used for detecting aromatic amino acids, *e.g.*, in cases of phenylketonuria.

For the determination of methylated basic amino acids, we used the automatic amino acid analyzer. With this instrument, we could well separate the three guanidino-methylated arginines (MMA, DMA and DMA′) from arginine; this is shown in Fig. 3, a chromatogram obtained for normal human urine. It is especially interesting

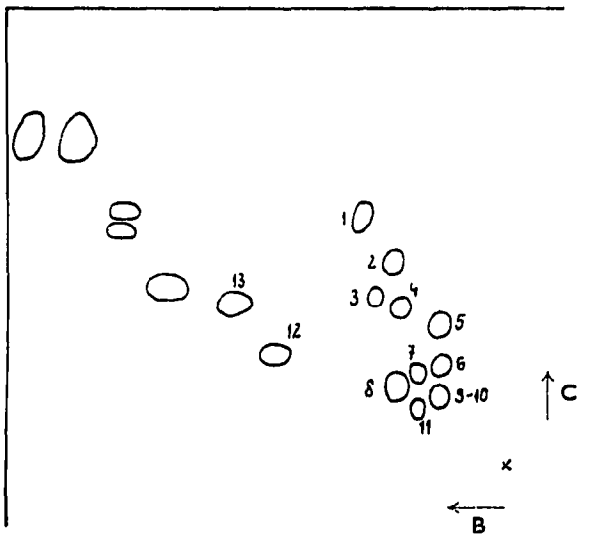

Fig. 1. Two-dimensional separation of aromatic and basic amino acids on a Fixion 50-X8 chromatoplate, with buffer solution B as solvent for the first dimension and buffer C as solvent for the second dimension. 1, Lys; 2, His; 3, MML; 4, 1-MeHis; 5, Arg; 6, MMA; 7, 3-MeHis; 8, DML; 9–10, DMA and DMA′; 11, TML; 12, Phe; 13, Tyr (each 2 μg).

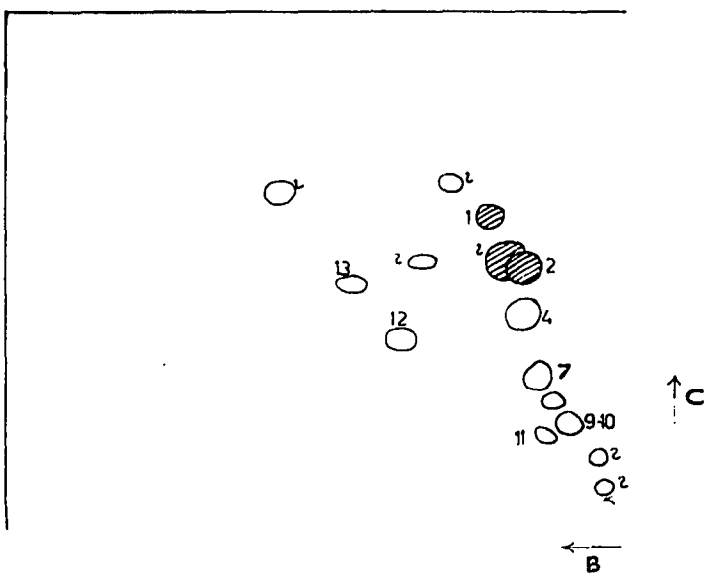

Fig. 2. Two-dimensional separation of aromatic and basic amino acids from normal human urine on a Fixion 50-X8 chromatoplate; conditions and abbreviations as for Fig. 1. The amounts used for analysis corresponded to 0.2 ml of urine after lyophilization and dissolution in methanol–1 *N* HCl (4:1).

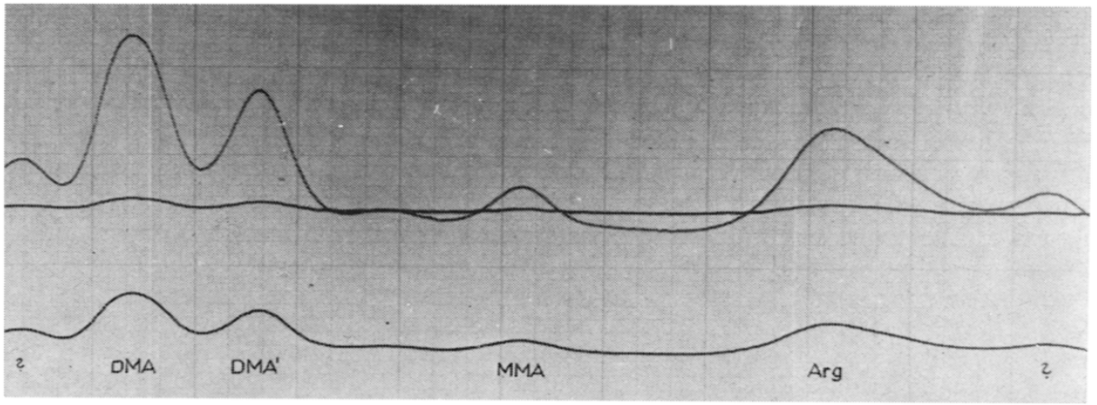

Fig. 3. The separation of guanidino-methylated arginines and arginine from normal human urine on the automatic amino acid analyzer. Retention times: Arg, 325 min; MMA, 275 min; DMA', 235 min; DMA, 215 min.

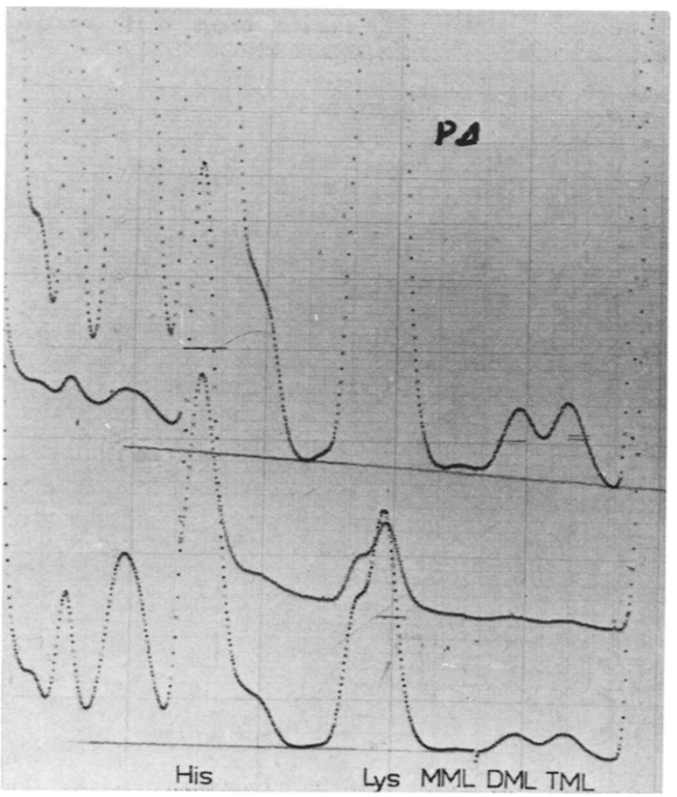

Fig. 4. The separation of N-methylated lysines and lysine from normal human urine on the automatic amino acid analyzer. Retention times: Lys, 75 min; MML, 85 min; DML, 90 min; TML, 95 min.

to note the separation of MMA from arginine; Kakimoto and Akazawa[11] could not separate these two amino acids.

In Fig. 4 is shown the separation of N^{ε}-methylated lysines (MML, DML and TML) from human urine. We have found that only a small amount of MML is present in normal urine.

We have so far not found an analyzer method suitable for the simultaneous separation of all the methylated basic amino acids. However, the proposed ion-exchange TLC method, used in conjunction with an automatic analyzer, achieves this object.

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