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RAPID SEPARATION OF HISTONES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON C_4 REVERSED-PHASE COLUMNS

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SUMMARY

A very rapid separation of H1 and core histones by reversed-phase high-performance liquid chromatography using a Bio-Rad Hi-Pore butyl (C_4) silica-based column in a single run is reported. The histones were dissolved in water containing 0.1% trifluoroacetic acid and fractionated within 20 min by means of a linear gradient system consisting of water–acetonitrile containing 0.1% trifluoroacetic acid. For the detection of histones the eluate was monitored at 210 nm. The identity and purity of eluted proteins were confirmed (1) by acid–urea and Triton–acid–urea polyacrylamide gel electrophoresis, and (2) by comparison with the retention times of pure histone markers. Despite the short elution time, a high resolution of the different histone fractions could be obtained. The eluted histones were recovered in the following order: H1 (LHP), H1 (MHP), H2B, H2A (LHP), H4 plus H2A (MHP), H3 (LHP), and H3 (MHP) (where LHP and MHP refer to less hydrophobic and more hydrophobic histone variants). The reported system is preferable to time-consuming electrophoretic systems for the separation of histones.

INTRODUCTION

Histones are the most intensively studied group of basic nuclear proteins and are of great importance with regard to the organization of chromatin structure and control of gene activity[1]. In the nuclei of all eucaryotic cells, DNA is packed in basically the same way: two each of the histones H2A, H2B, H3 and H4 form the fundamental chromatin subunit, the nucleosome; an additional part of DNA acts as linker between adjacent nucleosomes, which may be associated with histone H1[2,3].

It is apparent that four of the five major histone classes contain different variants[4–8]. Furthermore, the histones and their variants undergo several postsynthetic modifications such as acetylation, phosphorylation, methylation and ADP-ribosylation, which are thought to be important for regulatory functions[9,10]. To study these histone functions it is essential to isolate and fractionate the histones, and different kinds of gel electrophoresis are generally used. Sodium dodecyl sulphate gel electrophoresis[11,12] separates the five major histone fractions, but the modified forms of the

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histones are not resolved. Acetic acid–urea gel electrophoresis, as described by Panyim and Chalkley[13], can also resolve the five major fractions and in addition may also discriminate some of the modified forms. According to Franklin and Zweidler[14,15], the use of non-ionic detergents in acetic acid–urea gels enables the separation of histones and some non-allelic variants as well as modified forms.

However, these methods are laborious and time-consuming. It seemed desirable, therefore, to replace it by a more rapid and simplified technique.

The application of high-performance liquid chromatography (HPLC) to histones has been described by several authors[16–22]. Certa and von Ehrenstein[16] tried, as did Gurley *et al.*, to separate whole histone preparations by reversed-phase HPLC. Employing a relatively long time of analysis (80 min) they were able to fractionate the histones and some histone variants. However, irreversible adsorption of histones on the column packing resulted in a low recovery and, moreover, H3 variants were not separated.

Using a new gradient system (water–acetonitrile–trifluoroacetic acid) Gurley *et al.*[17] fractionated the histones within 2 h, except H2A from H4. They found two H3 variants but no H1 variant. Their conditions allowed a 90% recovery of all proteins applied to the column. By varying the type of column and the TFA concentration in the gradient, Gurley *et al.*[18,19] succeeded in separating H4 from H2A during 2 h. To differentiate between H1 and $H1^0$, Gurley *et al.*[20] and D'Anna *et al.*[21] requires 5 h and a tandem arrey consisting of μBondapak cyanopropyl silane and C_{18} columns.

Kurokawa and McLeod[22] recently reported a reversed-phase HPLC system that permits the fractionation in one step (40 min) of five H1 variants, and in another step (60 min) the core histones and different variants.

Despite all these efforts, however the separation of histone modifications by HPLC has not yet been successful. Even in cases where HPLC would be a good alternative to an electrophoretic separation of histones the long time of analysis still remains unsatisfactory.

Studies in our laboratory have been concerned with attempts to get more information on the biological consequences of changes in the extent of histone modification[23,24]. In the electrophoretic systems, as described above, all histone variants and most histone modifications can be separated, but owing to the complexity of gel electrophoresis it is almost impossible to identify and quantify each band exactly. Thus it would be advantageous to be able to fractionate the histones readily before electrophoresis.

In this report we describe a simple and rapid HPLC system. Not only two H1 variants but also the core histones, except H2A (MHP) from H4, can be separated in a single run within 20 min.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile and water were obtained from Merck (Darmstadt, F.R.G.), trifluoroacetic acid (TFA) and standard histone H2A (calf thymus, type VI-S) from Sigma (Munich, F.R.G.). The other histone reference substances H1 and H2B (calf thymus) were purchased from Boehringer-Mannheim (Mannheim, F.R.G.).

Calf thymus chromatin was prepared as described by Goodwin *et al.*[25]. The chromatin was treated with 0.35 *M* sodium chloride, the residual pellet extracted with 0.4 *N* sulphuric acid and the histones obtained according to Gurley *et al.*[17].

High-performance liquid chromatography

All HPLC experiments were performed on a Bio-Rad HPLC-gradient system equipped with two Model 1330 pumps, a microprocessor-controlled solvent programmer (Apple), and a Model 1305 A variable-wavelength UV–VIS monitor. The detection signal was documented on a Bio-Rad Model 1322 dual channel recorder.

Reversed-phase HPLC was carried out on histone preparations using a Bio-Rad Hi-Pore RP-304 C_4 column (250 × 4.6 mm I.D.) packed with 5-μm spherical particles (pore size 330 Å).

The lyophilized histones were dissolved in water (2 mg/ml) containing 0.1% TFA. To remove insoluble material, the solution was filtered through a 0.2-μm Sartorius Minisart filter. The pure histone solutions were prepared in the same manner (0.5 mg/ml). After equilibration of the column with the initial chromatography sol-

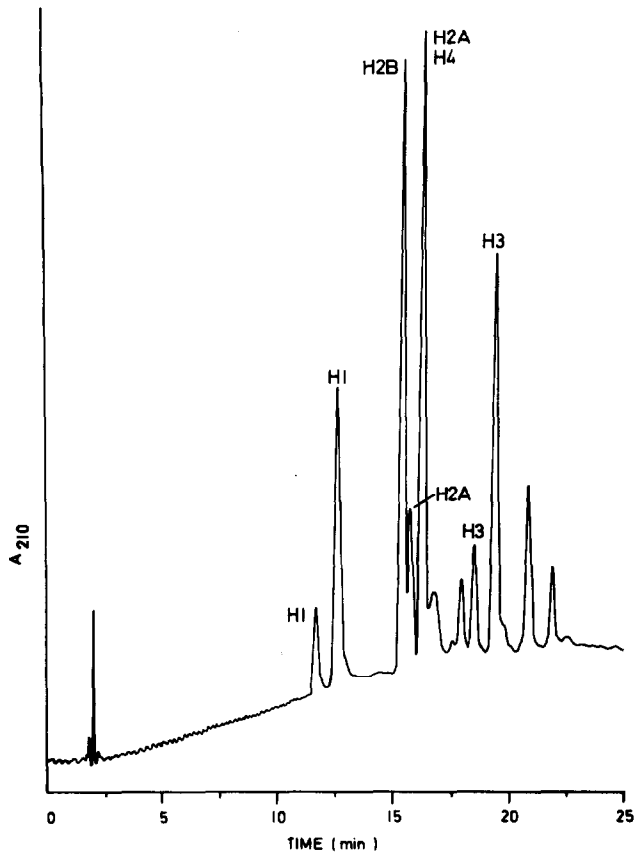
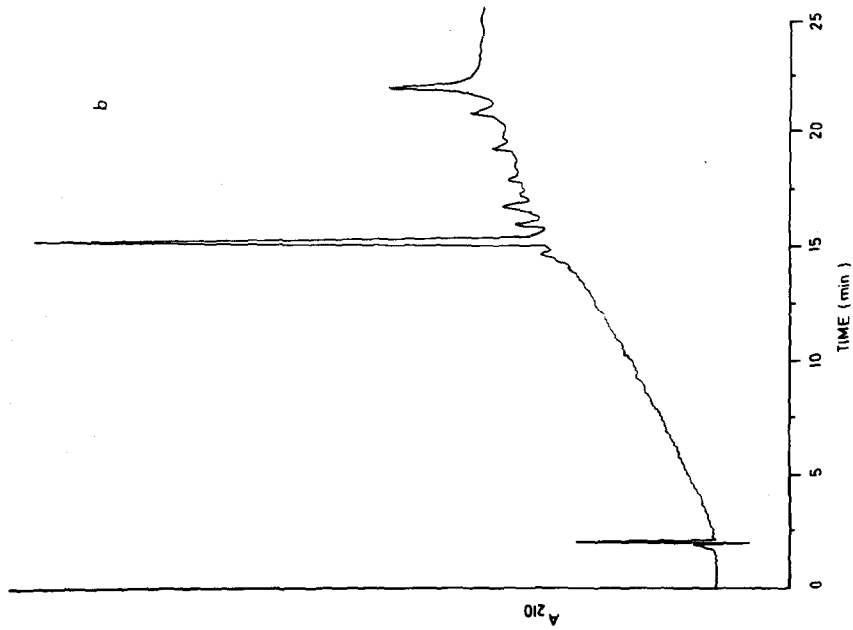


Fig. 1. Separation of calf thymus histones in an analytical run. 40 μg of protein were added to a Bio-Rad Hi-Pore RP-304 C_4 column (250 × 4.6 mm I.D.). Flow-rate, 1.5 ml/min; monitoring wavelength, 210 nm.


b
A 210
0
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10
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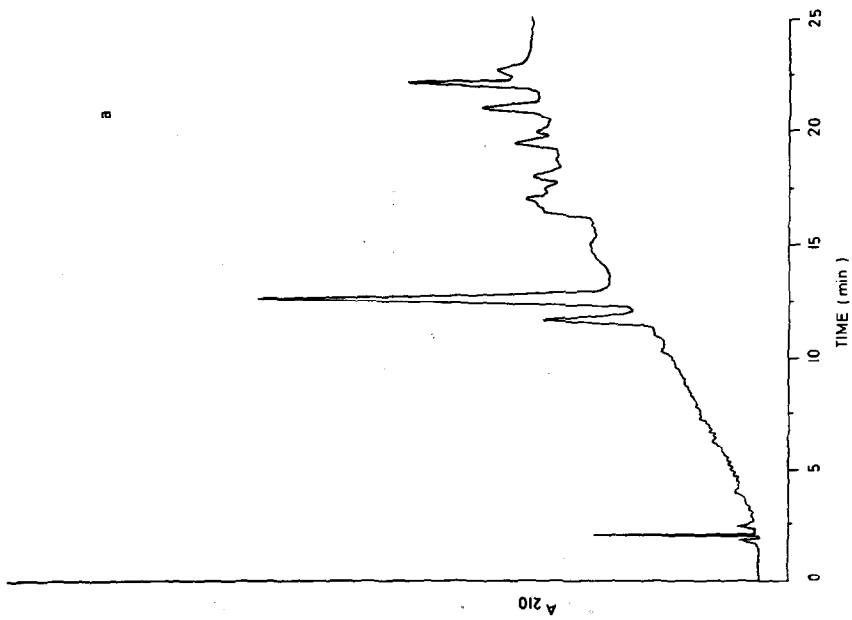
20
25
TIME (min)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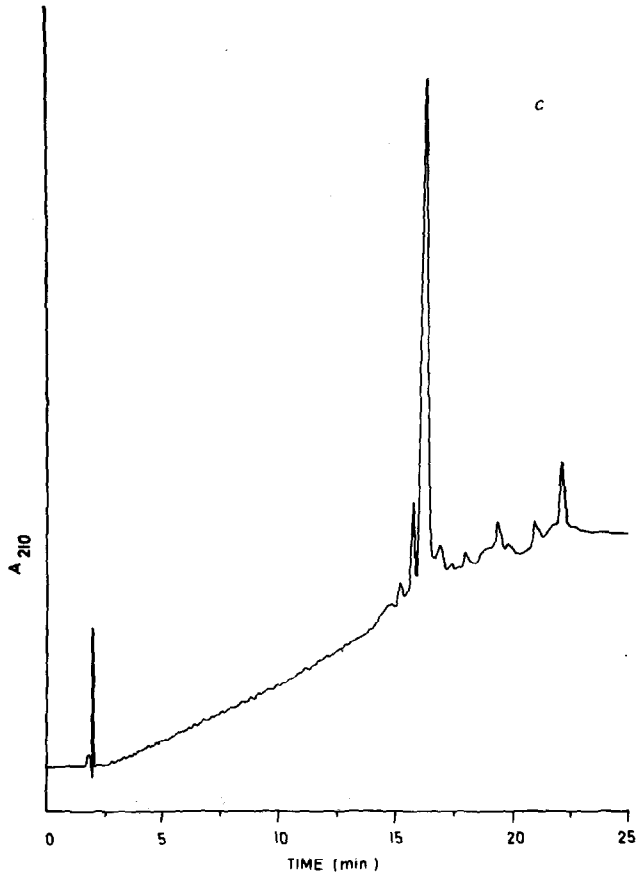


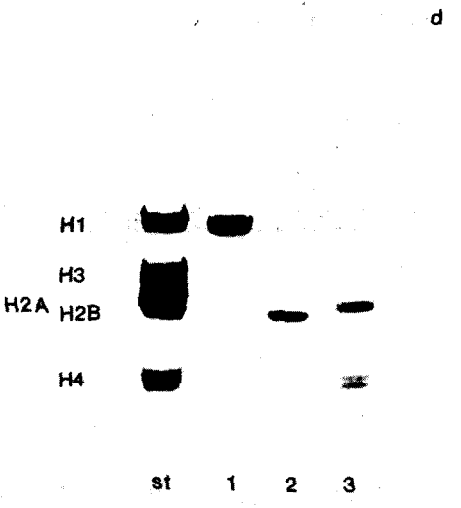
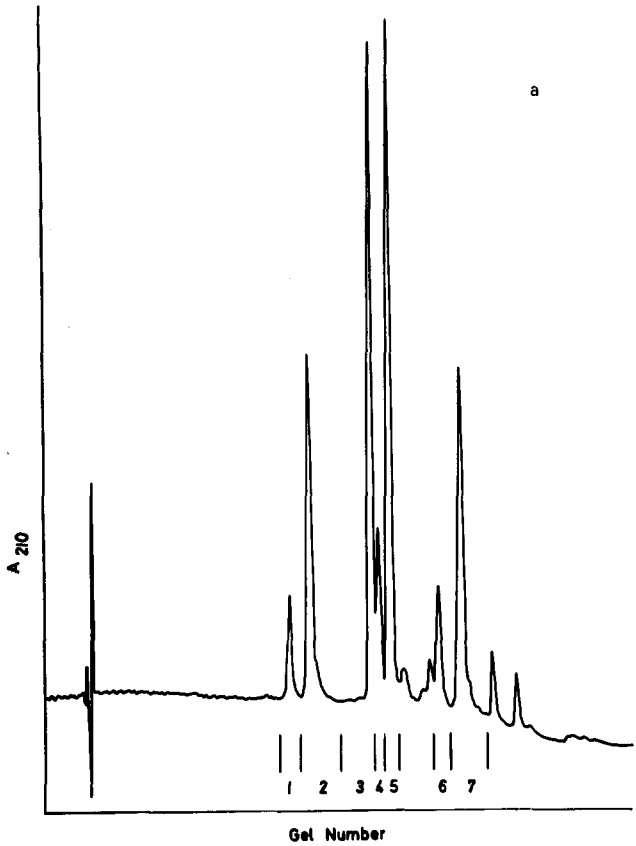


Fig. 2. Chromatograms and electrophoresis of histone markers. The retention times of (a) H1 (b) H2B and (c) H2A are shown. Each histone marker (10 μg) was subjected to HPLC, under the same conditions as for Fig. 1, including UV detection. As another control (d), the samples were submitted to electrophoresis on an acid–urea slab gel in the following order: H1 (lane 1), H2B (lane 2) and H2A (lane 3). The histone fractions were identified by comparison with a calf thymus histone standard (st) (total histones). Lane 3 (H2A) shows contamination with H4 (two minor bands).


a
A 210
1 2 3 4 5 6 7
Gel Number

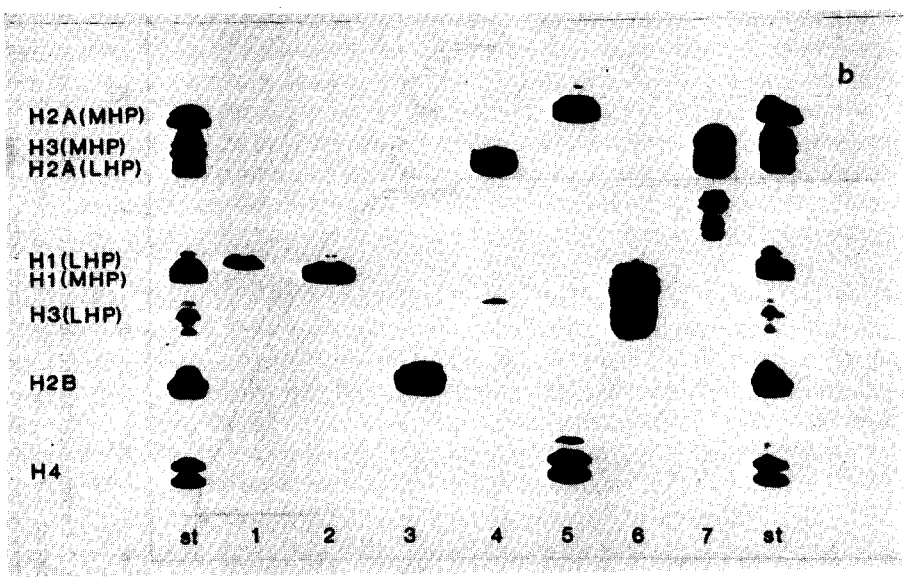

b
H2A(MHP)
H3(MHP)
H2A(LHP)
H1(LHP)
H1(MHP)
H3(LHP)
H2B
H4
st 1 2 3 4 5 6 7 st

Fig. 3.

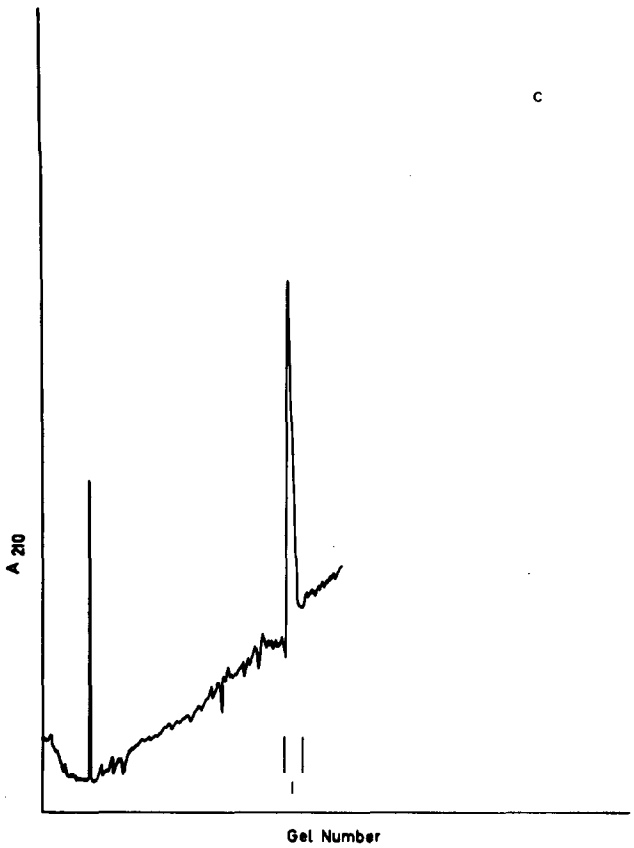


Fig. 3. Separation of calf thymus histones by HPLC and identification of the isolated fractions by Triton–acid–urea polyacrylamide slab gel electrophoresis. (a) The analysis of 1 mg of calf thymus histones (whole histones) in the HPLC system described in Fig. 1. Fractions 1–7 were lyophilized and subjected to electrophoresis. (b) Triton–acid–urea polyacrylamide slab gel electrophoresis of the HPLC fractions 1–7 as indicated on the UV absorbance profile in (a). The histone fractions were identified by comparison with a calf thymus histone standard (st). In (c), 0.5 μg of protein from fraction 1 was rechromatographed in the same HPLC system.

vent, the histones were injected on to the sample loop (20 μl). For preparative runs a more concentrated protein solution (10 mg/ml) was prepared and 100 μl were injected (1 mg of protein). The histones were eluted at room temperature by means of a linear gradient from 80:20 A–B to 20:80 A–B (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in 70% acetonitrile) for 20 min. The flow-rate was 1.5 ml/min and the eluent was monitored at 210 nm. The preparative runs were performed under the same conditions as the analytical separations.

Electrophoretic analysis of histone fractions

On the basis of the UV absorption, each HPLC peak was collected separately in a tube as indicated. Because of the short analysis time the use of a fraction collector was not required. The fractions were lyophilized to dryness and stored at −20°C

until used for electrophoretic analysis. Electrophoresis of each HPLC fraction as well as of pure histone reference substances was performed on polyacrylamide slab gels (160 × 0.75 mm) of two different types: (1) the acid–urea gel system of Panyim and Chalkley[13], and (2) the Triton X-100 gel system according to Zweidler[15]. The special conditions for the acid–urea gel were 15% polyacrylamide–0.9 *M* acetic acid–6.25 *M* urea, and for the Triton X-100 gel were 12% polyacrylamide–0.9 *M* acetic acid–8 *M* urea–0.37% Triton X-100, respectively. The gels were stained for 1 h with 0.1% Serva Blue R in 40% ethanol–5% acetic acid and destained in 20% ethanol–5% acetic acid.

RESULTS AND DISCUSSION

Several column types were tested for the rapid fractionation of the histones: Bio-Sil TSK™ columns, DEAE-cellulose, ion-chromatography and RP columns (C_3 and C_4 types). In addition, various solvent combinations and gradients were checked. Poor resolution, long processing times, and low recoveries were the main problems encountered.

The best results were obtained with a Bio-Rad RP-304 C_4 column and a linear gradient system from 80:20 A–B to 20:80 A–B. Fig. 1 shows the separation of histone proteins by HPLC and the retention times achieved under these conditions. We found eleven peaks on separating the calf thymus histones prepared as described under Experimental, seven of which were identified as individual histones. The unlabelled peaks in Fig. 1 were contaminants and non-histone proteins (data not shown). The individual histone peaks were identified (1) from retention times in comparison with purchased individual histone standards (Fig. 2a–c), and (2) by preparative runs and subsequent electrophoretic analysis of the various fractions (Fig. 3a–c). Fig. 2a shows the chromatogram from the H1 standard. Two peaks were detectable, and were in accordance with the ratios of the areas and the retention times of the first two peaks of Fig. 1. Electrophoresis of the H1 standard showed (Fig. 2d, sample 1) the uniformity of the reference. Therefore, we conclude that these two peaks contain at least two H1 variants. The minor peak with the higher mobility in the chromatogram was characterized as H1 (LHP) the second one as H1 (MHP), where LHP and MHP refer to the less hydrophobic and more hydrophobic variant, according to the nomenclature of Gurley *et al.*[17].

The separations of the H2B and H2A standards are shown in Fig. 2b and c. H2B was identified in the same manner as described for H1. The purity of the H2B standard was also determined by electrophoresis (Fig. 2d, sample 2). However, by electrophoretic control of the H2A reference, some contamination with H4 was detected and, therefore, it was impossible to determine exactly the location of the H2A histone among the HPLC fractions. For this reason, and the non-availability of individual H3 and H4 standard substances, preparative runs characterizing these proteins were performed.

For the separation of histone proteins by HPLC, 1-mg protein samples were loaded on the Bio-Rad C_4 column. The corresponding fractions of the eluate indicated on the abscissa of Fig. 3a were collected, lyophilized and subjected to Triton–acid–urea slab gel electrophoresis (Fig. 3b). Compared with the mobilities of calf thymus standard histones, the HPLC fractions were identified as follows: No. 1, H1; No. 2, H1; No. 3, H2B; No. 4, H2A; No. 5, H2A plus H4; No. 6, H3; No. 7, H3

(*cf.* Fig. 1). Fractions 1 and 2 in the electrophoresis of Fig. 3b show two H1 proteins with different electrophoretic mobilities. Sample 1, the protein with the lower mobility in the electrophoresis and the higher mobility in the HPLC system, is characterized as H1 (LHP), and the protein of fraction 2 as H1 (MHP). Fraction 3 contains only H2B. However, two proteins were detectable in fraction 4; the main component was an H2A variant H2A (LHP) and there were traces of a non-classified protein. In fraction 5 two histone species, H2A (MHP) and H4, were found.

In contrast to the application of the H2A reference alone (contaminated with H4 histone), an unambiguous specification of H2A and H4 was possible in this case. Moreover, Fig. 3b shows the identity and purity of the protein in fraction 6 with an H3 variant H3 (LHP). In the last fraction of the HPLC separation (number 7), H3 (MHP) was detectable as the main product.

The advantage of our HPLC separation method (using a Bio-Rad C_4 column) is demonstrated in Fig. 3c: Fraction 1 was rechromatographed without additional treatment (enrichment), and within 12 min the separation of H1 (LHP) from the residual proteins could be checked. H1 (LHP) shows the same retention time as in the first run and no contamination with the other H1 variant H1 (MHP).

This approach offers some advantages over currently used methods for separating and analysing the histone proteins: our method is very fast (20 min); not only two H1 variants, but also the core histones, except H2A (MHP) from H4, and some variants, H2A (LHP), H3 (LHP) and H3 (MHP) could be separated in a single run. Furthermore, the recoveries of the total histones from the column were more than 85%.

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