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Column-friendly reversed-phase high-performance liquid chromatography of peptides and proteins using formic acid with sodium chloride and dynamic column coating with crown ethers

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ABSTRACT

Reversed-phase high-performance liquid chromatography of proteins, traditionally carried out with strong acids like trifluoroacetic acid or phosphoric acid, which can damage reversed-phase columns, can be performed with excellent results using the far milder formic acid in the presence of salt. For certain separations, dynamic coating of the column with crown ethers can bring added resolution. Examples given are for peptides from a digest of methionine growth hormone, protein separations from whey proteins containing α-lactalbumin and the β-lactoglobulins A and B, and bovine and porcine insulins. The separation of methionine-growth hormone from growth hormone is also described.

INTRODUCTION

In a previous publication [1] we demonstrated the fact that it is not necessary to use a strong acid like trifluoroacetic acid (TFA) to elute peptides successfully from a reversed-phase column, but that a weaker acid (formic acid) which is less damaging to that column [2] can be used instead and may, in fact, give a better resolution. In this publication we will show that separations can be further improved by the addition of a moderate amount of salt. While for work with small peptides this may not always be necessary, it is demonstrated to be necessary in many cases for the separation of the larger peptides and proteins when using the formic acid system.

For especially difficult separations it has proven useful to change the character of the column somewhat by coating it with some compound of greater hydrophobicity than acetonitrile or some other organic eluent. If a compound less polar than the eluting solvent is added to this eluent, the column will become impregnated with it since the hydrophobic interaction between the column and the "non-polar" additive is stronger than that between the column and the bulk of the organic solvent. Among the solvents which have a relatively large effect even if added in small quantities are the ethers tetrahydrofuran and dioxan [3]. To a large extent the influence of the additive

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can be attributed to the hydrophobic interactions with the alkyl groups of the reversed-phase stationary phase, but they can also be bound to the free silanol groups of the stationary phase by a strong hydrogen bond formation (silanophilic interaction) [4]. This effect could perhaps be called "dynamic endcapping". The result of these two effects is that the column retains as much of the non-polar additive as it possibly can in a dynamic equilibrium with the surrounding buffer. Since this additive although non-polar is still more polar than the alkyl groups of the stationary phase the character of the column has now been modified. It may be unnecessary to add more than 0.1% of such a compound to obtain the maximum effect. If the organic modifiers are added in larger quantities, the unique difference between column and buffer would be diminished.

Other cyclic ether compounds of interest are the crown ethers, which have been used for dynamic coating of reversed-phase columns for the separation of alkali metals [5] and racemic amino acids [6]. Crown ethers have also been used for the chromatography of proteins and nucleic acids. In this case [7] the crown ethers were chemically bound to the silica. The authors describe the importance of the choice of salt in connection to the metal-binding properties of the crown ether. They demonstrate that to achieve the desired chromatographic effect it is imperative to use potassium chloride instead of the sodium salt. Another family of compounds displaying the dynamic coating phenomenon is the paired ion chromatographic (PIC) reagent group [8]. This paper presents the results from a comparison of crown ether addition *versus* sodium chloride addition to a mobile phase containing formic acid.

EXPERIMENTAL

Apparatus

Two sets of apparatus were used in this study.

A Waters Assoc. high-performance liquid chromatography (HPLC) system was used and consisted of two M-6000A pumps, a Model 680 gradient programmer, a Rheodyne Model 7125 injector with 2-ml loop, a 450 variable-wavelength detector and an Omniscribe (Houston Instruments) recorder.

An LKB HPLC system was also used interchangeably and consisted of a LKB 2249 gradient pump, a LKB 2141 variable-wavelength monitor, a Rheodyne Model 7125 injector and a Kipp & Zonen (Delft, The Netherlands) Model BD 41 recorder.

In all cases presented here a Vydac-Protein C_4 (250 × 4 mm) HPLC column was used.

Reagents

The following reagents were used: Milli Q water, acetonitrile (Mallinckrodt, ChromAR HPLC grade), sodium chloride (May & Baker, reagent grade), formic acid (May & Baker, Pronalys AR grade), 12-crown-4 (Aldrich), 18-crown-6 (Sigma), dioxan and tetrahydrofuran (Ajax, Unilab), diglyme, diisopropyl ether, methyl-cellosolve, glycol and *tert.*-butanol (BDH).

The following proteins were studied: methionine human growth hormone (Met-hGH) and recombinant human growth hormone (rhGH, Genentech), albumin (bovine, Sigma) and insulin (bovine and porcine, Sigma), acid whey protein isolate

containing mainly α-lactalbumin and β-lactoglobulins A and B was obtained from D. F. Elgar [9].

Buffer preparation

When buffer systems without salt were used the composition was as follows. Buffer A: 0.1% formic acid in water–acetonitrile (49:1) for peptides (digests) or 0.1% formic acid in water–acetonitrile (9:1) for proteins, to which the small amount of crown ether or other coating material may be added. Buffer B: 0.1% formic acid in water–acetonitrile (1:9) (+ the additive).

Salt-containing buffers were prepared as follows. A 10.0-g amount of sodium chloride was dissolved in water, 1.0 ml of formic acid and the desired quantity of crown ether or other modifier were added after which the volume was made up to 100 ml. Buffer B (500 ml) was prepared by filtration of 300 ml of acetonitrile through a 0.2-μl filter, followed by 150 ml of water and 50 ml of the above salt solution.

Attention: During the preparation of this buffer the mixture will become quite cold and will separate into two layers. Gentle warming will bring it to room temperature where the solution will be homogeneous. The buffer containing 1% sodium chloride can not contain more than 60% acetonitrile if a single layer at room

TABLE I

CONDITIONS FOR CHROMATOGRAMS

Separation of	Chromatogram No.	Buffers used for gradient	Linear gradient			
			Initial % B	Duration of the programme (min)	Final % B	Flow-rate (ml/min)
Tryptic digest of Met-hGH	1A	IA and IB	0	60	70	1
	1B	IIA and IIB	0	60	70	1
	1C	IIIA and IIIB	0	60	100	1
	1D	IVA and IVB	0	45	100	1
rhGH and Met-hGH	2A	VA and VB	100% B[a]			
	2B	VIA and VIB	60	45	80	1[b]
	2C	VIIA and VIIB	70	45	90	1
	2D	VIIIA and VIIIB	75	45	85	1
Bovine and porcine insulin	3A	VA and VB	20	45	30	1
	3B	VIA and VIB	15	45	30	1
	3C	VIIA and VIIB	30	45	50	1
	3D	VIIIA and VIIIB	30	45	50	1
Whey proteins	4A	VA and VB	30	45	50	1
	4B	VIA and VIB	25	45	50	1
	4C	VIIA and VIIB	60	45	80	1
	4D	VIIIA and VIIIB	60	45	80	1
All proteins	5	VIIIA and VIIIB	30	60	90	1
Whey proteins	6	IXA and IXB	50	45	70	1

[a] Isocratic.

[b] Gradient changed to 100% B after 15 min.

temperature is to be maintained. Buffer A is made similarly but does not require warming up.

Final composition of buffer A (for the chromatography of the digests): 0.1% formic acid + 1% sodium chloride (+ x% of the desired additive) in water–acetonitrile (49:1); for chromatography of the proteins the preferred ratio of water–acetonitrile is 1:9.

Composition of buffer B (for peptides as well as proteins): 0.1% formic acid + 1% sodium chloride (+ x% of the desired additive) in water–acetonitrile (2:3).

The gradients used were all linear and are described in Table I. Whenever possible for comparisons of chromatograms with or without an additive, comparable gradients were used. This however was not always possible because of the changed characteristics of the column. In general, as may be expected, the more polar the additive tested for dynamic coating, the less acetonitrile is needed to elute the proteins.

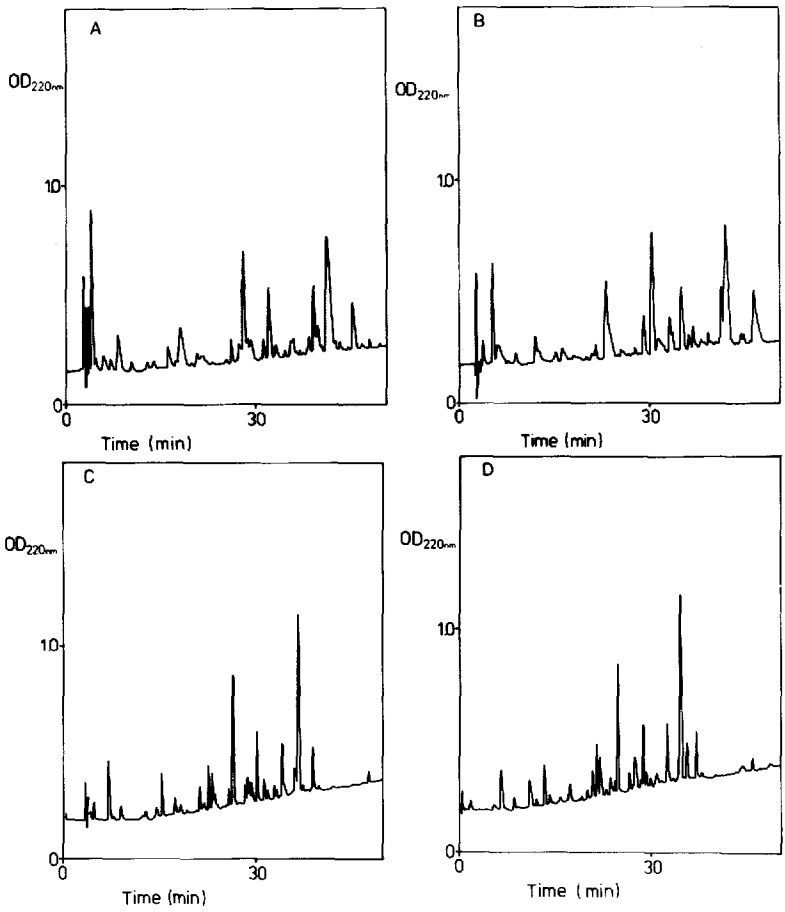


Fig. 1. Tryptic digest of Met-hGH. Elution (A) 0.1% formic acid, (B) + crown ether, (C) + NaCl, (D) + crown ether + NaCl. For buffer composition and gradient see Tables I and II.

Digests

The tryptic digests were prepared as previously described [1].

RESULTS AND DISCUSSION

Hodgin *et al.* [3] have reported the use of tetrahydrofuran and dioxan in reversed-phase HPLC. Previous uses of crown ether have involved the chromatography of alkali metals [5] and amino acids [6] or the immobilization of the crown ether [7]. The following results illustrate further potential uses of crown ethers with and without the addition of sodium chloride.

A tryptic map of Met-hGH (Fig. 1) illustrates the effectiveness of 0.1% formic acid plus NaCl (Fig. 1C) or NaCl with 18-crown-6 ether (Fig. 1D) in the separation of the peptides. The crown ether with 0.1% formic acid (Fig. 1B) effects little improvement over 0.1% formic acid alone (Fig. 1A).

A similar trend is seen in the attempted separation of Met-hGH and rhGH (Fig. 2) except in the case of intact proteins the combined effect of crown ether and NaCl produces the best separation (Fig. 2D). Note that formic acid alone does not appear to elute these proteins in the times used in this study (60 min or less).

In the examples of bovine and porcine insulin separations (Fig. 3) the effect is reversed, *i.e.*, even with identical gradients the better separation is achieved in the 0.1% formic acid plus NaCl case (Fig. 3C).

The situation reverses again for the chromatograms of a whey protein isolate containing largely α-lactalbumin and β-lactoglobulins A and B, with both additives effecting the best separation in the presence of formic acid (Fig. 4D). All the proteins

TABLE II

COMPOSITION OF BUFFERS

Buffer	Formic acid (%)	Sodium chloride (%)	18-Crown-6 (%)	Water–acetonitrile
IA	0.1	–	–	49:1
IB	0.1	–	–	1:9
IIA	0.1	–	0.05	49:1
IIB	0.1	–	0.05	1:9
IIIA	0.1	1	–	49:1
IIIB	0.1	1	–	2:3
IVA	0.1	1	0.05	49:1
IVB	0.1	1	0.05	2:3
VA	0.1	–	–	9:1
VB	0.1	–	–	1:9
VIA	0.1	–	0.05	9:1
VIB	0.1	–	0.05	1:9
VIIA	0.1	1	–	9:1
VIIB[a]	0.1	1	–	2:3
VIIIA	0.1	1	0.05	9:1
VIIIB[b]	0.1	1	0.05	2:3
IXA	0.1	1 (KCl)	0.05	9:1
IXB	0.1	1 (KCl)	0.05	2:3

[a] Identical to IIIB.

[b] Identical to IVB.

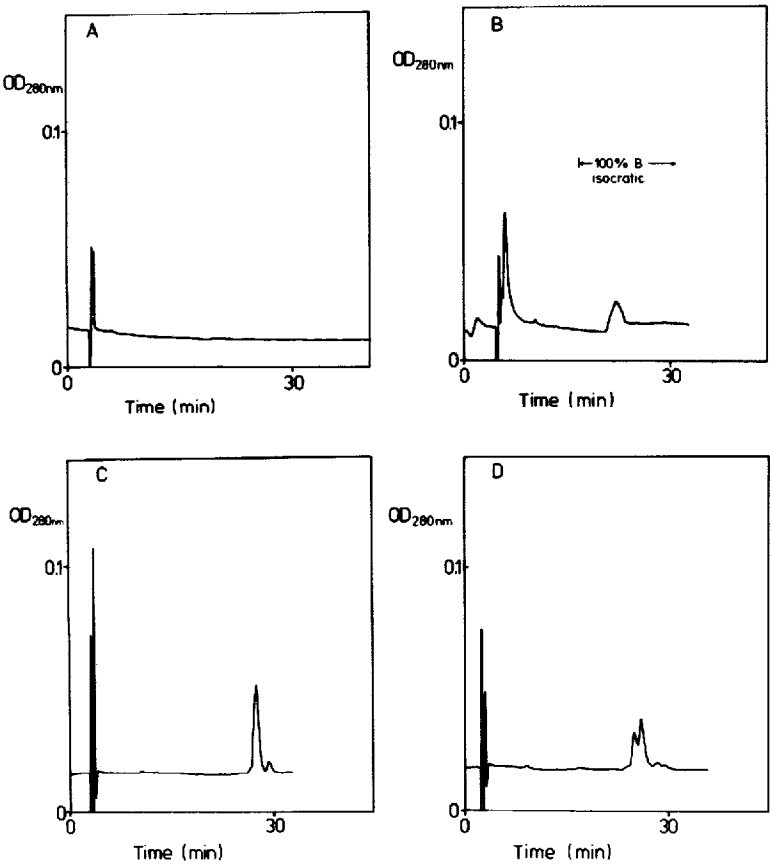


Fig. 2. Met-hGH and rhGH. Elution as in Fig. 1. For buffer composition and gradient see Tables I and II.

described in this publication were combined and chromatographed together (Fig. 5). This chromatogram shows the effectiveness of the combination of 0.1% formic acid, 18-crown-6 ether and sodium chloride in separating a mixture of proteins. For a good separation of proteins of very close polarity like the β-lactoglobulins or the two growth hormones, it is often not only important to use a shallow gradient, but also to start the chromatogram at the highest possible percentage of buffer B that still allows retention. Too low a percentage of buffer B will tend to bind the proteins too tightly to the column and may not allow quantitative and/or discreet elution.

Crown ethers react strongly with the different alkali and earth alkali metals [5,7]. This has prompted us to investigate the differences between sodium chloride and potassium chloride in the reversed-phase chromatography as well. Instead of the normal 1% of sodium chloride in the buffers, 1% of potassium chloride was used in one set of experiments. The results (Fig. 6) indicate that the proteins elute at a lower % buffer B, although the resolution is comparable.

Whether the dynamic coating will improve a certain separation is unpredictable from the examples shown here. The combination of the crown ether and sodium

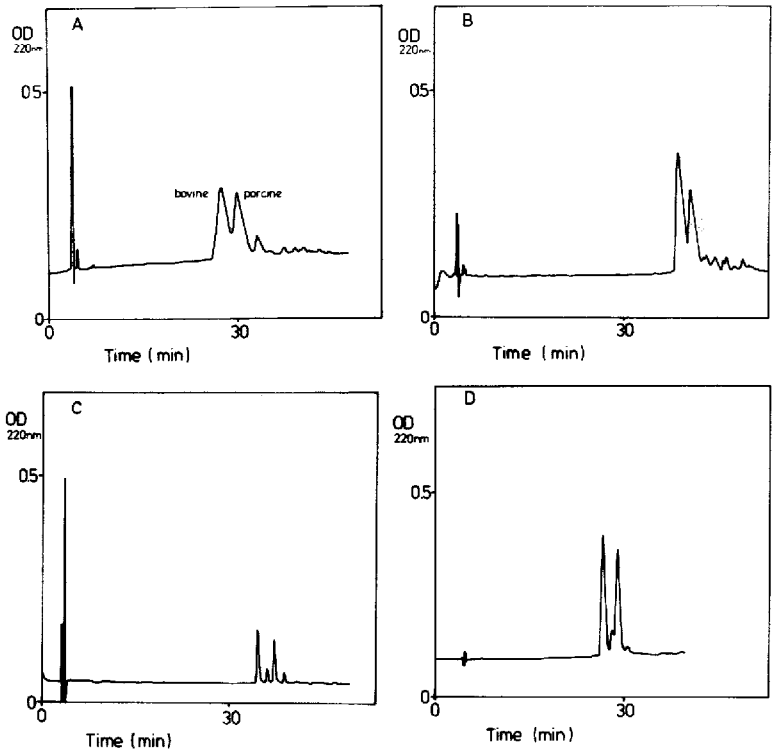


Fig. 3. Bovine and porcine insulin. Elution as in Fig. 1. For buffer composition and gradient see Tables I and II.

chloride certainly seems to be a powerful one in some cases. While it definitely improved the separation of the β-lactoglobulins and the surrounding small peaks (Fig. 4D) and of the growth hormones (Fig. 2D), the situation is reversed for the separation of the insulins (Fig. 3D). In the chromatograms with formic acid and crown ether but without sodium chloride, the effect of the crown ether is noticeable but nowhere does it seem as beneficial as the addition of the salt with or without crown ether.

All the examples of chromatograms given here containing a crown ether were carried out with 18-crown-6. Chromatograms prepared in the presence of 12-crown-4 were virtually identical to the 18-crown-6 examples shown here. The amount of crown ether added has been studied in a number of cases and it was found that there was no significant advantage to addition of more than 0.05% of these compounds. All other compounds tested for "dynamic coating", tetrahydrofuran, dioxan, diglyme, diisopropylether, methylcellosolve, glycol and *tert.*-butanol did not produce the beneficial effect shown here when used in conjunction with sodium chloride. In a few cases they could be used in separations without any salt and could effect improved separations when used in very low concentration (0.05%). At higher concentrations the effects were again counter-productive.

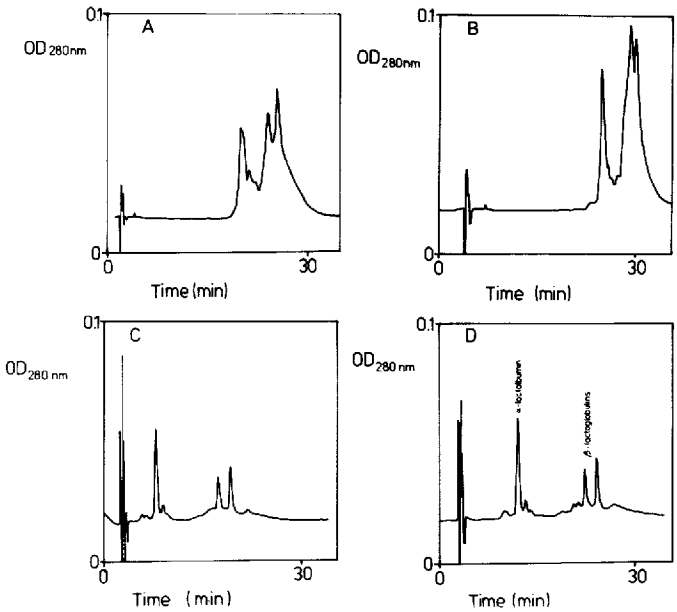
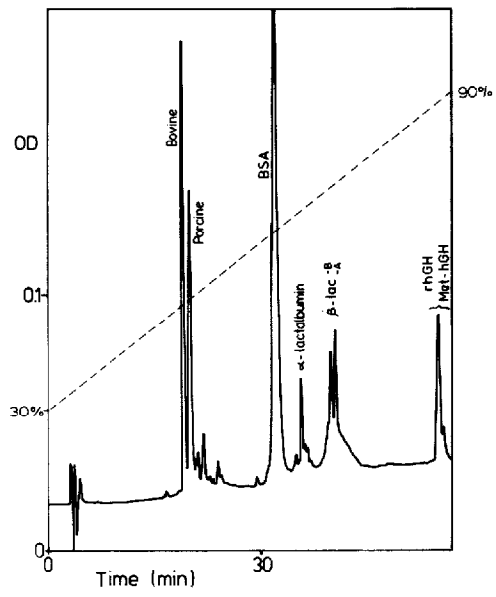


Fig. 4. Whey proteins. Elution as in Fig. 1. For buffer composition and gradient see Tables I and II.



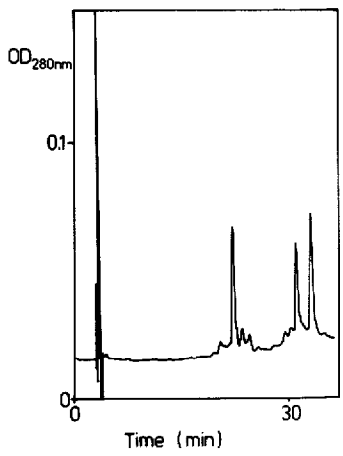


Fig. 5. All proteins. Elution as in Fig. 1D. For buffer composition and gradient see Tables I and II. lac = Lactoglobulin.

Fig. 6. Whey proteins. Elution as for Fig. 1D except KCl replaces NaCl. For buffer composition and gradient see Tables I and II.

The possible use of guanidine hydrochloride instead of sodium chloride was also investigated. Guanidine hydrochloride has the advantage that it is much more soluble in the acetonitrile than sodium chloride, to the extent that solutions of 2% of this compound can be prepared in 80% acetonitrile. (A maximum of 1% of sodium chloride in 60% acetonitrile is possible!) The examples studied above, when run with guanidine hydrochloride buffer did not show the resolution that the sodium chloride containing buffers were capable of. With guanidine hydrochloride it was found that the addition of 1% of the salt is necessary to effect elution. A composition of 0.5% was not enough while there was no significant difference between additions of 1% or 2%.

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