FORMIC ACID AS A MILDER ALTERNATIVE TO TRIFLUOROACETIC ACID AND PHOSPHORIC ACID IN TWO-DIMENSIONAL PEPTIDE MAPPING

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SUMMARY

In reversed-phase chromatography of peptides, formic acid has been shown to successfully replace the stronger traditional trifluoroacetic and phosphoric acids. Detection of non-aromatic peptide at lower wavelength is not impaired and being volatile the acid is easily removed, enabling further studies of the peptides. Also in ion-exchange chromatography (the first step of a two-dimensional approach) formic acid works well with a sulphonic acid ion-exchange resin.

INTRODUCTION

Trifluoroacetic acid (TFA) is widely used in modern chromatography, particularly in the reversed-phase high-performance liquid chromatography (RP-HPLC) of peptides and proteins[1]. Nevertheless this acidic material (pK_a = 0.6) is not used without concern. A recent study[2] has shown that 0.1% TFA in water at pH 2 in the presence of methanol or acetonitrile can result in loss of up to 50% of the initial reversed-phase loading on the stationary phase. In addition, the complete removal of trifluoroacetate from potentially useful therapeutic peptides is not readily achieved by techniques such as gel filtration[3]. Thus, despite its extensive analytical use, TFA may not prove to be the mobile phase modifier of choice for preparative chromatography of peptides and proteins destined for pharmaceutical use.

Phosphoric acid either alone[4] or titrated with hydroxide[5] or amines like triethylamine[6] to a pH in the range 2–4 is another popular mobile phase ion pair reagent. However the usefulness of sodium, potassium or ammonium phosphate is limited by their solubility in reversed-phase buffers containing an organic solvent. For example, these salts begin to precipitate at approximately 40% acetonitrile. Triethylammonium or triethanolammonium phosphate is preferred in this respect. However these salts are not volatile and their use therefore often requires a further processing step.

A special place among the buffer components is held by ammonium bicarbonate[7]. It has been employed for (semi)preparative work because it can be removed by

freeze drying. This buffer however loses carbon dioxide to the atmosphere and conse-
quently the pH rises easily to values over 9. Buffers at this pH are detrimental to
expensive silica-based HPLC columns even with the use of precolumns.

Formic acid (pK_a = 3.75) also has been employed in the liquid chromatography
of peptides and proteins[1]. While it does not appear to enjoy the popularity of trifluo-
roacetic acid or phosphate it has nevertheless been used in the RP-HPLC of peptides
and proteins in a parallel fashion, i.e., alone[8] or with counter ions such as ammoni-
um[8], triethylammonium[9] or pyridinium[10]. Used alone formic acid should prove to be
a useful buffer component because of its volatility and decreased acidity. Criticisms
that are levelled at formic acid systems relative to TFA or phosphate systems include
lower degrees of resolution[11] and recovery[12] plus inapplicability of UV detection at
220 nm[10]. This paper presents results from peptide mapping and two-dimensional
chromatographic experiments that illustrate the usefulness of formic acid in these
areas of HPLC.

EXPERIMENTAL

Apparatus

Three sets of apparatus were used in this study. A Pharmacia fast protein liquid
chromatography (FPLC) system was used for HR 5/5 Mono Q, Mono S and PepRPC
columns (50 × 5 mm I.D.). Detection at 280 nm was carried out with a single-path
UV-1 monitor.

A Waters Assoc. HPLC system was used with a Vydac Protein C_4 column (250
× 4.6 mm I.D.). This system consisted of two M6000A solvent delivery units, an
M680 controller and a U6K universal liquid chromatographic injector, coupled to an
M450 variable-wavelength UV spectrophotometer (Waters Assoc.) and an Omni-
scribe two-channel chart recorder (Houston Instruments, Austin, TX, U.S.A.).

An LKB system using LKB 2150 pumps, an LKB 2152 LC controller and an
LKB 2157 autosampler were also used. An LKB 2151 variable-wavelength detection
monitor was used with the LKB 2134-216 Ultropac column (250 × 4.6 mm I.D.,
TSK ODS-120, T5 μm).

Reagents

The following reagents were used: Milli Q water, acetonitrile (BDH HiPerSolv.,
Prod.No. 15252); formic acid [May & Baker, Pronalys AR (98–100%)]; ammonium
hydroxide (J. T. Baker, Analysed Reagent); sodium hydroxide (May & Baker, Volu-
con); hydrochloric acid (BDH, Convol); ammonium chloride was produced *in situ*.

All buffers were filtered and degassed through 0.45-μm filters (Millipore) for
HPLC, while the buffers for FPLC were filtered using 0.2-μm filters. Samples were
filtered through Gelman ACRO™ TC 13 filters (0.2 μm). Reversed-phase buffers
were prepared in the usual way. For the Mono S and Mono Q experiments, buffer A
was prepared as described in Figs. 8 and 9. The resultant apparent pH readings were
as follows: Mono Q, buffer A, pH 10.72; Mono S, buffer A, pH 3.04. Buffer B was
prepared by titrating the appropriate component to neutrality. The designated
amount of acetonitrile was added. No adjustment was made for apparent pH shift. A
few ml of water was added to achieve the final desired volume. The resultant apparent
pH readings were as follows: Mono Q, buffer B, pH 6.95; Mono S, buffer B, pH 8.50.

Note that 30% acetonitrile with 1 *M* sodium chloride is close to the limit of this one phase system. Currently we use 20% acetonitrile without any problems.

HSA was normal serum albumin (human) U.S.P. Albutein® 25% from Alpha Therapeutic Co.; lysozyme (egg white) was obtained from Sigma (Prod. No. 6876); bovine serum albumin (BSA) was also obtained from Sigma (Prod. No. A7638); methionine human growth hormone (Met-hGH) was obtained from Genentech; sheep liver phosphofructokinase (PFK) was obtained from Mrs. K. J. Rutherfurd (Massey University) and trypsin was obtained from Sigma (Prod. No. T8003).

Methods

The tryptic digestions were performed as follows: trypsin was added (1 mg trypsin/100 mg protein) to the protein dissolved in 1% ammonium bicarbonate solution. The digest was then left for 4 h at 37°C, after which it was treated again with another dose of trypsin and left for 16 h at 37°C. The chromatographic conditions are reported in the figure legends.

RESULTS AND DISCUSSION

Glajch *et al.*[2] have recently presented more evidence as to the detrimental effect of TFA on reversed-phase columns and the subsequent effects it can have on protein separations. Nevertheless, TFA and phosphoric acid are widely used. In the area of thin-layer chromatography, it is customary to check purity by running more than one eluent system. Obviously this concept carries over to HPLC. The results discussed below go some way to furthering this concept with respect to formic acid as a viable
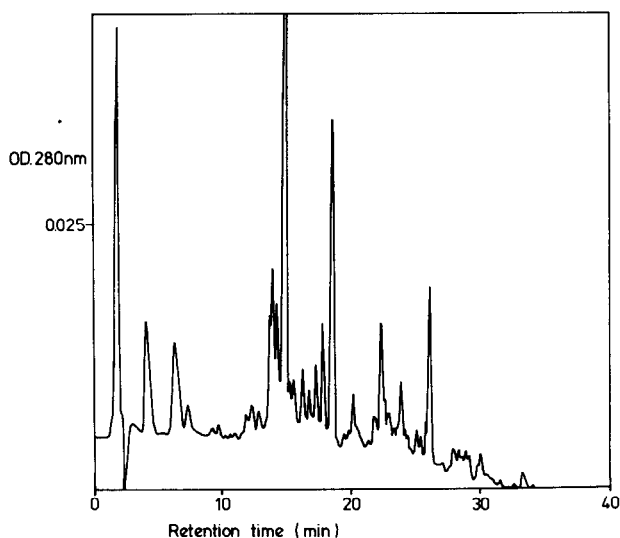

Fig. 1. HSA digest on a PepRPC HR 5/5 column. Conditions: buffer A, 0.1% formic acid–acetonitrile (19:1) and buffer B, 0.1% formic acid–acetonitrile (1:4); gradient, 0–100% B in 60 min at 0.5 ml/min; chart, 0.5 cm/min.
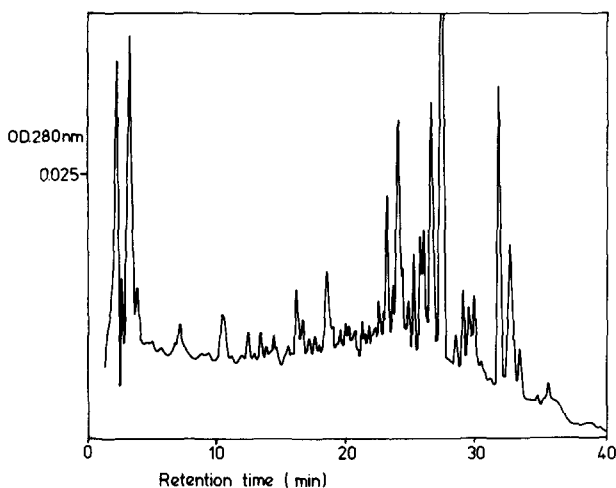
Fig. 2. Sheep liver PFK digest on PepRPC HR 5/5 column. Conditions as in Fig. 1.

alternative to other acids. Its applicability to the two-dimensional mapping of protein hydrolysates is also presented.

Figs. 1 and 2 illustrate the use of formic acid with trypsin hydrolysates of a commercial blood product and a sheep liver enzyme at 280 nm with a PepRPC column. Similarly the mapping of a recombinant protein in Fig. 3 illustrates good resolution on a Vydac C_4 column at the same wavelength. Figs. 4–6 show that signif-
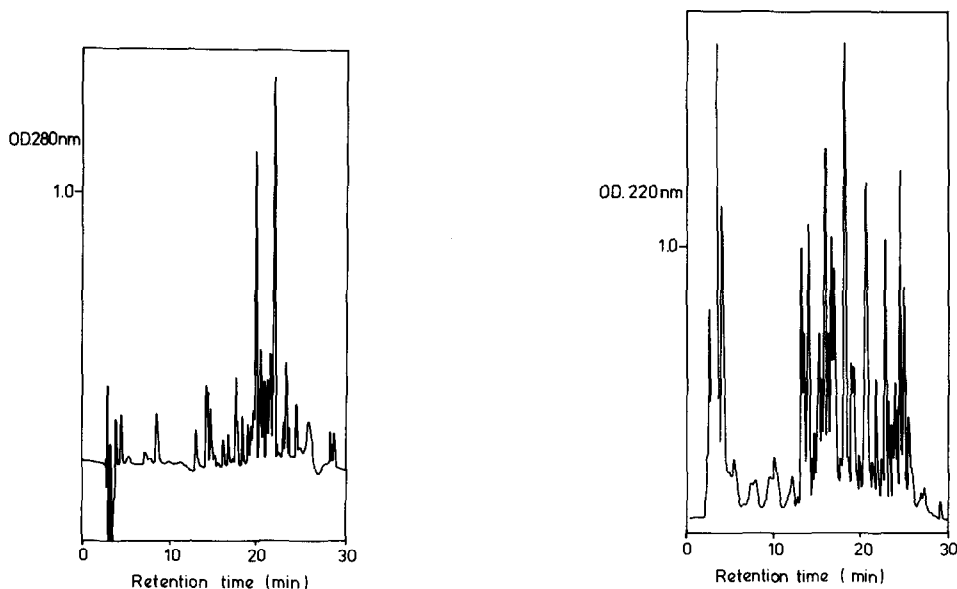
Fig. 3.Met-hGH digest on a Vydac Protein C_4 column (250 × 4.6 mm I.D.). Conditions: buffers and chart as for Fig. 1. Gradient: 0–100% B in 45 min at 1 ml/min.

Fig. 4. BSA digest on a Vydac Protein C_4 column (250 × 4.6 mm I.D.). Conditions as in Fig. 3.
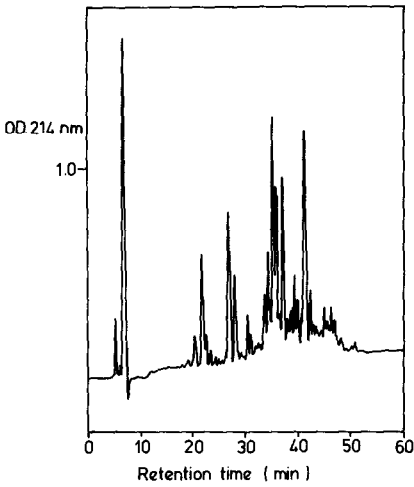
Fig. 5. Lysozyme digest on an Ultropac column (250 × 4.6 mm I.D.). Conditions: buffers as in Fig. 1; gradient 0–60% B in 60 min; chart, 2 mm/min.
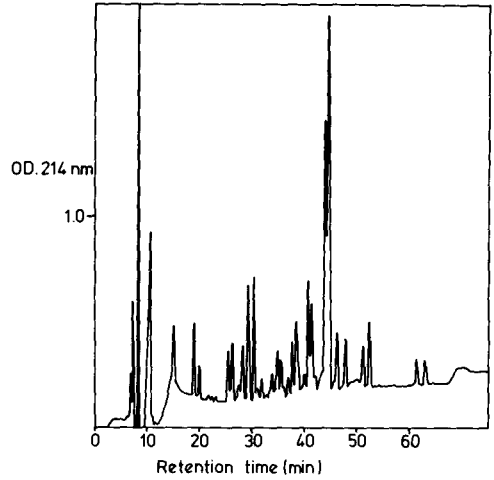
Fig. 6. Met-hGH digest on an Ultropac column (250 × 4.6 mm I.D.). Conditions as in Fig. 5 except gradient, 0–60% B for 60 min then 60–100% B for 15 min.

icant resolution is maintained at lower wavelengths with a variety of hydrolysates on either a C_4 or a C_{18} column. Fig. 7 compares the performance of formic acid and phosphoric acid in a system that has all other components identical. The similarities are apparent and thus in this case illustrate the comparability of the two acids with the exception of volatility in favour of formic acid.
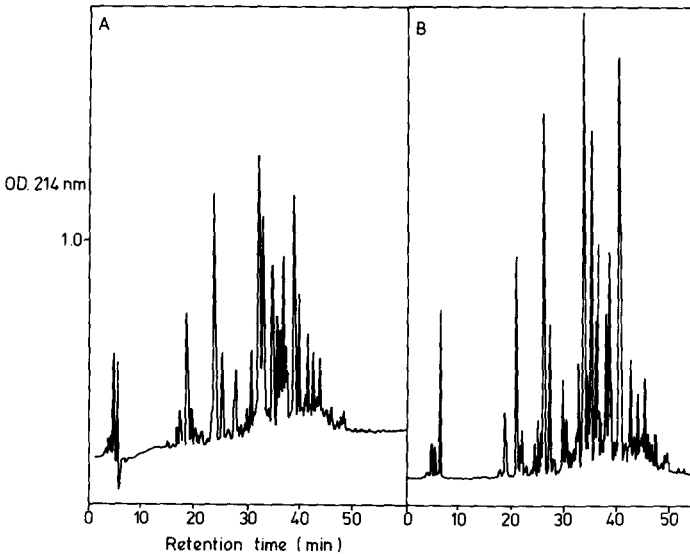

Fig. 7. HSA digest on an Ultropac column (250 × 4.6 mm I.D.). Conditions: (A) as in Fig. 6; (B) identical except for buffer A, 0.1% H_3PO_4 in water–acetonitrile (19:1) and buffer B 0.1% H_3PO_4 in water–aceto-nitrile (1:4).
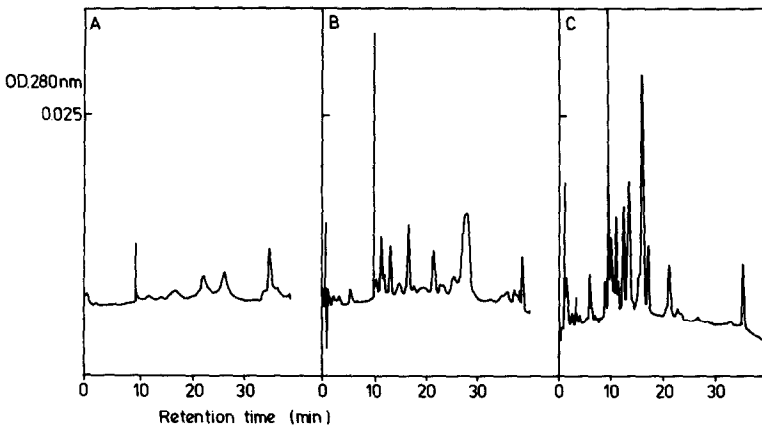
Fig. 8. HSA digest on a Mono S HR 5/5 column. Conditions: (A) as in (C) without acetonitrile; (B) as in (C) but 15% acetonitrile; (C) buffer A, 0.05 M formic acid in water–acetonitrile (7:3) and buffer B, 0.05 M sodium formate + 1 M sodium chloride in water–acetonitrile (7:3); gradient, 0–100% B in 30 min at 1 ml/min; chart, 2 mm/min.

Fig. 8 outlines the beneficial effect of using a formic acid system in conjunction with acetonitrile on a Mono S cation-exchange column. With the limitation noted above as to the concentration limit of the acetonitrile, the effect of organic modifier in this case is obvious. This effect has been noted in other systems employing ion exchange media for peptide separations[13,14]. Similarly a Mono Q anion-exchange column (Fig. 9) can be used for mapping experiments of lysozyme and HSA. Fig. 10
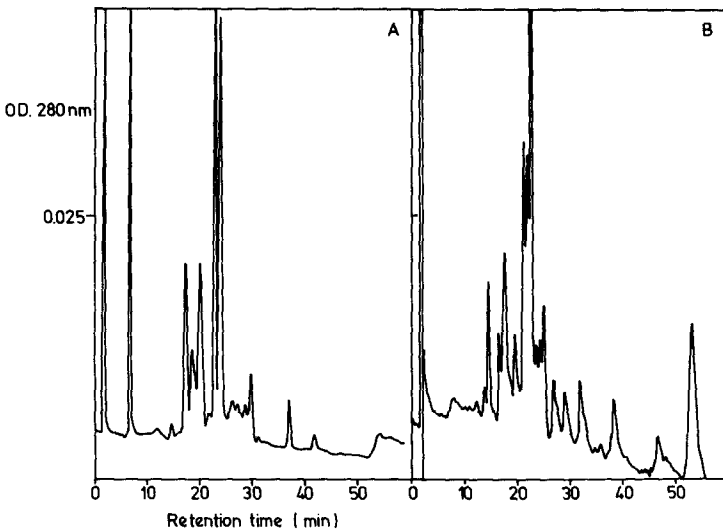

Fig. 9. Lysozyme (A) and HSA (B) digests on a Mono Q HR 5/5 column. Conditions: buffer A, 0.05 M ammonium hydroxide–acetonitrile (7:3) and buffer B, 0.05 M ammonium chloride + 1 M sodium chloride–acetonitrile (7:3); gradient, 0–25% B in 60 min then to 100% B in 10 min at 1 ml/min; chart, 2 mm/min.
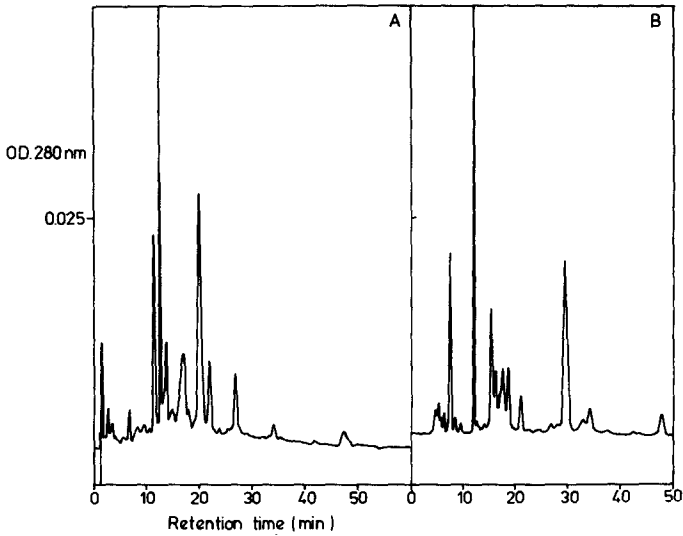
Fig. 10. HSA (A) and lysozyme (B) digests on a Mono S HR 5/5 column. Conditions: buffer A, 0.05 *M* formic acid–acetonitrile (7:3) and buffer B, 0.05 *M* sodium formate + 1 *M* sodium chloride–acetonitrile (7:3) gradient and chart as in Fig. 9.

illustrates the separation of the same materials on a Mono S column. With the idea of combining two-dimensional chromatography with peptide mapping, a digest of BSA was run on the Mono S column (Fig. 11A) using the formic acid–acetonitrile system. The success of this analytical run led to scaling-up and trapping of six peaks (Fig. 11B). These peaks were then analysed under reversed-phase conditions (Fig. 12).
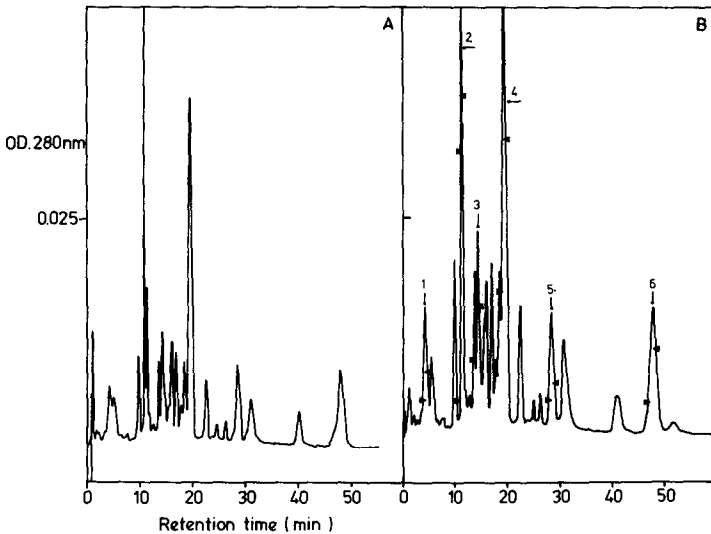

Fig. 11. BSA digest on a Mono S HR 5/5 column. Conditions: as in Fig. 10. (A) 300 μg BSA digest; (B) 2 mg BSA digest with peaks trapped as indicated.
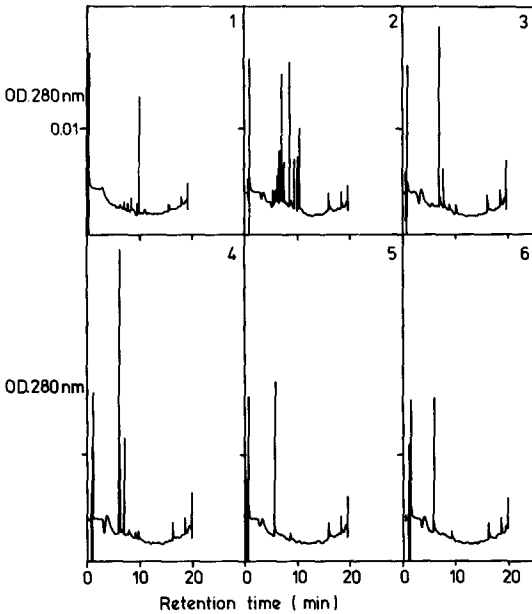
Fig. 12. Peaks 1–6 from Fig. 11B BSA digest run on a PepRPC HR 5/5 column. Conditions: buffers and chart as in Fig. 1; gradient, 0–100% B in 20 min at 1 ml/min.

With the exception of peak 2 all peaks were relatively clean and could be used for other analytical techniques such as amino acid analysis, sequencing or electrophoretic gels. The similarity of the retention times in Fig. 12 compared to the distinctly different retention times of Fig. 11B points out the obvious advantages of the two-dimensional approach.

In summary, this paper goes some way toward addressing the criticisms against the use of formic acid in high-performance chromatography. The figures above illustrate that formic acid can be used at low wavelengths with good resolution. With the exception of the MonoQ experiment, in all of the above experiments formic acid was used. Of parcticular importance with respect to Fig. 12 is the fact that even if formic acid had not been used in the ion-exchange step of the two-dimensional mapping, its use for the reversed-phase step constitutes a desalting method with a volatile buffer.

ACKNOWLEDGEMENTS

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