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FACTORS INFLUENCING THE RETENTION OF INSULINS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEMS

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SUMMARY

The effects of the concentrations of methanol, buffer and neutral salt, of pH and temperature, as well as of the type, chain length and concentration of ion-pair reagents added to aqueous phosphate buffer–methanol eluents upon the retention of bovine and porcine insulins have been investigated in detail. A possible explanation for the retention-influencing effects of both the cationic and anionic ion-pair reagents is proposed.

INTRODUCTION

Traditionally, gel chromatography has been used for the analysis of proteins, and large poly- and oligopeptides including insulins, *e.g.*, refs. 1–4. Chemically bonded controlled porosity glass[5], deactivated silica, etc., have also been tried with various degrees of success[6]. These techniques, at best, allow the separation of insulins from other much larger or much smaller polypeptides, but not the separation of insulins of various species, and of related compounds. Since then, reversed-phase (RP) and ion-pair high-performance liquid chromatography (HPLC) have been introduced for the analysis of proteins and polypeptides (for a comprehensive review see, *e.g.*, ref. 7). Most RP separations of insulins published so far are based on one of two principles: (a) control of the degree of ionization of the insulins; (b) use of ion pair reagents.

In the first group the extent of retention is controlled by altering the degree of ionization of the insulin molecule. It was shown[8,9] that at pH < 2 all terminal and side-chain carboxyl groups of insulins are protonated, along with the terminal and side-chain NH groups. Between pH 2 and 4 only the side-chain carboxyl and the terminal and side-chain NH groups are protonated. Above pH 4 even the side-chain carboxyl groups become dissociated (the isoelectric point of insulins is at 5.3). Therefore, adjustment of the eluent pH allows control of the ionization of the insulin molecule, *i.e.*, its actual hydrophobicity. This is the basis of the separation schemes described in refs. 10–15. Hancock and co-workers[10–13] postulated that insulins formed hydrophilic ion pairs with $H_2PO_4^-$ present in the eluent. Similar separations were obtained with sulphuric acid[14], hydrochloric acid[15] and perchloric acid[16] even though

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they are generally not considered strong hydrophilic ion-pairing agents. Low pH and high salinity were mandatory in each case for good peak shape and reproducible retention. Acetate and formate anions were not as effective. The organic solvent added to the eluent also had a profound effect upon the capacity factor (k') of insulin: k' values increased in the somewhat unexpected order acetonitrile, tetrahydrofuran, dioxan and methanol.

Dinner and Lorenz[14] described an elegant and simple isocratic method for the analysis of bovine and porcine insulins, and their monoarginine and monodesamido derivatives.

In the second general method, insulins are separated on a hydrophobic packing (alkyl silicas) by a buffered hydroorganic eluent containing either cationic[16–18] or anionic[11,19] ion pair reagents. Rivier[17] used a trialkylamine–phosphoric acid buffer system in acetonitrile, Biemond *et al.*[16] added a tetramethylammonium hydroxide–phosphoric acid buffer to methanol and Damgaard and Markussen[18] used an ethanolamine–phosphoric acid system. It was claimed that the small tetraalkylammonium ion could block the residual OH groups of the RP packing and also possibly act as an ion-pairing agent interacting with the carboxylate groups of the insulins. Identical retentions were obtained with a pH 2 phosphate eluent without and with tetramethylammonium ions. However, in the last case the peak shape was better[16].

Terabe *et al.*[19] added butanesulphonate anions to pH 3.0 tartrate buffer–acetonitrile as eluent. Again, the peak shape of the insulins was better in the presence, rather than in the absence, of the butanesulphonate anion. Changes in the eluent pH had only a slight effect upon the k' values. It was assumed that the RSO_3^- anions blocked the residual OH groups of the RP packing, and also possibly formed ion pairs.

Apparently, acceptable separations can be obtained with both cationic and anionic ion pair reagents. However, to our knowledge, there are no quantitative data relating to the effects of the type and concentration of the ion pair reagent, the ionic strength, pH, temperature and methanol concentration of the eluent upon the retention of insulins. The aim of the work reported here was the procurement of such data, and the explanation of the somewhat unexpected effects of the cationic and anionic ion pair reagents.

EXPERIMENTAL

Experiments were carried out on a Varian LC 5020 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) equipped with an LC55 variable-wavelength detector (Perkin-Elmer, Norwalk, CT, U.S.A.) set to 220 nm, and custom-made stainless-steel columns (250 × 4.0 mm I.D.) packed with 10-μm RP-18 silica (E. Merck, Darmstadt, G.F.R.). The columns were jacketed and thermostatted[20] by a circulating U10 water-bath (MLW, Medingen, G.D.R.).

In preliminary experiments it was found that the k' values of insulins were extremely sensitive to the methanol concentration of the eluents. Therefore, great care was taken in eluent preparation. The calculated amount of methanol was weighed into a 1000-ml volumetric flask followed by the calculated amount of buffer components, ion-pair reagent and salt used for adjustment of the ionic strength. Then the contents of the flask were brought almost to the mark with double distilled water,

leaving a free space of about 500 μl. The temperature of the flask was adjusted to 25 ± 0.2°C, and the pH of the eluent was measured with a combined glass electrode, calibrated with aqueous buffers, and a precision digital pH meter (Radelkis, Budapest, Hungary). If necessary, (minor) final pH adjustment was achieved by adding a few drops of concentrated phosphoric acid. Then the flask was brought to the mark with distilled water. As an ultimate check on the actual elution strength of the eluent, members of the nitroalkane homologous series, benzene and toluene were also separated and their k' determined, since their retentions are primarily dependent on the methanol content of the eluent. With these precautions, the k' values of insulins were reproducible within 2% relative.

The ion-pair reagents were obtained from BDH (Poole, Great Britain), Fluka (Buchs, Switzerland) and Ferak (West Berlin, G.F.R.). Methanol, phosphoric acid, sodium bromide and sodium dihydrogen phosphate were obtained from Reanal (Budapest, Hungary). The insulin samples tested were from Gedeon Richter (Budapest, Hungary) and NOVO (Copenhagen, Denmark).

RESULTS AND DISCUSSION

Effect of the methanol concentration of the eluent

Fig. 1 shows the k' values of porcine and bovine insulins and the neutral reference compounds as a function of the methanol concentration in a pH 3.2 eluent containing 0.05 mole/l tetramethylammonium hydroxide and 0.1 mole/l phosphoric acid at 30°C. The dead volume of the column was determined by injecting a saturated KI solution as described in ref. 21. A 5% (w/v) increase in the methanol concentration causes a ten-fold decrease in the k' values of insulins, while the k' of "regularly behaving" nitroalkanes and other small molecules changes only by a factor of 1.1–1.3. In the narrow methanol concentration range where insulin separation can be achieved,

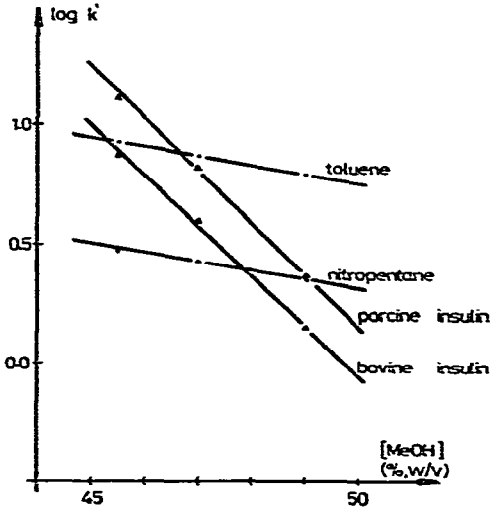


Fig. 1. Dependence of the k' values of bovine and porcine insulins upon the methanol (MeOH) concentration in 0.05 mole/l tetramethylammonium hydroxide, 0.1 mole/l phosphoric acid eluent, pH 3.2, at 30°C.

there is a linear relationship between log k' and the methanol concentration. The slopes obtained for the bovine and porcine insulins and their monodesamido derivatives are identical, -0.22, and differ significantly from those of the nitroalkanes, -0.04.

Similar curves and similar slopes were obtained when the methanol concentration of pH 3.0 0.05 mole/l tetraethylammonium hydroxide–0.1 mole/l phosphoric acid and pH 2.6 0.005 mole/l tetrabutylammonium hydroxide–0.01 mole/l phosphoric acid eluents was changed, -0.19 and -0.21. When heptanesulphonic acid was used as ion pair reagent the slope was -0.25.

Thus, it can be concluded that, independently of the type of ion-pair reagent used, the k' values of insulins are extremely sensitive to the methanol concentration of the eluent. Therefore, the methanol concentration range available for practical insulin separations is very narrow and reproducible separations require extreme care in the preparation of the eluents.

Effect of the salt concentration of the eluent

A number of papers[8–19] have emphasized that high salinity is mandatory for good insulin peak shape. Therefore, eluents which contained 49% (w/v) methanol, 0.05 mole/l NaH_2PO_4, 0.05 mole/l H_3PO_4 and increasing amounts of sodium bromide (0–0.8 mole/l) were prepared.

The k' of insulins is plotted against the sodium bromide concentration in Fig. 2. The increase in k' caused by the 0–0.8 mole/l increase of the sodium bromide concentration is equivalent to that caused by a 1% decrease in the methanol concentration of the eluent. Such a change in methanol concentration would result in an increase the k' of benzene, toluene and the nitroalkanes as described above. However, no such change can be detected, *i.e.*, the increase in the k' of insulins can be attributed to the presence of NaBr.

It was noted that the pH of the eluents decreased about 0.3 units from pH 3.2 when the concentration of NaBr increased from 0 to 0.8 mole/l. This is due to a change in the dissociation of phosphoric acid with the sodium bromide concentration. The apparent first pK values of H_3PO_4 taken from potentiometric titration

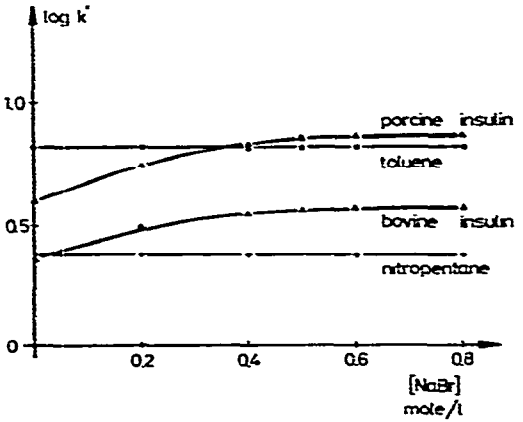


Fig. 2. Dependence of the k' values of bovine and porcine insulins upon the NaBr concentration in a 49% (w/v) methanol, 0.05 mole/l H_3PO_4, 0.05 mole/l NaH_2PO_4 eluent at 30°C.

curves of 0.05 mole/l H_3PO_4 dissolved in the different eluents were: 3.25 in 0.4 mole/l NaBr in water; 2.95, 3.05 and 3.30 in 1.0, 0.5 and 0.1 mole/l NaBr in 49% (w/v) methanol, respectively. It will be shown later that the change in k' caused by a pH change of 0.3 units is negligible compared with the overall change observed.

Effects of the ion pair reagents

Anionic ion pair reagents

Phosphate anion. In preliminary experiments we found that the k' of insulins was sensitive to the concentration of the phosphate buffer. Therefore, the effects of the overall phosphate concentration of the eluent upon the k' of insulins was investigated. The concentration ratio of NaH_2PO_4 and H_3PO_4 was kept at about 1:1, and the overall phosphate concentration was changed from 0.04 to 0.7 mole/l in pH 3.2, 49% (w/v) eluents. The results obtained are shown in Fig. 3.

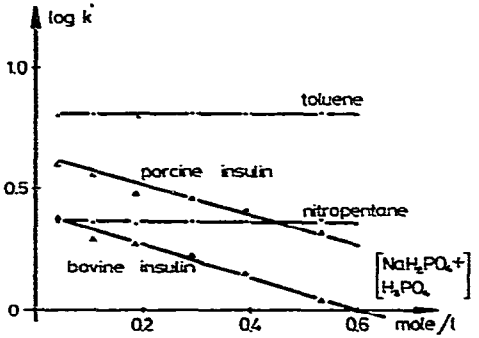


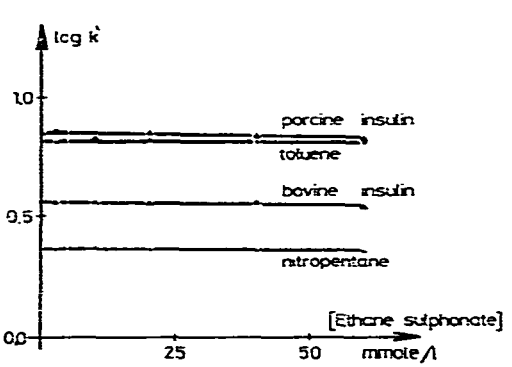
Fig. 3. Influence of the overall phosphate concentration of the eluent upon the k' of insulins. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, pH 3.2, at 30°C.

The log k' of insulins decreases strongly with increasing overall phosphate concentration, slope −0.6. The increase in overall phosphate concentration also means an increase in ionic strength, which, as shown above, results in greater retention of insulins. Thus, the overriding effect is the decrease in retention caused by $H_2PO_4^-$. Since the eluent pH was constant, 3.2, and changes in the methanol concentration could also be ruled out (as indicated by the constant k' values of nitroalkanes, benzene and toluene), the most plausible explanation is that the $H_2PO_4^-$ anion interacts with the positive charges of the insulin molecule and forms hydrophilic ion pairs. The effect of the concentration of $H_2PO_4^-$, about 0.1 log k' units, is much larger than, in the case of other ion pair reagents (see below). This means that the rôle of phosphate is dual: it acts as pH-controlling buffer, and also as a retention-controlling ion pair reagent. Therefore, its concentration has to be kept strictly constant for reproducible separations.

Alkanesulphonic acids. As mentioned in the Introduction, butane-, hexane- and heptanesulphonic acids have been used in various eluents, but no data relating to the effects of chain length and concentration of the ion-pair reagent are available. Therefore, a water-soluble alkanesulphonic acid, ethanesulphonic acid, and a much less

soluble acid, heptanesulphonic acid, were selected and their concentration effects were studied.

The pH 3.1 eluents contained 49% (w/v) methanol, 0.5 mole/l NaBr–0.05 mole/l phosphate buffer (1:1) and various amounts of ethanesulphonic acid and heptanesulphonic acid. The results are shown in Figs. 4 and 5. It is seen that the k' values of the neutral components are practically constant (or decrease very slightly) over the reagent concentration range tested. The retention of insulins is practically constant with ethanesulphonic acid as ion pair reagent. With the more hydrophobic heptanesulphonic acid, the k' of insulins first increases, then begins to level off. Unfortunately, the *n*-heptanesulphonic acid concentration could not be increased past the point indicated, because our supply ran out.



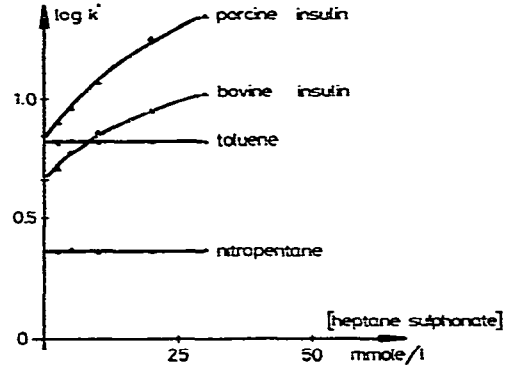


Fig. 4. Influence of the ethanesulphonate concentration of the eluent upon the k' of insulins. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l phosphate buffer, pH 3.1, at 30°C.

Fig. 5. Influence of the heptanesulphonate concentration of the eluent upon the k' of insulins. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l phosphate buffer, pH 3.1, at 30°C.

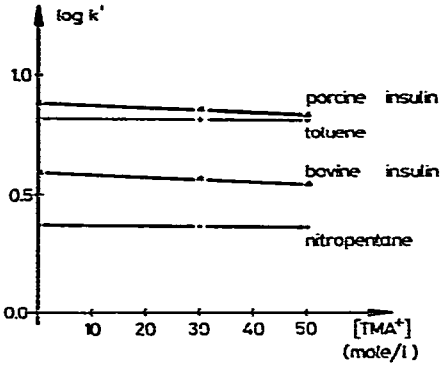
In contrast to the effect of the similarly negatively charged $H_2PO_4^-$ anion, the ethanesulphonate anion apparently does not influence k', while the heptanesulphonate anion increases the retention of insulins. This means that the hydrophobic anions interact with the positive charges of the insulins (which, at this pH, are in excess with respect to the negative charges) and increase their retention in the usual way.

The peak shapes obtained with the base eluent in the presence and absence of alkanesulphonate anions were practically identical.

Changing the methanol concentration of the eluent resulted in a log k' *vs.* methanol concentration relationship of slope −0.25 a value similar to those obtained without ion-pair reagents and with cationic ion-pair reagents.

Cationic ion pair reagents. Tetramethylammonium (TMA) bromide and tetrabutylammonium (TBA) bromide were used as positively charged ion pair reagents. Once again 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l phosphate buffer, pH 3.1, was the base eluent. The log k' *vs.* reagent concentration relationships obtained are shown in Figs. 6 and 7 for TMA^+ and TBA^+, respectively.

It is seen that, contrary to the effects of the alkanesulphonates, the k' of in-



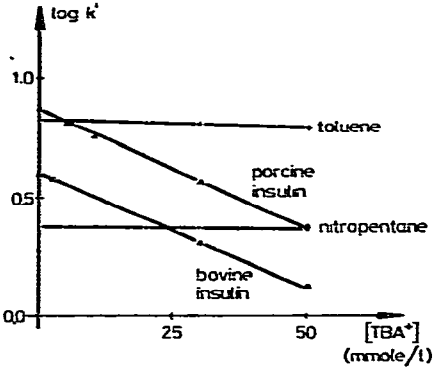


Fig. 6. Influence of the tetramethylammonium bromide concentration upon the k' of insulins. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l phosphate buffer, pH 3.1 at 30°C.

Fig. 7. Influence of the tetrabutylammonium bromide concentration upon the k' of insulins. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l phosphate buffer, pH 3.1 at 30°C.

sulins decreases with increasing tetraalkylammonium ion concentration. The decrease is much more pronounced for the tetrabutylammonium ion (Fig. 7).

A possible explanation of this behaviour is that the reversed-phase material adsorbs the positively charged tetraalkylammonium ions, so the packing repels the insulin molecules on which positive charges are in excess at this pH. This behaviour is similar to that noted by Knox and Hartwick[22] for smaller zwitterions, the opposite charges of which were sufficiently separated in space. Melin *et al.*[23] and Sokolowski and Wahlund[24] also noted that the tetrabutylammonium cation decreased the retention of amines in phosphate buffers.

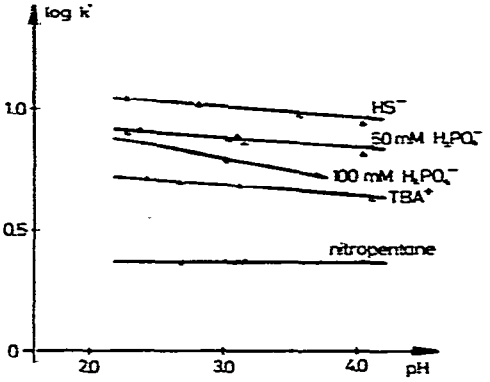
Effect of the eluent pH

Using the 49% (w/v) methanol, 0.5 mole/l NaBr and 0.05 mole/l phosphate eluent, the effects of the pH upon the retention of insulins was investigated. Successive eluents contained $H_2PO_4^-$ only, heptanesulphonate and tetrabutylammonium as ion pair reagents. The results obtained are shown in Fig. 8. For the sake of clarity, only the k' values of porcine insulin are shown, but those of bovine insulin run parallel.

Independently of the type of ion-pair reagent, $\log k'$ decreases with pH, and the slopes are identical, -0.05. Since all three eluents contained identical amounts of the phosphate buffer, and the ratio of $H_2PO_4^-/H_3PO_4$ changed in the same manner, the apparent pH dependence was attributed to the effects of increasing $H_2PO_4^-$ concentration. To verify this, another eluent was prepared which contained twice as much phosphate buffer. The k' values obtained are also shown in Fig. 8. This time the slope was -0.10. This indicates that in the pH range 2.3–4.2 the change in the k' of insulins is not due to the change in pH, but to the increasing concentration of $H_2PO_4^-$ as ion-pairing agent.

Effect of the eluent temperature

The effects of eluent temperature upon the k' values of insulins and nitroal-



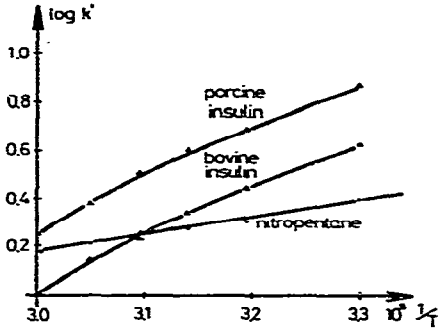


Fig. 8. Apparent influence of eluent pH upon the k' of porcine insulin. Eluent: 49% (w/v) methanol, 0.5 mole/l NaBr, 0.05 mole/l NaH_2PO_4, 0.05 mole/l H_3PO_4, 0.005 mole/l heptanesulphonic acid (HS^-) and 0.01 mole/l tetrabutylammonium bromide (TBA^+).

Fig. 9. Effect of the eluent temperature upon the k' values of nitropentane, bovine and porcine insulins. Eluent: 45.9% (w/v) methanol, 0.05 mole/l tetramethylammonium hydroxide, 0.1 mole/l H_3PO_4, pH 3.2.

kanes are shown in Fig. 9. The log k' *vs.* $1/T$ curves of insulins are not linear as those of the nitroalkanes, or those of other simple, non-ionic solutes examined earlier[25]. The curvature can be attributed to changes with temperature of the protonation constants of both the insulins and the components of the buffer[26].

CONCLUSIONS

A review of the RP-HPLC separations of insulins shows that although a number of successful separations have been published in the last 3 years, quantitative data relating to the retention-influencing parameters are lacking. Therefore, we investigated the effects of methanol, phosphate buffer, neutral salt and ion pair reagent concentration upon k' of bovine and porcine insulins. All experiments were carried out in the pH range 2.4–4.1.

The practical methanol concentration range is extremely narrow; a 5% (w/v) change results in a ten-fold increase in log k'. Increasing NaBr concentration results in an increase of log k' which, at high molarity, tends to level off. The $H_2PO_4^-$ anion greatly influences the k' of insulins; k' decreases with increasing phosphate concentration, indicating the formation of hydrophilic ion pairs. Alkanesulphonate ion-pair reagents increased the retention of insulins, the magnitude of the increase depending on the chain length of the reagent. The relationship between log k' and heptanesulphonate concentration is not linear. Tetraalkylammonium ion pair reagents greatly decrease the k' of insulins, the extent of the decrease again depending on the chain length of the reagent. The slight apparent pH dependence could be traced back to the dependence of k' upon the $H_2PO_4^-$ concentration.

All these results indicate that reproducible separations require extreme care in the preparation of the eluents.

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