SPECTRAL PROPERTIES OF SOME ION-PAIRING REAGENTS COMMONLY USED IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS AND PEPTIDES IN ACETONITRILE GRADIENT SYSTEMS

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SUMMARY

Electronic spectra of some anionic and cationic ion-pairing reagents in aqueous solution and in solvent mixtures with various contents of acetonitrile were recorded between 200 and 300 nm, and optimum conditions for highly sensitive detection using linear solvent gradients in reversed-phase high-performance liquid chromatography were proposed. Butane-, hexane and octanesulphonic acids showed no remarkable absorbance and recorded baseline drifts are therefore mainly caused by the increasing contents of acetonitrile. For trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFB), the baseline trace of a linear acetonitrile gradient system is the result of a discontinuous change from the $\pi \rightarrow \pi^*$ band of the anion in aqueous solution to the $n \rightarrow \pi^*$ transition of the undissociated acid in the organic solvent and therefore depends strongly on the wavelength used. For both acids, there exists an optimal recording point below 220 nm (TFA: 215 nm, HFB: 219 nm). Cationic hydrophobic ion-pairing reagents such as alkylated amines are weakly protonated at a higher pH and show relatively strong absorbance below 250 nm. The ionization is further reduced by the addition of acetonitrile, leading to an extreme baseline drift. At low pH, however, complete ionization is achieved even at high contents of organic solvent, but ion-pairing with the carboxylic group of peptides is then impossible. In contrast to the amines, the tetraalkylammonium cation is a relatively strong Lewis acid and the pH dependency as well as the organic solvent dependency of the electronic spectra is therefore less marked. Nevertheless, in the case of tetrabutylammonium phosphate the baseline drift shows a minimum at pH 5 and therefore this pH is highly recommended for sensitive detection using acetonitrile gradients.

INTRODUCTION

The rapid progress in high-performance liquid chromatography (HPLC), and especially in column technology, has rendered reversed-phase chromatography one of the most versatile tools for micropreparative and microanalytical separations of proteins and peptides. In fact, following the introduction of small-diameter silica particles and microbore columns, the required quantities of polypeptides could be reduced to low nanomole and even to picomole levels. In order to record such small amounts of underivatized proteins, it is necessary to use a low detector range setting and a wavelength below 225 nm. However, using gradient elution, which is often inevitable, the sensitivity of detection can be severely limited by baseline drift. Indeed, even when the aqueous solvent and the organic modifier have nearly the same absorbance, e.g. water and acetonitrile, the use of essential mobile phase additives may cause serious problems. This is especially true for hydrophobic ion-pairing reagents, which are commonly used in reversed-phase separations of amino acids, peptides and proteins in order to improve the resolution of highly-charged and therefore unretained molecules[1-4].

Ion-pairing reagents belong to different classes of chemical compounds but an essential common feature is the amphiphilic structure. Usually, alkylated acids or amines and their respective salts are used. Although most of these reagents show negligible absorbance of ultraviolet light in aqueous solution, drastic increases in absorbance may occur during development of the gradient. This is due to a shift of the dissociation equilibrium towards the uncharged molecule with decreasing polarity of the solvent mixture. In a previous paper[5], we discussed this effect using trifluoroacetic acid in a water–acetonitrile model system. Moreover, we have shown that the spectra meet closely at a certain point and optimal adjustment of the wavelength considerably reduces the baseline drift. This demonstrates that a knowledge of the spectral properties of a mobile phase additive is of great importance if highly sensitive detection is to be achieved. In the following report, we have therefore investigated several commonly used ion-pairing reagents and the results show that in most cases the baseline drift can be considerably lowered if optimum recording conditions are chosen.

EXPERIMENTAL

Ion-pairing reagents (Ionate®) were purchased from Pierce (Rockford, IL, U.S.A.) and acetonitrile was of HPLC grade (J. T. Baker, Deventer, The Netherlands). All solutions were made up with HPLC-grade water (Pierce).

Spectra were recorded with a DU-8 spectrophotometer (Beckman, Berkeley, CA, U.S.A.).

RESULTS AND DISCUSSION

Two principle mechanisms of ion-pairing are discussed; the formation of hydrophobic ion-pairs in solution prior to adsorption, and the covering of the stationary phase surface leading to a dynamic ion-exchange process[6-8]. In both cases, the retention of a polar peptide is increased. The actual change of the retention time

then strongly depends on the alkyl chain-length of the ion-pairing reagent and the number of oppositely charged functional groups on the peptide. In the case of anionic ion-pairing, a negatively charged compound interacts with the protonated amino group[1] of the sample, whereas a cationic ion-pairing reagent shows high affinity to carboxylic side-chains[2]. In the present paper, we deal with both types of ion-pairing reagents. We have analysed alkylsulphonates and perfluorinated carboxylic acids as representatives for anionic ion-pairing reagents, and alkylamines and tetraalkylammonium salts for cationic ion-pairing reagents.

Alkylsulphonates

The homologous series of alkylsulphonates offers a wide spectrum of anionic ion-pairing reagents with gradually changing hydrophobicity[1,3,9−11]. The retention of polar peptides can thus be exactly controlled by the use of the correct alkyl chain-length of the sulphonate[12]. We have examined the spectral properties of the sodium salts of 1-butane-, 1-hexane- and 1-octanesulphonic acids. In all three cases, the absorbance of a 0.01 M aqueous solution, measured between 200 and 300 nm, did not exceed 0.04 a.u. The small variations observed with the three homologues did not show any regularity and we assume that differences in the purification grade are responsible for this effect. In any case, the predicted, small baseline drift of a linear gradient is caused by the greater absorbance of the organic solvent and not by the ion-pairing reagent. Therefore, in reference to the spectral behaviour, alkylsulphonic acids seem to be ideal for highly sensitive detection using solvent gradient systems.

Perfluorinated carboxylic acids

The great advantages of perfluorinated carboxylic acids over other ion-pairing reagents are their strong resolving power for proteins, their low UV cut-off, their strong ion-pairing tendency[13,14] and their excellent volatility, which is of great convenience for preparative work. Salt-free protein can thus be easily recovered by lyophilization without further steps of purification which may involve considerable losses of material. In addition, they are strong organic acids due to the inductive effect of the halogen atoms. This makes them highly favourable for low pH separations that cannot be achieved with underivatized carboxylic acids because of their strong absorbance below 225 nm. Due to these convenient properties, the popularity of perfluorinated carboxylic acids is rapidly increasing and in the following paragraphs the spectra of two commonly used representatives are described.

Trifluoroacetic acid (TFA). The electronic spectra of TFA were discussed in detail in a previous paper[5]. In short, the 200–260 nm region is dominated by the relatively weak $n \rightarrow \pi^*$ transition of the undissociated molecule in acetonitrile, with a maximum at 216 nm, and the strong $\pi \rightarrow \pi^*$ band of the anion in aqueous solution. The transition between these two spectra proceeds discontinuously (see also Fig. 2) due to the strong dependency of the one-step dissociation equilibrium on the solvent polarity. Up to 60% acetonitrile there is a slight increase of absorbance with increasing acetonitrile concentrations, but at higher concentrations of organic modifier the influence of the free acid becomes more prominent. This is clearly reflected by the slope of the baseline which is ascending at higher wavelengths (Fig. 1, 225 and 220 nm) but largely descending at lower wavelengths (Fig. 1, 210 and 205 nm). However, the spectra meet closely at 215 nm and the comparison of the baselines in Fig. 1
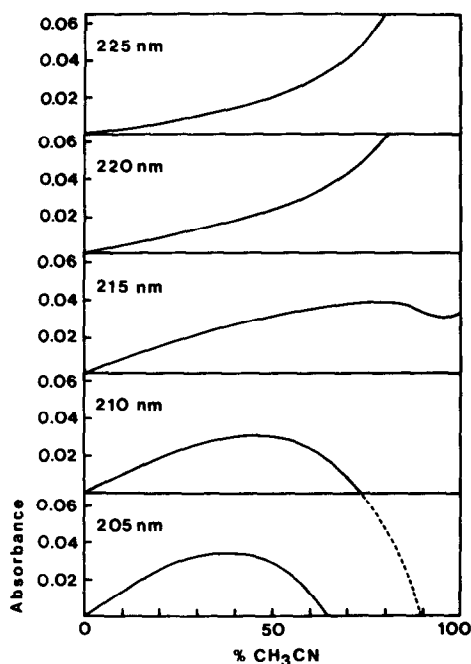
Fig. 1. Baselines of a linear solvent gradient using 0.01 M aqueous TFA *versus* 0.01 M TFA in acetonitrile at different wavelengths. The curves were constructed using point by point measurement of the absorbance of solvent mixtures with increasing contents of acetonitrile and careful interpolation.

demonstrates that the best result is achieved at this wavelength when the whole range between 0 and 90% or even 100% acetonitrile is exploited. At lower concentrations of acetonitrile, the influence of the undissociated TFA is small and therefore limited gradients usually show flatter baselines at higher wavelengths, but the absorbance strength of the peptide is rapidly decreasing above 215 nm and the higher detection sensitivity achieved is then largely ineffective.

Heptafluorobutyric acid (HFB). In principle, all aspects that were discussed in connection with TFA are valid also for HFB. However, some minor differences are caused by the increased number of carbon and fluorine atoms. The maximum of the $n \rightarrow \pi^*$ band shows a bathochromic shift and is now located at 219.2 nm (Fig. 2). At the same wavelength, the spectra meet closely and, again, this is the optimum point to record extended acetonitrile gradients (Fig. 3). In general, the absorbance of HFB is higher than that of TFA and the differences within a gradient are more prominent, which make HFB less suitable for highly sensitive detection.

Although we have shown that an optimum wavelength exists for each of the perfluorinated carboxylic acids, which allows sensitive detection of gradient eluted proteins, the impairment of detection sensitivity caused by the baseline shift is an inherent and serious problem. Some authors therefore propose the use of unequivalent concentrations of the ion-pairing reagent in the aqueous and organic solvents, or the modification of the aqueous solution by the addition of organic acids, which show higher absorbance than the ion-pairing reagent[15]. This method of gradient flattening may be helpful if it does not impair the quality of the separation by un-
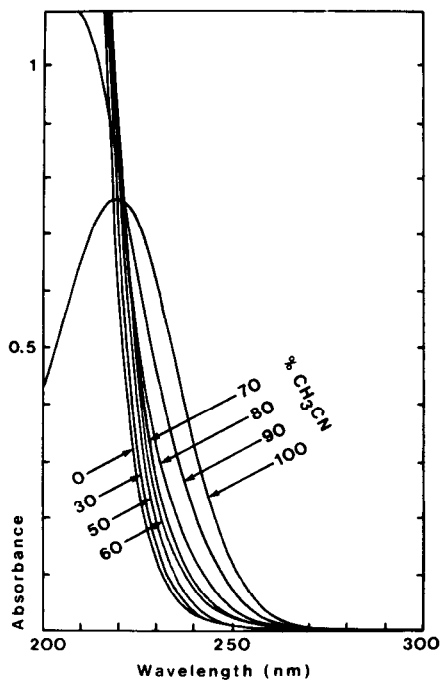
Fig. 2. Electronic spectra of 0.01 M HFB in different mixtures of water and acetonitrile. The distribution of the spectra is the result of a transition between the strong $\pi \rightarrow \pi^*$ band of the anion (0% acetonitrile) with a maximum below 200 nm and the weak $n \rightarrow \pi^*$ band of the undissociated molecule (100% acetonitrile) with a maximum at 219.2 nm.
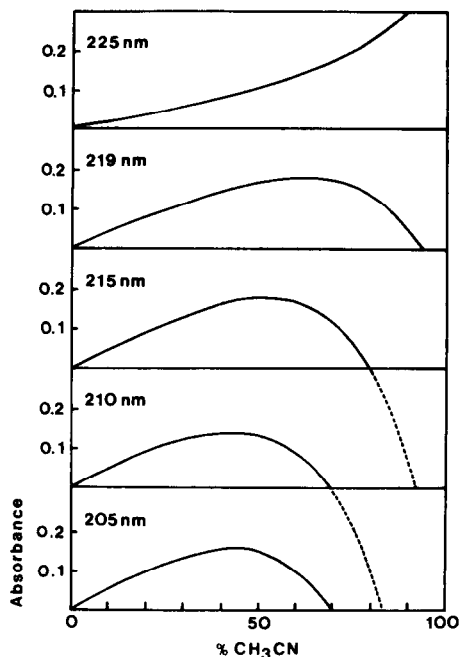
Fig. 3. Baseline of a linear solvent gradient using 0.01 M aqueous HFB versus 0.01 M HFB in acetonitrile at different wavelengths (see also legend to Fig. 1).

balanced equilibrium of ion-pairing and could best be applied to the optimal wavelength (*cf.* Figs. 1 and 3). However, since the dissociation equilibrium of TFA is not affected by different concentrations of ion-pairing reagents, the descent of the curve cannot be eliminated at wavelengths below the optimum.

Alkylamines

Hydrophobic cations such as tertiary alkylamines are used as counter-ions for carboxylic groups of peptides[16-18]. Depending on the composition of the mobile phase and the type of additive, the nature of interaction can range from exclusive ion-pair formation over mixed modes to dynamic ion-exchange. Cationic ion-pairing therefore allows a good control of the retention behaviour of a peptide on the reversed-phase HPLC column. The use of the free base, however, is often impossible, because of the high pH which would damage alkyl-modified silica. As an alternative, one may use a polymer support[12] that is resistant to basic solutes or reduce the pH by the addition of an acid. In our experiments, we have adjusted the pH with *o*-phosphoric acid because if shows little absorbance compared with the alkylammonium ions used. In addition, this background was blanked out by measuring the absorbance of the ion-pairing reagent against an equivalent amount of phosphoric acid. The spectra

recorded at different pH values can thus be directly compared and lines connecting points of identical wavelengths reflect the situation found during real gradient performance.

Triethylamine. The spectra of triethylamine[19] (0.01 M) in water and triethylamine (0.01 M) in a mixture of 90% acetonitrile and 10% water and several intermediates are shown in Fig. 4a. The great differences in absorbance, which are due to different stages of protonation, do not allow sensitive detection when using acetonitrile gradients. At a lower pH, however, a different situation is observed. The greater ionization of the molecule leads to a drastic reduction of the absorbance in the aqueous solvent. Nevertheless, at pH 7.2 (Fig. 4b) the formation of the uncharged molecule is enhanced when acetonitrile is added. This results again in a considerable baseline drift. At pH 2 (Fig. 4c) this effect is then supressed by almost complete ionization, even in the presence of 90% organic solvent. Unfortunately, a low pH is not suitable for cationic ion-pairing because of the low concentration of dissociated carboxylic groups. Even at pH 4, a peptide would be predominantly protonated and thus show minimal interaction with the ammonium ions. For this reason it is necessary to balance the pH exactly in order to achieve good ion-pairing and recording conditions, which allow highly sensitive detection. It is possible to improve the detectability of some amino acids, *e.g.* tyrosine or cystein, by optimal setting of the
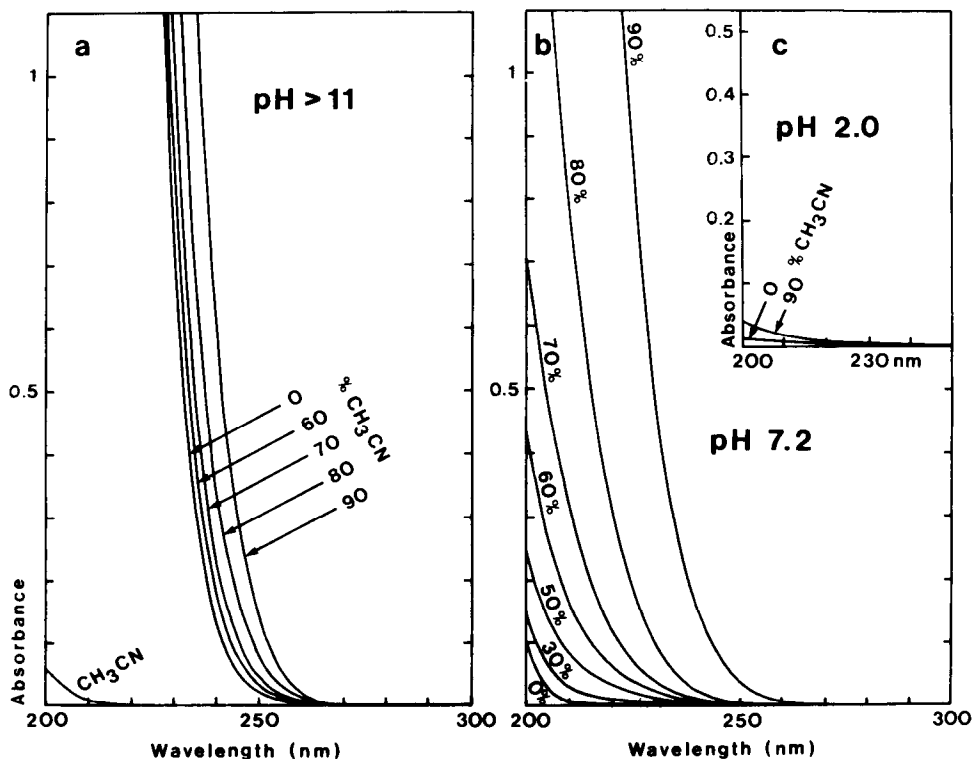

Fig. 4. Electronic spectra of 0.01 M triethylamine in different mixtures of water and acetonitrile at (a) pH > 11, (b) pH 7.2 and (c) pH 2.0. The pH was adjusted with *o*-phosphoric acid and an aqueous solution with the same concentration was used as a blank in order to eliminate the interfering absorbance.
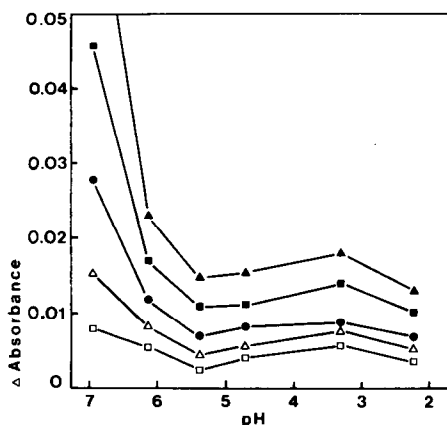
Fig. 5. Numerical differences of the absorbances of 0.01 M triethylamine in water and 0.01 M triethylamine in 50% acetonitrile determined at different pH values. The solutions were easily prepared by titrating 0.02 M aqueous triethylamine with o-phosphoric acid and diluting 1:1 either with water or with acetonitrile. The curves offer a good estimation for the expected baseline drift of a limited gradient (max. 50% ace-tonitrile) at a certain pH and thus allow the determination of optimal conditions for highly sensitive detection. ($\blacktriangle$) 205 nm; ($\blacksquare$) 210 nm; ($\bullet$) 215 nm; ($\triangle$) 220 nm; ($\square$) 225 nm.

pH[20,21], but the most effective and universally applicable way of enhancing the de-tection sensitivity is to reduce the baseline drift. A fast and convenient method of estimating the expected baseline drift at a certain pH is the establishment of 50% acetonitrile titration curves (Fig. 5). In the case of triethylamine, a solution with a pH between 5 and 5.5 is obviously the optimum for highly sensitive detection of a linear solvent gradient.
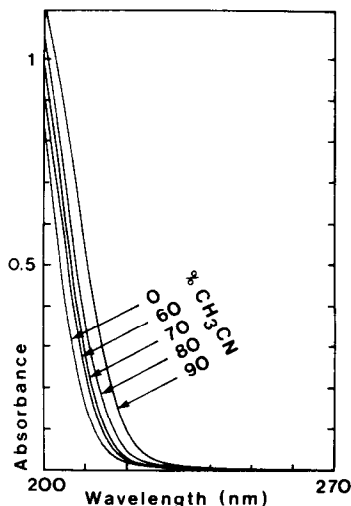

Fig. 6. Electronic spectra of 0.01 M tetrabutylammonium phosphate in different mixtures of water and acetonitrile.
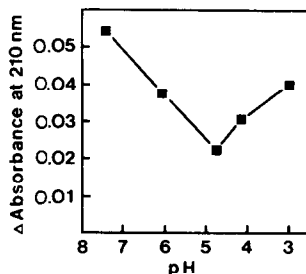
Fig. 7. 50% Acetonitrile titration curve of 0.01 M tetrabutylammonium phosphate performed as described in the legend to Fig. 5. Optimal recording conditions for a limited gradient are achieved at approximately pH 5.

Tetraalkylammonium salts

Quarternary ammonium ions are also used as cationic ion-pairing re-agents[2,3,12], but unlike amines the positive charge is obligatory and thus they are relatively strong Lewis acids. The addition of an organic solvent and the reduction of the pH is therefore not expected to have the same drastic effect as that observed with alkylamines.

Tetrabutylammonium phosphate. A 0.01 M aqueous solution has a pH of 7.4 and the spectra of several mixtures with acetonitrile at that pH are shown in Fig. 6. The differences in absorbance are rather small compared with triethylamine and a relatively sensitive detection using acetonitrile gradients can be achieved. Neverthe-less, a further improvement is possible by acidifying with *o*-phosphoric acid. Again, the 50% acetonitrile titration curve (Fig. 7) is helpful for estimating the expected baseline drift. Fig. 7 shows that a minimum of absorbance exists at approximately pH 5, which is a convenient pH because it allows good ion-pairing with the carboxylic group without affecting the stationary phase.

ACKNOWLEDGEMENTS

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