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Indirect UV detection of hydrophilic ionized compounds in reversed-phase liquid chromatography by use of a UV-absorbing ion of the same charge*

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Abstract: Ion-pair reversed-phase liquid chromatographic systems have been developed that allow the detection and quantitation of low amounts of hydrophilic ionized substances, which otherwise display little or no capability for optical detection. The mobile phase contains a UV-absorbing ion of the same charge and hydrophobic character as the samples, most often together with a hydrophobic counter-ion to give the samples suitable retention; the stationary phase consists of octadecylsilica. Chromatograms featuring two system peaks are usually obtained, and these give rise to some deviations from the usual response pattern.

In the present work the influence on retention and detection sensitivity of the non-absorbing counter-ion, the UV-absorbing ion and the uncharged organic modifier have been studied systematically. Principles for optimizing the detector response by changing the mobile phase composition have been deduced. Examples of applications of the indirect detection technique to the analysis of hydrophilic compounds of pharmaceutical and biological interest, such as amino acids, water-soluble vitamins and piperazine, are presented.

Keywords: *Indirect UV detection; reversed-phase ion-pair liquid chromatography; UV-absorbing ions in the mobile phase; amino acids; water-soluble vitamins; piperazine.*

Introduction

The indirect UV-detection technique has been used increasingly during the past few years in high-performance liquid chromatography and it has been shown to be a very simple and general way of improving the possibilities for optical detection. The principle can be exploited in various kinds of liquid chromatographic system, provided that the

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stationary phase is a solid with limited binding capacity and the mobile phase contains at least one optically detectable component. This latter component in the mobile phase is distributed to a certain extent onto the solid phase. When equilibrium is reached, the mobile phase gives a constant and rather high background absorption signal. After injection of the sample, the distribution of the detectable compound is disturbed in the starting zone and this gives rise to changes in its concentration in the eluent. Deviations from the constant background signal are then detected in the chromatogram as positive or negative peaks.

Two kinds of peak are normally observed. Some peaks have capacity ratios (k') which correspond to those of sample components (sample peaks), whose area is proportional to the amount of the given component in the sample; these peaks are therefore suitable for quantitative determinations. A second type of peak, of which there may be one or two (the system peak), is characteristic of the chromatographic system itself. Although the system peak's retention is constant, its area and polarity can vary considerably as it is affected not only by sample components, but also by mobile phase components, when the latter are present in the sample at higher or lower concentrations than in the eluent. In principle, each mobile phase component can give rise to a system peak, but often the chromatographic conditions are chosen so that only one system peak is retained and easily observed; that is the one which is given by the optically detectable component itself.

In liquid–solid systems with a detectable component in the eluent, it is thus possible to detect and quantify substances which otherwise display little or no capability for optical detection. Most applications of the principle have been developed so far with UV-absorbance detectors, in ion-pair reversed-phase systems with hydrophobic bonded silica as the stationary phase [1–12]. It has been shown that in these systems, UV-absorbing ions could be used not only for the indirect detection of both cationic and anionic compounds, but also for uncharged substances [2, 3, 13, 14].

Recently the technique has been utilized in normal-phase systems where the organic mobile phase contained the UV-absorbing ion as ion pairs and the stationary phase consisted of a polar, chemically-modified silica [15, 16]. Moreover eluents with UV-absorbing ions have also been used in ion-exchange chromatography, mainly for the visualization of inorganic anions and cations [17–20].

Chromatograms with sample and system peaks have also been obtained in cases where there were no ionic components in the mobile phase [21–22]. The applicability of the indirect detection technique when the UV-absorbing component of the mobile phase is non-ionic has been recently demonstrated: compounds such as aliphatic alcohols and carbohydrates have been detected photometrically in both normal-phase and reversed-phase systems ([5]; P. Herné, M. Renson and J. Crommen, to be published). The technique can be applied not only in different chromatographic systems but also with types of detection other than UV photometry, such as, for example, fluorimetry [2] or refractometry [21–22].

This paper describes ion-pair reversed-phase systems suitable for the indirect UV detection and quantitation with high sensitivity of non-absorptive hydrophilic compounds. Studies on the relationship between the nature and concentration of the different mobile phase components and the detector response are presented. Principles for the optimization of detection sensitivity by changing mobile phase composition are given. Some examples of applications in pharmaceutical and biomedical analysis are described.

Experimental

Apparatus

The equipment for high-performance liquid chromatography consisted of an Altex model 110 A pump, an Altex model 210 injector with a 20-μl loop and a Pye-Unicam model 4020 variable-wavelength UV-absorbance detector with an 8-μl cell. The columns (125 × 4.0 mm i.d.) were of stainless steel with a polished inner surface. They were thermostatted in a Heto model 02 PT 923 TC water-bath (Birkeröd, Denmark).

Chemicals and reagents

Nicotinamide and L-tryptophan were obtained from E. Merck (Darmstadt, FRG). L-Dopa (3,4-dihydroxyphenylalanine) was from BDH (Poole, UK). Sodium thymolsulphonate was supplied by Prophac (Evreux, France).

Sodium salts of *n*-octyl sulphate and 1-octanesulphonate were from E. Merck while those of 1-pentane and 1-hexanesulphonate were from Chrompack (Middelburg, The Netherlands). Methanol, orthophosphoric acid and sodium phosphate were of Pro Analysi quality from E. Merck. The L- or D,L-amino acids were obtained from BDH. The pharmaceuticals used as samples were of pharmacopoeial grade. All other substances were of analytical reagent grade.

Chromatographic systems

Nucleosil C_{18} (5 μm) from Macherey-Nagel (Düren, FRG) and LiChrospher 100 CH-18 (5 μm) from E. Merck were used as solid stationary phases.

The mobile phases were aqueous buffered solutions of a UV-absorbing ionized compound with the same charge as the samples. In most cases a hydrophobic ion of opposite charge was added to the eluent; in some cases some methanol was added.

Chromatographic technique

The columns and the connecting tubes preceding the column were thermostatted at 35.0 ± 0.1°C by immersion in a water-bath. The flow rate was 0.5 ml/min. Equilibrium was usually reached after passage of 20–30 ml of eluent.

The samples were dissolved in the mobile phase prior to injection, unless otherwise stated. The hold-up volume of the column, V_m, used to calculate the capacity ratios, was obtained from the leading peak in the chromatogram. Peak areas were determined by triangulation.

Results and Discussion

Detector response with a non-absorbing hydrophobic counter-ion in the eluent

Hydrophilic ionized compounds can be retained in reversed-phase chromatographic systems if a hydrophobic ion of opposite charge (counter-ion) is added to the mobile phase. Under these conditions the samples are distributed onto the solid phase as ion pairs with the hydrophobic counter-ion; retention can be regulated by changing the nature and/or the concentration of this counter-ion [23–25].

In order to be able to apply the indirect detection technique to ionic samples, a UV-absorbing ion has to be included in the mobile phase. In principle, this ion can be either of the same or of the opposite charge with respect to that of the samples [1–6]. However, as a hydrophobic counter-ion is required to give these compounds a suitable retention, it is often more convenient to choose a UV-absorbing ion of the same charge.

Indeed, if the hydrophobic counter-ion were the UV-absorbing component of the mobile phase, the possibilities for regulating retention by the counter-ion concentration would be limited by the requirement that the background absorbance of the eluent cannot be much higher than one absorbance unit [1, 3, 5]. Moreover, the large difference in hydrophobicity between the counter-ion and the samples might have a detrimental effect on the detector response [5]. On the other hand, the use of two different counter-ions, one chosen to be non-absorbing and hydrophobic for increasing retention, and the other being UV-absorbing and more hydrophilic for detection, would certainly lead to a system with low sensitivity, due to the very low distribution of the UV-absorbing ion onto the solid phase [2, 3].

However, when the mobile phase contains a hydrophobic counter-ion with no absorbing properties, the addition of a UV-absorbing ion of the same charge as the analyte has been found to give rise to two retained system peaks: one is given by the detectable ion (S_1), the other by the non-detectable counter-ion (S_2). The other mobile phase components, being much more hydrophilic, did not usually yield detectable system peaks. Under these conditions, the response pattern has been shown to be dependent on the retention order of the two system peaks.

For example, Fig. 1 shows a chromatogram obtained at 254 nm with hydrophilic amino acids as samples. At the pH used (1.8) the amino acids were mainly present in the mobile

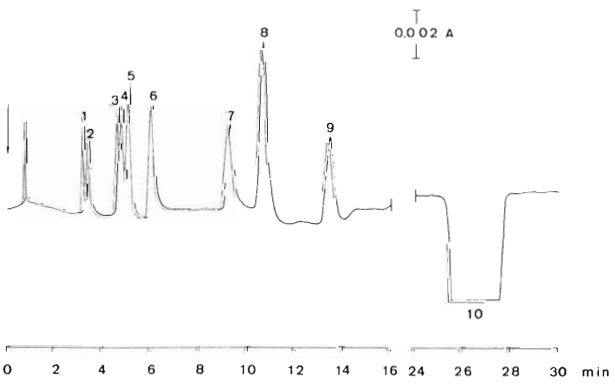

Figure 1
Indirect UV detection of hydrophilic amino acids. Mobile phase: $2 \cdot 10^{-4}$M nicotinamide and 10^{-3}M sodium *n*-octyl sulphate in 0.05 M phosphoric acid. Solid phase, Nucleosil C_{18}; detection wavelength, 254 nm. Key to samples (0.5–1.5 μg): 1, glycine; 2, aspartic acid; 3, threonine; 4, glutamic acid; 5, alanine; 6, cysteine; 7, proline; 8, α-aminobutyric acid. System peaks: 9, *n*-octyl sulphate; 10, nicotinamide.

phase as cations and were retained on the solid phase as ion pairs with *n*-octyl sulphate as the counter-ion. Nicotinamide, which was also mainly present in cationic form at that pH, was used as the UV-absorbing mobile phase component because it was found to have about the same retention as the most hydrophilic amino acids. It has been demonstrated in previous papers [1–6] that maximum sensitivity is obtained in the indirect detection method when sample retention relative to S_1 is close to unity.

As can be seen in Fig. 1, positive peaks are given by amino acids less retained than S_1, which is in agreement with the usual response pattern observed when the samples and the UV-absorbing component are of the same charge [1–6]. The strongly negative S_1 peak is attributable to the fact that the amino acids were dissolved in water prior to injection.

In principle samples more strongly retained than S_1 should give negative peaks, but in cases where two system peaks are observed, this response pattern is only obtained when S_2 is more strongly retained than S_1. In order to detect the maximum number of samples, it is thus advisable to modify the mobile phase composition in such a way that S_2 has the highest possible retention relative to S_1.

In practice, with *n*-octyl sulphate as counter-ion, the addition of methanol had a favourable effect, as the changes in the capacity ratio of S_2 were much less pronounced than those of S_1. With methanol concentrations of 5% v/v and higher, the elution order of S_1 and S_2 was reversed, S_2 being then the most strongly retained system peak. A capacity ratio higher for S_2 than for S_1 was also obtained by replacing the alkyl sulphate by aliphatic sulphonates, even in the absence of methanol. These compounds thus seem to be more suitable as counter-ions for the analysis of hydrophilic cations by the indirect detection technique.

Influence of the nature of the non-absorbing counter-ion

Figure 2 illustrates the effect of the alkyl chain length of the non-absorptive alkanesulphonate used as counter-ion, on the retention of samples and system peaks. As can be expected in ion-pair reversed-phase systems, the capacity ratios (k') of the cations used as samples and of the cationic UV-absorbing component increase with increasing number of alkyl carbons in the counter-ion (N_C).

Most amino acids tested as samples gave about the same slope except the basic amino acids, such as arginine. The latter is a divalent cation at the pH used and its slope was found to be much higher. The two system peaks had somewhat lower slopes than those of the monovalent cations. In the case of nicotinamide, this is probably due to incomplete ionization of the heterocyclic nitrogen at the pH of the eluent.

For monovalent amino acids less strongly retained than S_1, an increase of the chain

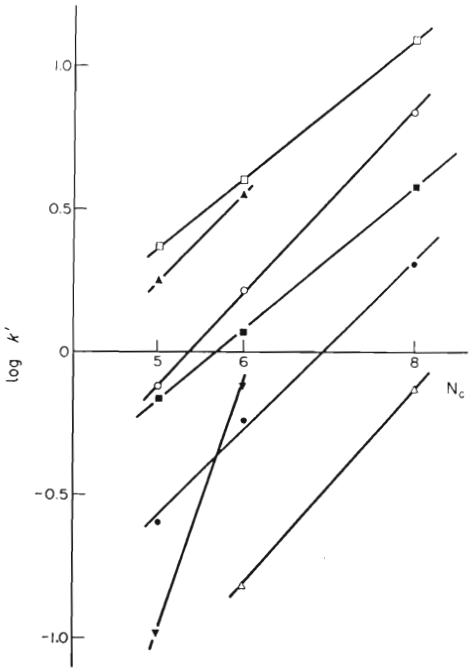

Figure 2
Dependence of retention on the alkyl chain length of the non-absorbing counter-ion. Mobile phase: $2.5 \cdot 10^{-4}$M nicotinamide and 10^{-3}M sodium alkanesulphonate in 0.05 M phosphoric acid. Solid phase, Nucleosil C_{18}; detection wavelength, 268 nm. Key to samples: △ Glycine; ● Proline; ○ Methionine; ▲ Leucine; ▼ Arginine. System peaks: ■ Nicotinamide; □ Alkanesulphonate. N_C is the number of alkyl carbons in the alkanesulphonate counter-ion.

length had a favourable effect on the detector response, as their retention relative to S_1 ($\alpha_S = k'_{sample}/k'_{system\ peak\ S_1}$) was increased (cf. Table 1). The retention of the divalent arginine was so strongly affected by the nature of the counter-ion that quite different responses were obtained with the three sulphonates used. The detection sensitivity was much higher with hexanesulphonate than with pentanesulphonate, due to the fact that α_S was much closer to unity. With octanesulphonate, however, arginine could no longer be detected, as its capacity ratio was then higher than that for S_2. For the same reason, no response was obtained for leucine in the octanesulphonate system.

Table 1
Influence of relative retention and the nature of the non-absorbing counter-ion on detection sensitivity*

	Number of alkyl carbons in the alkanesulphonate counter-ion					
	5		6		8	
Compounds	Log (ϵ^*)†	Log (α_S)‡	Log (ϵ^*)	Log (α_S)	Log (ϵ^*)	Log (α_S
Glutamic acid	—	—	1.19	−0.59	1.57	−0.51
Cysteine	0.96	−0.85	1.31	−0.52	1.68	−0.45
Proline	1.80	−0.44	2.03	−0.38	2.34	−0.27
Methionine	2.23	0.04	2.43	0.15	2.70	0.26

* For chromatographic conditions, cf. Fig. 2.
† ϵ^* = 'apparent molar absorptivity' in the chromatographic system, defined as the quotient between the sample peak area (AU-l) and the amount of analyte injected (mole); ϵ^* is the peak area per mole of analyte.
‡ $\alpha_S = k'_{sample}/k_{system\ peak\ S_1}$; α_S is the relative retention of an analyte peak with respect to the system peak S_1 for the optically-absorbing ion.

Table 1 illustrates the relationship between detection sensitivity and relative retention in systems with different aliphatic sulphonates as counter-ions. The sensitivity for a given sample can be expressed quantitatively by its 'apparent molar absorptivity', ϵ^*, in the particular chromatographic system. This is the quotient between the sample peak area (in absorbance unit-litres) and the amount of compound injected in moles [2, 3, 16], i.e. the sample peak area per mole.

As mentioned above, the gain in sensitivity with increase in the hydrophobic character of the counter-ion can be related to the concomitant increase in α_S, as far as samples with capacity ratios lower than that of S_1 are concerned. However, for methionine, which was eluted between S_1 and S_2, a decrease in ϵ^* with increasing α_S would normally have been expected, as α_S values were higher than unity in this case [1–6]. However, an increase in sensitivity was observed, which seems to indicate that the improvement of detection sensitivity with increasing number of alkyl carbons in the counter-ion is not entirely due to changes in relative retention.

Influence of the counter-ion concentration

Figure 3 shows the changes in capacity ratios of samples and system peaks observed by altering the concentration of the hydrophobic counter-ion octanesulphonate ($[X^-]_m$) in the mobile phase. The retention of all cations increases with the counter-ion concentration; the shape of the curves obtained are similar to those usually observed in ion-pair reversed-phase systems with an alkylbonded solid phase [23, 25].

The main difference with reference to the results in Fig. 2 is that the capacity ratio of S_2 *decreases* with increasing counter-ion concentration. This means that in this case, the

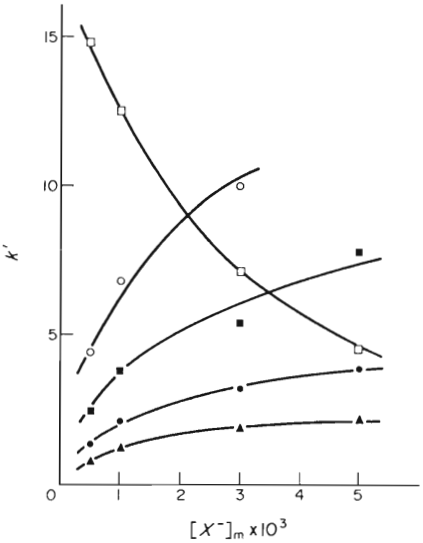

Figure 3
Dependence of retention on counter-ion concentration. Mobile phase: $2.5 \cdot 10^{-4}$M nicotinamide and $0.5–5 \cdot 10^{-3}$M sodium 1-octanesulphonate in 0.05 M phosphoric acid. Solid phase, Nucleosil C_{18}; detection wavelength, 268 nm. Key to samples: ▲ Glutamic acid; ● Proline; ○ Methionine. System peaks: ■ Nicotinamide; □ Octanesulphonate. $[X^-]_m$ = molar concentration of octanesulphonate counter-ion in mobile phase.

retention of the two system peaks changes in opposite directions; indeed, the elution order of S_1 and S_2 is reversed at higher octanesulphonate concentrations. Consequently, only the most hydrophilic samples can still be detected at higher counter-ion concentrations. For example, methionine gave a very low response with an octanesulphonate concentration of $3 \cdot 10^{-3}$M and was completely undetectable at a concentration of $5 \cdot 10^{-3}$M. This observation limits considerably the applicability of eluent systems with high concentrations of this counter-ion.

As can be seen from data in Table 2, the relative retention (α_S) values are almost unaffected by changes in counter-ion concentration. Nevertheless the ϵ^* values have a tendency to increase with increasing counter-ion concentration, provided that sample retention remains lower than that of S_2. This increase in detection sensitivity with both the concentration and hydrophobicity of the counter-ion (cf. Tables 1 and 2) might be related to the fact that the loading of the UV-absorbing cation nicotinamide on the solid phase also increases under these conditions.

Influence of the UV-absorbing ion concentration

The effect on retention caused by an increase of nicotinamide concentration ($[Q^+_{UV}]_m$) in the mobile phase was in accordance with the general rules for ion-pair reversed-phase chromatography [23–25]. The capacity ratios of cationic samples and of nicotinamide itself (S_1) were reduced due to a competitive effect, while the retention of the counter-ion octanesulphonate (S_2) was increased. Again changes in k' occurred in opposite directions for the two system peaks, but this time the effect was favourable with respect to detector response since the difference in k' between S_1 and S_2 increased with nicotinamide concentration. However, retention changes were rather limited and α_S values remained fairly constant.

The relationship between detection sensitivity and UV-absorbing ion concentration is illustrated in Fig. 4. The ϵ^* values were determined at 268 nm in the lower range of nicotinamide concentration ($1–2.5 \cdot 10^{-4}$M) and at 279 nm in the higher concentration range ($0.5–5 \cdot 10^{-3}$M) in order to avoid background absorbances higher than 1.3 unit in the mobile phase.

Table 2
Influence of counter-ion concentration on detection sensitivity*

	Concentration of 1-octanesulphonate (mM)							
	0.5		1		3		5	
Compounds	Log (ϵ^*)†	Log (α_S)‡	Log (ϵ^*)	Log (α_S)	Log (ϵ^*)	Log (α_S)	Log (ϵ^*)	Log (α_S)
Glycine	0.90	−0.74	0.92	−0.72	—	—	0.99	−0.74
Proline	2.30	−0.28	2.34	−0.27	2.50	−0.22	2.70	−0.30
Methionine	2.52	0.26	2.70	0.26	(a)	—	(a)	—

* For chromatographic conditions, cf. Fig. 3.
† ϵ^* = 'apparent molar absorptivity' (see Table 1 and text).
‡ α_S = relative retention of analyte with respect to the system peak S_1 (see Table 1 and text).
(a) no response.

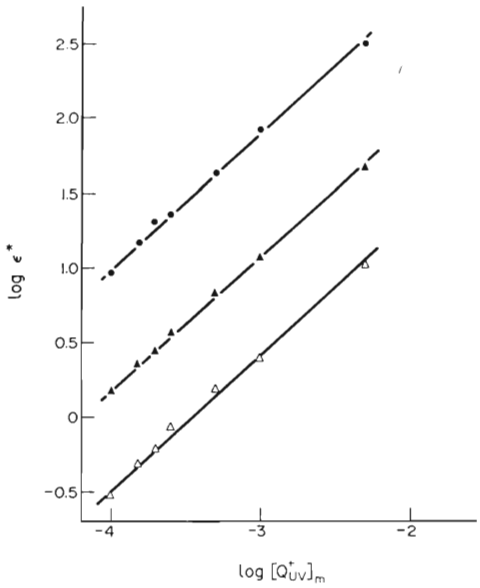

Figure 4
Dependence of detection sensitivity on UV-absorbing ion concentration. Mobile phase: $1–50 \cdot 10^{-4}$M nicotinamide and 10^{-3}M sodium l-octanesulphonate in 0.05 M phosphoric acid. Solid phase: Nucleosil C_{18}; detection wavelengths, 279 nm ($5 \cdot 10^{-4}–5 \cdot 10^{-3}$M nicotinamide) and 268 nm ($1–2.5 \cdot 10^{-4}$M nicotinamide). Key to samples: △ Glycine; ▲ Glutamic acid; ● Proline. $[Q^+_{UV}]_m$ = molar concentration of nicotinamide in mobile phase.

The ratio of the apparent molar absorptivities obtained at 268 and 279 nm respectively ($\epsilon^*_{268}/\epsilon^*_{279}$) was calculated, using a mobile phase with a nicotinamide concentration of $2.5 \cdot 10^{-4}$M and various amino acids as samples. The ratio had a fairly constant value of 10.3 and was used to estimate ϵ^*_{279} values in the lower range of nicotinamide concentration, as the response at 279 nm was generally too low in that concentration range to enable precise peak area measurements.

As can be seen from Fig. 4, there is a considerable improvement in sensitivity when the concentration of the UV-absorbing compound (nicotinamide) is increased. The logarithmic plots of ϵ^* versus $|Q^+_{UV}|_m$ gave similar slopes for all amino acids tested. These slopes are not much lower than unity (around 0.9), which means that any change of the nicotinamide concentration in the eluent will give rise to an almost equivalent change in detection sensitivity, at least in the concentration range studied. This is another indication that the magnitude of the detector response at a given wavelength is dependent on the amount of detectable component adsorbed onto the solid phase.

Preliminary determinations of adsorption isotherms for nicotinamide in systems with octanesulphonate as counter-ion and octadecylsilica as the solid phase seem to confirm that the concentration of the UV-absorbing mobile phase component on the solid phase may well be a parameter of prime importance for optimizing sensitivity when using the indirect detection technique (J. Crommen and P. Herné, in preparation). However, further experimental studies should be performed with other kinds of chromatographic systems before firm conclusions about the role of this parameter can be drawn.

From a practical point of view, the use of higher UV-absorbing ion concentrations in the mobile phase for improving sensitivity seems to be of little interest, as the background absorbance of the mobile phase can only be increased to a limited extent. Still, by using a variable-wavelength detector, wavelengths other than that which corresponds to the maximum absorptivity can be selected, which may result in an increase in specificity of detection. The loss in sensitivity due to the use of a non-optimal wavelength can then be compensated for by increasing the concentration of the UV-

absorbing compound. Following the same principle, substances with rather poor chromophoric properties could be used successfully as UV-absorbing mobile phase components if they were present in sufficiently high concentration.

Influence of methanol concentration

Methanol is often used in reversed-phase chromatography to regulate sample retention. In the systems described above, methanol was found to reduce the capacity ratios of amino acids and system peaks to approximately the same extent, so that only very slight changes in selectivity (α_S) occurred.

As shown in Fig. 5, the addition of methanol to the eluent gave rise to a slight but significant decrease of sensitivity. Similar observations have been made earlier [3, 14]. This effect of methanol might again be related to changes in the loading of the UV-absorbing ion on the solid phase; a decrease in the loading of nicotinamide was obtained by increasing the methanol content of the mobile phase. The decrease in ϵ^* caused by the presence of methanol could eventually be counterbalanced by an increase of the nicotinamide concentration in the eluent.

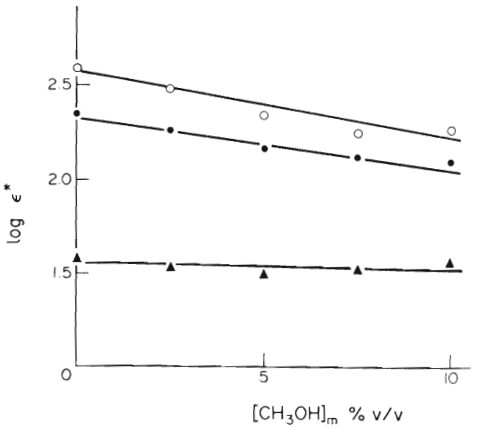

Figure 5
Dependence of detection sensitivity on methanol concentration. Mobile phase: $2.5 \cdot 10^{-4}$M nicotinamide and 10^{-3}M sodium 1-octanesulphonate in 0.05 M phosphoric acid containing 0–10% v/v methanol. Solid phase: Nucleosil C_{18}; detection wavelength: 268 nm. Key to samples: ▲ Glutamic acid; ● Proline; ○ Methionine. $[CH_3OH]_m$ = molar concentration of methanol in mobile phase.

Applications in pharmaceutical analysis

Systems with nicotinamide and octanesulphonate in the mobile phase have been used for the determination at 262 nm or higher wavelengths of the most hydrophilic amino acids in pharmaceutical preparations such as perfusion solutions and coated tablets. For the analysis of basic amino acids, which are present in the eluent as divalent cations, hexanesulphonate was chosen as counter-ion in order to obtain a lower retention than the system peak S_2 (cf. above). The same kind of system has permitted the quantitation of the basic drug piperazine in the nanogram range; this is also a divalent cation at low pH. As can be seen from the chromatogram in Fig. 6, a concentration of hexanesulphonate was selected at which the capacity ratio for piperazine was about the same as that for the system peak S_1.

Another drug, methylmethionine sulphonium iodide or vitamin U, has also been analysed in solutions for injection by means of the indirect detection technique. The concentration of methionine, which is the main degradation product of this substance, also had to be estimated. Octanesulphonate was the preferable counter-ion used in this case, as it increased the retention of the highly polar and divalent methylmethionine

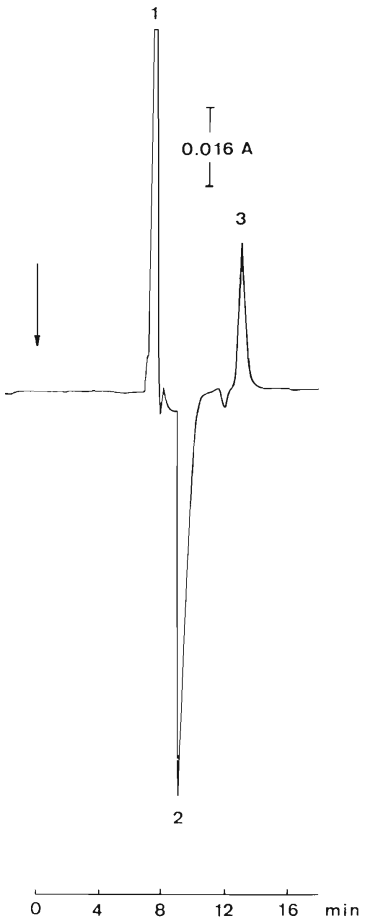

Figure 6
Indirect detection of piperazine. Mobile phase: $2.5 \cdot 10^{-4}$M nicotinamide and $2 \cdot 10^{-3}$M sodium 1-hexanesulphonate in 0.05 M phosphoric acid. Solid phase: Nucleosil C_{18}; detection wavelength: 262 nm. Key to sample: 2. piperazine. System peaks: 1, nicotinamide; 3, hexanesulphonate.

sulphonium more effectively than that of the monovalent methionine. The two compounds could then be determined simultaneously in the isocratic mode as demonstrated in Fig. 7. The concentration of octanesulphonate in the mobile phase was rather low, so that the system peak S_2 had a much higher capacity ratio than the substances to be analysed.

For the most hydrophobic amino acids and for small peptides, suitable retention could be obtained on octadecylsilica without adding a hydrophobic anion to the eluent. These compounds have been detected with high sensitivity by using solutions of a highly UV-absorbing amino acid, such as L-Dopa or tryptophan in 0.05 M phosphoric acid as mobile phases [5, 6].

Chromatographic systems analogous to those described for cations can be developed for the analysis of non-absorbing anionic samples. For example, the water-soluble vitamin biotin (vitamin H), which contains a carboxy group in its molecule, has been separated in a system with a phosphate buffer pH 7.0 containing the UV-absorbing anion thymolsulphonate as eluent, as shown in the typical chromatogram (Fig. 8). As little as 50 ng of biotin could be quantified with good precision in this system.

Very hydrophilic anions can be analysed by indirect detection if a hydrophobic

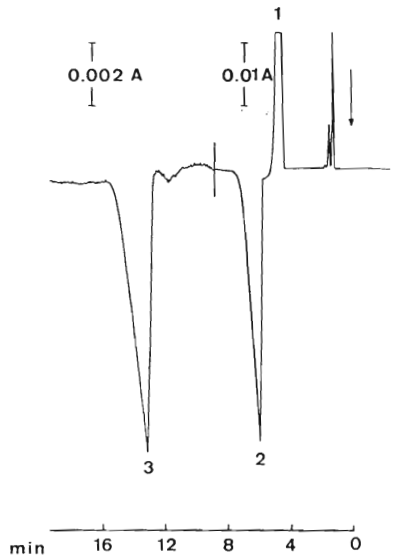

Figure 7
Indirect detection of methylmethioninesulphonium. Mobile phase: $2.5 \cdot 10^{-4}$M nicotinamide and $4 \cdot 10^{-4}$M sodium 1-octanesulphonate in 0.05 M phosphoric acid containing 8% v/v methanol. Solid phase: LiChrospher 100 CH-18; detection wavelength: 273 nm. Key to samples: 2, methylmethionine sulphonium (40 μg); 3, methionine (20 μg). System peak: 1, nicotinamide.

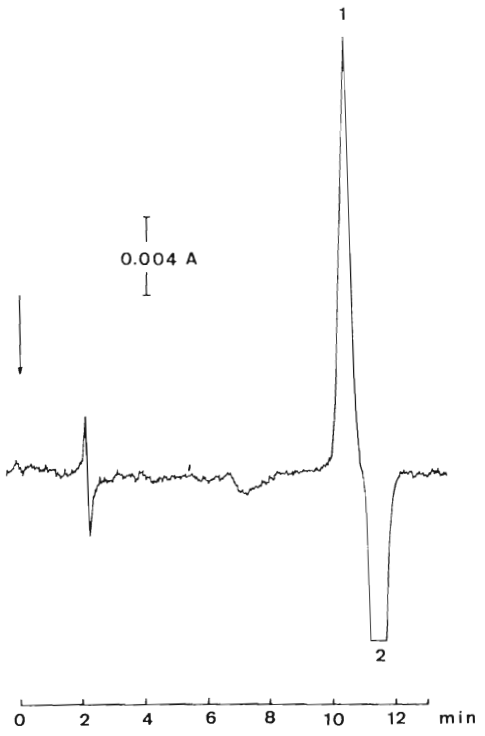

Figure 8
Indirect detection of biotin. Mobile phase: $1.5 \cdot 10^{-4}$M sodium thymolsulphonate in phosphate buffer pH 7.0. Solid phase: Nucleosil C_{18}; detection wavelength: 237 nm. Sample: 1, biotin (2 nmol), system peak: 2, thymolsulphonate.

counter-ion, such as the tetra-*n*-alkylammonium ion, and a UV-absorbing anion of high polarity, such as, for example, sulphanilic acid, are included in the mobile phase, in exactly the same way as for cationic samples.

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