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# Ion-pairing detection technique in reversed-phase high-performance liquid chromatography of drugs and related compounds*

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**Abstract**: The principles and practice of UV-detection methods based on the ion-pairing technique are discussed with respect to reversed-phase high-performance liquid chromatography. The detection of amino acids, aliphatic alcohols and vitamins is described together with the rationale for optimizing sensitivity of detection in using the ion-pairing technique for pharmaceutical analysis.

**Keywords**: *Ion-pairing detection; amino acids; aliphatic alcohols; vitamins; reversed-phase HPLC; UV-detection.*

## Introduction

Detection problems are sometimes encountered in high-performance liquid chromatography because of the lack of universality of sensitive detectors. Different kinds of chemical derivatization techniques (pre- or post-column) have been developed to increase detection possibilities. The ion-pairing technique represents another way of improving detection and its main advantages are simplicity, non-destructiveness and wide scope of application.

This technique was initially applied in liquid–liquid systems with an organic mobile phase [1–5]. In these systems, an aqueous solution of a UV-absorbing ion is adsorbed on a hydrophilic support. A non-absorbing sample of opposite charge elutes in the organic mobile phase as an ion pair with the UV-absorbing ion and can thus be monitored with high sensitivity by a UV-detector. In the case of hydrophilic samples, however, the applicability of these normal-phase systems is rather limited.

Schill *et al.* and several other authors [6–12] have shown that an improvement in detection can also be obtained by ion-pairing in reversed-phase systems with chemically bonded stationary phases, to give the technique a considerably higher flexibility. A UV-absorbing ion is added to the aqueous mobile phase and is adsorbed to a certain extent on the solid phase. The injection of an ionic compound of opposite charge will cause

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local changes in the distribution of the UV-absorbing ion, to give a consequent detector response as the compound elutes from the column, even if it originally lacks UV-absorbing properties.

It has been further demonstrated that the addition of a UV-absorbing ion to the mobile phase also enables the detection of samples of the same charge [6, 7, 13] as well as uncharged samples [7, 14, 15]. In such ion-pair systems, sample injection may thus give rise to a peak, positive or negative, for each sample component. In addition, a 'system peak' with constant retention for a given system is usually observed on the chromatogram.

The height or area of sample peaks can be used for quantitation in the low concentration range, irrespective of the direction of the peaks. The ion-pair chromatographic technique is thus well suited to the determination of drugs and related compounds with little or no detectable properties.

Chromatograms with anomalous peaks have also been obtained in other kinds of liquid–solid systems. In all instances, the mobile phase contains at least one detectable component. Inorganic or organic non-absorbing ions give negative peaks by UV-detection, after separation on an ion-exchange column, when a UV-absorbing ion with the same charge as the samples is included in the mobile phase [16–19].

System peaks similar to those mentioned above have also been observed by using a refractive-index detector in normal-phase [20] or reversed-phase [21] chromatographic systems with no ionic components in the mobile phase. Mixtures of solvents were used as eluents in both cases and other solvents were injected as samples. Recently the author has confirmed these observations by studying reversed-phase systems with aqueous solutions of a UV-absorbing non-ionic compound as mobile phases (P. Herné and J. Crommen, in preparation). On injection of a non-absorbing uncharged sample, a sample peak and a system peak appear on the chromatograms when using UV-detection, exactly in the same way as in ion-pair systems.

**Detection Principles**

Applications of the principle of improving detection by use of a UV-absorbing component in the mobile phase have been mainly performed so far in ion-pair systems and the discussion will therefore be focused on systems of this kind.

The samples give rise to positive or negative peaks according to their charge and retention relative to the UV-absorbing ion in the mobile phase, as can be seen from Table 1 [6, 7]. In these systems, the detectable ion is in general the most hydrophobic component of the aqueous mobile phase, which usually also contains buffer ions and sometimes an organic modifier such as methanol. When the sample and the UV-absorbing ion have opposite charges, the sample peak will be negative if it comes before the system peak, which has the same capacity ratio as the UV-absorbing ion, and positive if it comes after this peak.

The injection of samples with the same charge as the UV-absorbing ion will also give a chromatogram with both sample and system peaks, but the direction of the peaks is reversed: a positive peak is obtained for a sample less retained than the UV-absorbing ion, while a negative peak is given by a sample more retained than this ion. This is illustrated in Fig. 1, which shows the separation of amino acids in cationic form, the detector response at 280 nm being obtained by addition to the eluent of a UV-absorbing cationic compound, 3,4-dihydroxyphenylalanine (DOPA). A response pattern similar to

that given by ions of the same charge is observed in the case of uncharged compounds [7].

When there is only one sample peak, the system peak is always of the opposite direction, which indicates that the changes of the UV-absorbing ion concentration that occur in the sample zone are compensated by the system zone [7]. It should also be noted that in these systems, the sums of the areas of positive and negative peaks are equal, provided that the injected samples are dissolved in the mobile phase. Dissolution of the sample in water does not significantly affect the sample peak, but gives rise to a strongly negative system peak. Some early-eluting additional peaks are also observed [6].

## Influence of the Properties of the Chromatographic System on Detection Sensitivity

The magnitude of the response given by the UV-detector is of course related to the molar absorptivity of the UV-absorbing component of the mobile phase, but it is also strongly dependent on the retention of the sample. An illustration of the effect of sample retention on detection sensitivity is given in Fig. 1: all samples were injected in equivalent amount (0.8 nmol). A rather low response is obtained for samples that elute early, but the sensitivity increases rapidly with increasing capacity ratio. A maximum sensitivity is reached when the sample and the UV-absorbing ion have about the same retention. More strongly retained samples give a lower but fairly constant response [6, 7].

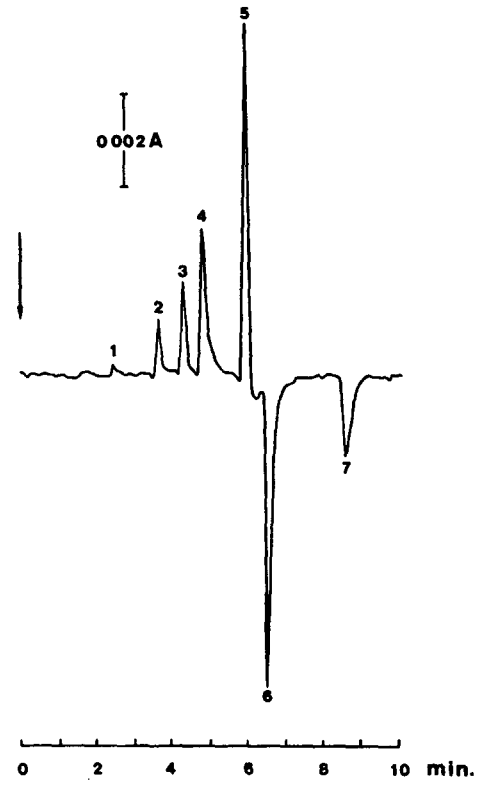


**Figure 1**
Separation of amino acids. Mobile phase: $2 \times 10^{-4}$ M DOPA in 0.05 M phosphoric acid; solid phase: Ultrasphere ODS (5 μm); detection wavelength: 280 nm.
Samples: 1, proline (92 ng); 2, valine (94 ng); 3, methionine (119 ng); 4, ε-aminocaproic acid (105 ng); 5, system peak (DOPA); 6, tranexamic acid (126 ng); 7, leucine (105 ng).

The response of a given sample can thus be optimized by changing the mobile phase composition in such a way that the capacity ratios of the sample and system peak are very close to each other. Under these conditions, the response is often higher than that which would be given by an equivalent amount of the UV-absorbing ion [6].

If the sample and the detectable ion have opposite charges, one can in principle change their retention in different directions by altering the concentration of detectable ion in the mobile phase; this might give rise to an increase in detection sensitivity. However, regulation of retention by the UV-absorbing ion concentration is possible only in a very limited concentration range, as a high background absorbance (more than 1.0 absorbance unit) should be avoided [6].

It is often more convenient to change the nature of the UV-absorbing ion. The best results should normally be obtained with an ion that has about the same hydrophobic character as the sample. In most cases, however, ionic samples will give rise to a fairly high response in a rather broad retention range around the capacity ratio of the system peak, which makes it possible to quantify about 0.1 nmol of non-absorbing compound. A lower response is usually obtained for uncharged samples and in this case the lowest amount that can be detected with good precision is approximately 1 nmol [7].

Detection sensitivity can also be strongly affected by the nature and concentration of non-detectable ions in the mobile phase. It seems that the presence of such ions, which are generally buffer components, is necessary to obtain the normal response pattern given in Table 1, as deviations from this pattern have been observed when the mobile phase only consists of an aqueous solution of a salt of the UV-absorbing ion [6, 7]. In these systems, the highest sensitivity has been obtained when the non-absorbing ions of the mobile phase are rather hydrophilic and not present in too great an excess.

**Table 1**
Response with a hydrophobic UV-absorbing ion (S) in the mobile phase

| | Direction of sample peak | |
|---|---|---|
| Charge of sample (X) | $k'_X < k'_S$ | $k'_X > k_S$ |
| Opposite to S | Negative | Positive |
| Same as S or uncharged | Positive | Negative |

Non-ionic modifiers, such as methanol, are very useful in regulating the retention of both sample and system peak. They should, however, be used with care as detection sensitivity has been found to decrease with increasing methanol content ([16], L. Hackzell and T. Rydberg, to be published). When very hydrophobic samples are to be analysed, it might be preferable to reduce their retention by use of a stationary phase with a lower binding capacity.

In order to detect very low amounts of samples, the baseline noise at the UV-detector should also be kept as low as possible. As the mobile phase absorbance is rather high (0.5–1.0 absorbance unit), this noise depends strongly on the stability of the experimental conditions. The whole chromatographic system should therefore be carefully thermostatted.

**Applications**

The indirect UV-detection of inorganic ions, such as bromide, has been reported by Schill and coworkers [6]. They used as mobile phase a solution of a UV-absorbing quaternary ammonium ion, 1-phenethyl-2-picolinium, in acetic acid. Dreux *et al.* [22] have also analysed non-absorbing inorganic anions by ion-pair reversed-phase chromatography. In this instance, the anions were retained as ion pairs with long-chain alkylammonium ions and the detector response was obtained by adding to the mobile phase a UV-absorbing ion of the same charge, *p*-toluene sulphonate.

Some amino acids and dipeptides have been separated and detected at 254 nm by using naphthalene-2-sulphonate as the UV-absorbing component of the mobile phase [6]. The separations were performed at pH 1.8, i.e. under conditions where the samples were mainly present in the mobile phase in cationic form and distributed to the solid phase as ion pairs with naphthalene-2-sulphonate. The amino acids were eluted before the system peak given by the UV-absorbing anion and gave negative peaks, which is in accordance with the general rules for sample peak direction given in Table 1.

The aromatic amino acids, however, do absorb at the detection wavelength, and it would thus be preferable to choose chromatographic systems where positive peaks are obtained, since an inherent absorbance can decrease the magnitude of a negative peak.

The author has developed ion-pair reversed-phase systems that permit the detection of most amino acids and peptides as positive peaks at 254 nm and higher wavelengths (P. Herné and J. Crommen, in preparation). For the most hydrophobic amino acids and for dipeptides, aqueous acidic solutions of a UV-absorbing amino acid, tryptophan or 3,4-dihydroxyphenylalanine (DOPA), were used as mobile phases and octadecyl-silica as the solid phase.

In these systems, tryptophan is more strongly retained than all other amino acids injected ($k' = 23$) and consequently the detector response obtained for the least retained amino acids is very low. The use of the more hydrophilic DOPA ($k' = 2.7$) enables the determination of nanogram amounts of α- or ω-amino acids, which have a low retention in the system, as shown in Fig. 1. The sample peaks are positive before the system peak (DOPA) and negative thereafter, as can be expected when the samples and the UV-absorbing ion have the same charge (cf. Table 1). The two ω-amino acids, ϵ-aminocaproic acid and tranexamic acid, are drugs with haemostatic properties.

For the analysis of the most hydrophilic amino acids, a hydrophobic counter ion, such as octyl sulphate or hexanesulphonate, was added to the eluent in order to obtain suitable retention. DOPA was too strongly retained under these conditions and a much more hydrophilic cation, nicotinamide, had to be used to improve detection sensitivity. The systems described above have been used for the determination of essential amino acids in perfusion solutions.

Aliphatic alcohols have been detected in the visible range (651 nm) by Freiser *et al.* [14, 15], who used methylene blue as the light-absorbing component of the mobile phase. Schill *et al.* [7] have analysed the same type of compounds with 1-phenethyl-2-picolinium as the UV-absorbing ion. As mentioned above (cf. Table 1), these uncharged samples give a response pattern similar to that obtained with ions of the same charge, but the magnitude of the response is lower.

The author has found that alkanols and alkanediols could also be detected by addition to the mobile phase of a UV-absorbing uncharged compound, such as salicylamide or nicotinamide. An example of such a separation is given in Fig. 2, which shows that the

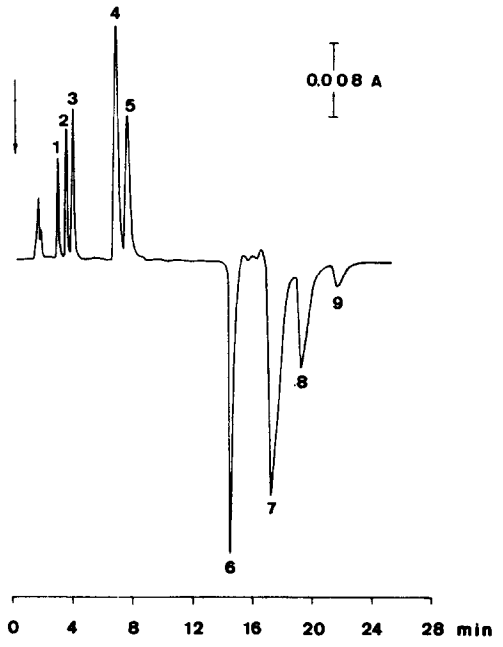


**Figure 2**
Separation of aliphatic alcohols. Mobile phase: $4 \times 10^{-4}$ M nicotinamide in water; solid phase: Ultrasphere ODS (5 μm); detection wavelength: 268 nm. Samples (10–20 μg): 1, methanol; 2, propylene glycol; 3, ethanol; 4, isopropanol; 5, 1-propanol; 6, system peak (nicotinamide); 7, 2-butanol; 8, isobutanol; 9, 1-butanol.

direction of the peaks is the same as for ionic compounds with the same charge. The detection sensitivity seems to be of the same order as that obtained for alcohols in ion-pair systems. The possibilities of using systems of this kind for the detection of carbohydrates are being investigated.

There are two water-soluble vitamins which have weakly absorptive properties, pantothenic acid and dexpanthenol. Various systems containing a UV-absorbing component in the mobile phase have been examined in order to improve the detection of these substances. The best results have been obtained with a mobile phase containing *p*-aminobenzoic acid in phosphate buffer (pH 4) with a small percentage of methanol. Under these conditions, pantothenic acid and *p*-aminobenzoic acid are present as uncharged species.

The ion-pairing method is applicable to the detection of drugs and related compounds by UV-photometry, fluorimetry and refractive index measurement. The method can be used for charged and uncharged species and has been demonstrated as a useful detection method for those compounds which have little or no useful UV-absorption. It can therefore be seen that the ion-pairing technique has considerable scope for exploitation as a detection method in the separation of drugs and compounds of clinical interest by HPLC.

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