

유기 및 무기음이온에 대한 이온쌍크로마토그래피

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Ion-Pair Chromatography of Organic and Inorganic Anions

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요 약. 이온쌍크로마토그래피에 의한 유기 및 무기음이온을 분리하는데 반대이온으로서 양이온 염료인 메틸렌 블루(MTB⁺)를 사용하여 흡수가 되지 않는 음이온 시료를 간접적으로 분광광도 검출기로서 가시영역인 665nm에서 검출할 수 있었으며 혼합시료의 분리를 시도한 결과 좋은 분리도와 높은 감도를 보여주었다. 또한 여러 실험조건에서 분리인자를 측정하여 머무름 기구를 조사하였다. 본 연구에서 머무름 기구는 MTB⁺가 고정상에 일차층(primary layer)을 형성하고 반면 음이온 시료 또는 이동상에 존재하는 다른 음이온들이 경쟁적으로 이차층(secondary layer)을 형성하는 ion-interaction model임을 확인하였다.

ABSTRACT. A cationic dye, methylene blue (MTB⁺) was examined as a counter ion in the separation of organic and inorganic anions by ion-pair chromatography. Nonabsorbing anions could be indirectly detected by photometric detector with the assistance of MTB⁺ in visible range (665 nm). A mixture of anions was able to be separated with good base line resolution and high sensitivity. The capacity factors were also determined in various experimental conditions to study retention mechanism. The retention followed the ion-interaction model where the MTB⁺ occupies a primary layer at the stationary phase while the analyte anion and other anions in the system compete for forming the secondary layer.

INTRODUCTION

Ion-pair chromatography (IPC) has found wide applications in the separation of organic and inorganic anions. IPC as adapted to modern liquid chromatography is of comparatively recent origin, being first applied in the mid-1970s. The development of IPC as a new HPLC method is generally attributed to Schill and his coworkers.¹

The current popularity of IPC arises mainly from the limitations of ion exchange in handling

certain samples by the other LC methods (*e.g.* compounds that are very polar, multiply ionized and strongly basic).²

IPC can be carried out in either normal phase or reverse phase modes, each of which has its own advantages. The more popular mode involves separation in a reverse phase system on chemically bonded phase column.

The unique advantage of IPC is that it can offer excellent possibilities of a high response on photometric detector even for non-UV-absorbing compounds by using a counter ion of high

molar absorptivity at the measuring wavelength. Since this technique was first introduced by Schill *et al*¹, there have appeared a number of papers on the determination of non-absorbing organic or inorganic species using ion-pair chromatographic system.³⁻¹¹

Lagerström and Eksborg *et al*² used a quaternized tricyclic antidepressant, N,N-dimethyl propriptyline to separate three carboxylic acids of widely different molecular weight in normal phase system with organic mobile phase. They estimated that the detection limit would be at least one order of magnitude lower than RI detector. Parris^{3,4} used diisobutyl-ethoxyethyl-dimethylbenzylammonium and aromatic sulfonates as UV-absorbing counter ions in the aqueous mobile phase in separation of bile acids and ionic surfactants. Improvement in detection by using UV-absorbing counter ions in reverse phase system has also been obtained by Bidingmeger with phenethyl ammonium and cetylpyridinium as counter ions and alkylsulfonates as sample⁵.

Recently, DiNunsio and Freiser¹⁰ extended this idea to visible range, using brilliant green as the stationary phase and hexane-dichloromethane as eluent in normal phase chromatography and were able to separate aliphatic acids with high sensitivity at 630 nm wavelength. Gnana-sambandan and Freiser^{11,12} were also able to detect a series of aliphatic alcohols and sugars not possessing chromophores in visible range at submicrogram levels using methylene blue dye as counter ion in reverse phase chromatography.

This paper demonstrated that separation and detection of non-absorbing organic and inorganic anions, using cationic dye, methylene blue that absorbs in the visible range as the ion pairing reagent, can be accomplished with this visualization technique in reverse phase system. The chromatographic behaviors were also investigated

in various experimental conditions and the retention mechanism was discussed in this work.

EXPERIMENTAL

Apparatus. A modular high performance liquid chromatography consisting of an Altex pump (Model 110A), a variable wavelength, UV-visible detector (Schoeffel Model SP 770 spectroflow), and a strip chart recorder (Linear Instrument Model 261 M/M), was used. The sample injector was a 10 μ L loop injector (Spectra physics rotary valve injector SP-419-9410).

Column. A stainless steel column of 25cm length and 4.6mm inner diameter was used with a 2 μ m Altex filter in each [end fitting]. The column was slurry packed in the laboratory with 5 μ m particles of Spherisorb S5 ODS-2 at 6000 p.s.i. using octanoic acid and followed by methanol and finally water-methanol (50 : 50 V/V%). Column efficiency, expressed as the number of theoretical plates of the column, was calculated by the equation

$$N = 5.54 (T_r / W_{1/2})^2$$

where T_r is the retention time of the solutes and $W_{1/2}$ is the width of the peak at half height. The solutes chosen were *p*-xylene, naphthalene and anthracene. The mobile phase was methanol-water (80 : 20 V/V%). The theoretical plates of the column was 5000 ± 500 within experimental error.

Materials. The organic acids used in this study (acetic, propanoic, butanoic, pentanoic, hexanoic, chloroacetic, bromoacetic and iodoacetic acid) were obtained from Sigma Chemical Co. as reagent grade. The inorganic anions, sodium salts (halide, thiocyanate, sulfate, nitrate, chlorate and perchlorate) were obtained from Eastman Kodak Chemical Co. All compounds were used without further purification. Deionized water was degassed under vacuum at

50°C. Reagent grade methanol was used as received. Methylene blue, chloride form (MTB⁺ Cl⁻, mol. wt., 373.9 Baker Analyzed) was used after purification from ethanol-ether mixture.

Chromatographic Technique. The eluent and reservoir and column were thermostated at 30°C ± 0.1°C in a water bath. The mobile phase was prepared by dissolving an appropriate amount of methylene blue (MTB⁺) in solvent mixture of methanol and water. The solution was then adjusted to a suitable pH when necessary by addition of hydrochloric acid or sodium hydroxide. All mobile phases were filtered through a 0.45 μm Millipore filter and degassed before use in an ultrasonic bath. The average flow rate was measured periodically by measuring the volume of eluted solvent as a function of time. The flow rate was maintained at 1.0 mL/min. The column pressures ranged between 1500 to 2000 p. s. i.

Before injecting the analytes, the column was equilibrated with the mobile phase containing MTB⁺ by passing mobile phase through the column until the absorbance of the eluent equivalent to that of the original mobile phase is observed. When the plateau or background level absorbance was achieved, samples were injected.

The behaviors of the analytes were characterized by the capacity factor. The capacity factors were calculated by using the equation, $k' = (V_r - V_0) / V_0$ where V_r is the apparent retention volume of a solute and V_0 is the void volume of column. V_0 was obtained from water peak of the chromatogram.

RESULTS AND DISCUSSION

In IPC, it is usual to regulate and separation selectivity for the ionic samples by changing the nature and the concentration of the counter ion. The counter ion can, however, have dual functions in these systems and it can also be used

to improve the detectability to a considerable extent.

The technique has so far mainly been utilized on photometric detector, where IPC principle offers a high detector response even for non-absorbing compounds by using a counter ion of high molar absorptivity at the measuring wavelength. The principle was applied initially to normal phase system. The absorbing counter ion is applied in solution on a hydrophilic solid phase. A non-absorbing sample with the opposite charge will migrate with the organic mobile phase as an ion pair which, owing to the high absorptivity of the counter ion, will show a high absorbance.

The IPC principle can also be applied in reverse-phase system with a hydrophobic adsorbent as the stationary phase. The sample is distributed to the adsorbent which, owing to the prerequisite for electroneutrality in the phases, might give to rise changes in the concentration of the absorbing counter ion in the mobile phase. The injection of ionic sample causes the adsorption of additional reagent component from the eluent to the stationary phase. The adsorbed amount of ion-pairing reagent is released with the sample to maintain electrical neutrality where upon a reagent ion is again adsorbed onto the stationary phase and eventually coelutes with the sample. The detailed mechanism for this visualization technique has been suggested¹³.

In order to find the optimal chromatographic conditions, and the retention mechanism, the capacity factors were determined under various experimental conditions. The retention mechanism for reverse-phase IPC has been a topic of considerable discussion in recent years, and two retention mechanisms have been proposed. The ion pair mechanism is one where it is suggested that ion pairs are formed between

analyte ions and the hydrophobic counter ions prior to sorption on a hydrophobic bonded phase. Other workers suggested an ion-interaction mechanism, where the counter ions are first sorbed and these charge site serve as exchange sites for the analyte ions. If more hydrophobic counter ions are used, ion-interaction model is more favorable than ion-pair model.

Retention of Analytes as Function of Methanol Concentration. The capacity factor (k') is much affected in IPC by a change in pH. In this study, maximum k' values of organic anions exhibited above pH of 6.0, where they are completely ionized. But in the case of inorganic anions, k' values were almost not affected by changes of pH from 3.0 to 6.5. Therefore, all mobile phases used in this experiment were adjusted to a pH of 6.5 with sodium hydroxide. The samples were detected at 665 nm with maximum sensitivity. Capacity factor of some organic and inorganic anions determined in three methanol concentrations each containing 0.2mM MTB⁺ are listed in Table 1. As can be seen in Table 1, k' value was decreased with increasing the concentration of methanol. For reverse-phase system, as the percent of water is decreased, the solvent becomes stronger and sample k' values decrease.

The order of k' values of anions was increased with increase in molecular weight, size and their charge. In the case of organic anions, the heavier acids, being more hydrophobic than the lighter acids exhibited larger k' value. Therefore, the heavier hydrophobic acids are eluted after the lighter acids. For inorganic anions, the retention order is $\text{ClO}_4^- > \text{SO}_4^{2-} > \text{ClO}_3^- > \text{I}^- > \text{SCN}^- > \text{Br}^- > \text{NO}_3^- > \text{Cl}^-$ and is similar to the anion selectivity order found on a typical, strongly basic anion exchanger¹⁴. These data are consistent with the feature of ion-interaction model for reverse phase ion-pair chromato-

graphy.

Effect of the Concentration of MTB⁺ upon the Retention of Analytes. One of the feature of the ion-interaction model is that an initial equilibrium of the lipophilic counter ion is established between the stationary phase and the eluent.

As a result, a charged primary layer is formed on the packing surface. Therefore, the MTB⁺ in mobile phase is first sorbed onto the stationary phase as a primary layer and coanions occupy the secondary layer due to electrostatic attraction of opposite charged primary layer. The density of primary layer depends upon the concentration of MTB⁺ and its lipophilic attraction to the surface of stationary phase.

The plot of MTB⁺ adsorbed as a function of the concentration of MTB⁺ in mobile phase is shown in Fig.1. The amount of adsorbed on

Table 1. Retention of organic and inorganic anions

| Analytes | Capacity Factor, k' at MeOH % | | |
|--------------|---------------------------------|------|------|
| | 5% | 10% | 20% |
| Acetic | 3.39 | 1.56 | 0.88 |
| Propionic | 4.21 | 1.95 | 1.02 |
| Butyric | 6.37 | 2.99 | 1.45 |
| Valeric | 12.3 | 5.31 | 2.42 |
| Caproic | 18.5 | 8.75 | 3.63 |
| Chloroacetic | 8.64 | 3.95 | 1.82 |
| Bromoacetic | 9.77 | 4.21 | 2.05 |
| Iodoacetic | 13.6 | 5.34 | 2.84 |
| Chloride | 7.24 | 3.09 | 1.48 |
| Bromide | 9.07 | 4.12 | 2.18 |
| Iodide | 12.5 | 5.66 | 2.73 |
| Thiocyanate | 11.6 | 5.12 | 2.44 |
| Nitrate | 8.56 | 3.43 | 1.98 |
| Sulfate | 19.3 | 7.87 | 3.39 |
| Chlorate | 14.7 | 6.01 | 2.66 |
| Perchlorate | 21.5 | 9.89 | 4.16 |

Stationary Phase: Spherisorb S5 ODS-2, 25cm × 0.46cm. Mobile Phase: MeOH-H₂O solvent mixture containing 2.0 × 10⁻⁴M MTB⁺, pH=6.5. Flow rate: 1.0mL/min. Detection: 665nm

stationary phase was increased with increasing the concentration of MTB^+ and then levelled off at higher concentration above 10^{-2}M , because the column might be saturated with MTB^+ . After the formation of the equilibrium distribution of MTB^+ counter ion, the second feature of the ion-interaction model is that the retention of a sample of opposite charge to the counter ion depends upon both an electrostatic attraction and the adsorbed amount of counter ion on column. Thus, as shown in Fig. 2, retention increases as the MTB^+ concentration increases. However, retention reaches a maximum at a point where the accompanying anion, Cl^- (in this case, $\text{MTB}^+ \text{Cl}^-$ form) begins to exert a favorable competing effect over retention of the analyte anions and its retention begins to drop as the MTB^+ concentration increases.

Effect of Ionic Strength upon the Reten-

tion of Analyte Anions. Ionic strength might be expected to change retention by influencing the electrostatic attraction. A number of paper have been reported that increasing ionic strength in the mobile phase leads to reduce retention of sample having a charge opposite to that of ion-interaction reagent.¹⁵⁻¹⁷ Since the ion-interaction model implies that electrostatic interactions are essential for retention, it is necessary to determine the influence of ionic strength over a wide range of salt concentration. Fig. 3. shows the results of such an experiment. As the ionic strength is increased from 10^{-5} to 10^{-4}M NaCl, there is a slight effect upon the k' value. Beyond 10^{-4}M , as NaCl concentration increases, the k' value of analytes decrease rapidly because the competing effect of Cl^- over k' value of the analyte anions is greatly enhanced in the higher ionic strength eluent. The data in this work is consistent with ion-interaction model.

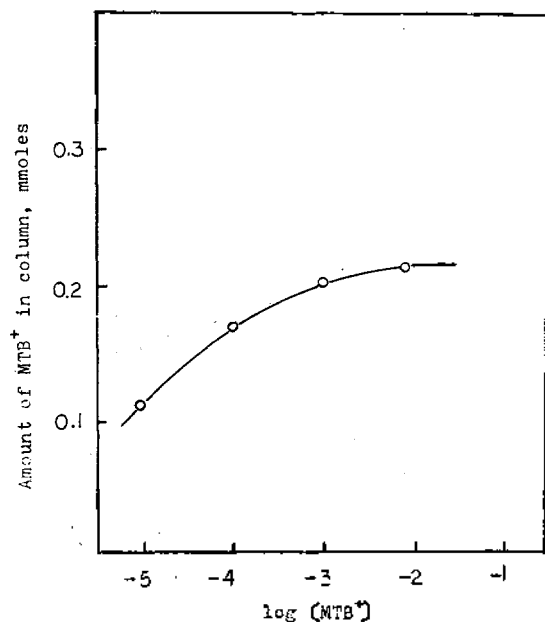


Fig. 1. Amount of MTB^+ adsorbed on column as a function of concentration of MTB^+ of mobile phase. Column: Spherisorb ODS-2, $25\text{cm} \times 0.46\text{cm}$. Mobile phase conditions are water containing MTB^+ and pH of 6.5. Flow rate: $1.0\text{mL}/\text{min}$. Detection: 665nm .

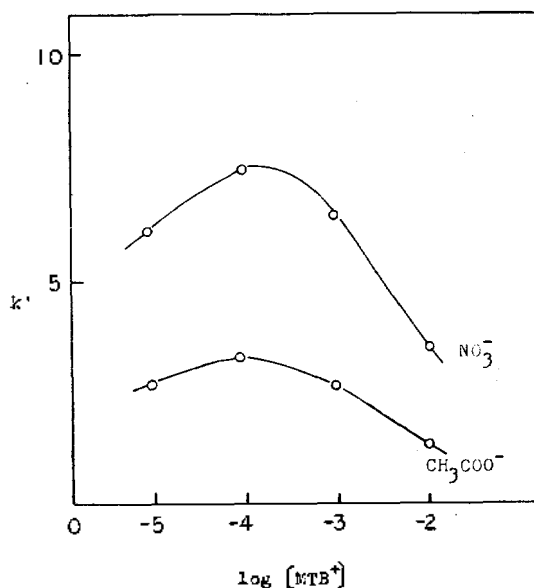


Fig. 2. Retention of anions versus concentration of MTB^+ of mobile phase. Mobile phase conditions are $5\%(\text{V}/\text{V})\text{MeOH}-\text{H}_2\text{O}$ and added MTB^+ . Other experimental conditions are same as in Fig. 1.

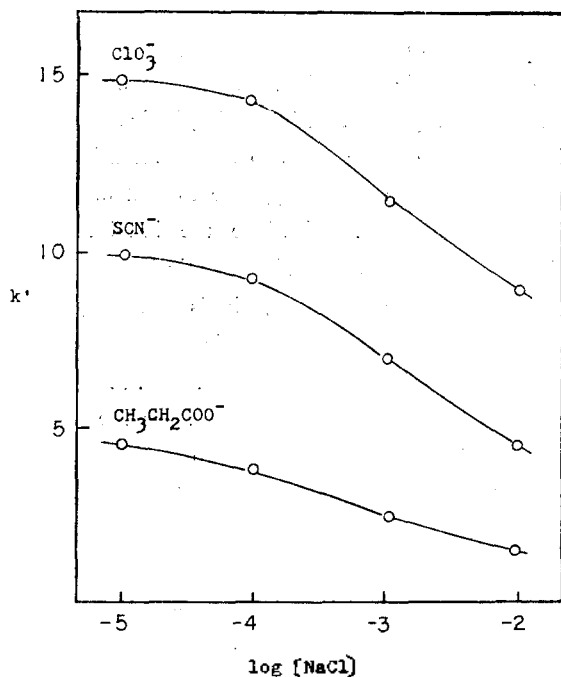


Fig. 3. The effect of ion strength of mobile phase on the retention of anions. Mobile phase conditions are 5% (V/V) MeOH-H₂O, 0.1mM MTB⁺ and added NaCl. Other experimental conditions are same as in Fig. 1.

Separation and Detection Limit. Fig. 4. and 5 represent the separation of a mixture of some organic and inorganic anions. Fig. 4 illustrates the separation of some organic acids using 10% methanol-water containing 0.1mM MTB⁺ and 0.1mM NaCl (pH=6.5). A mixture as can be seen, is chromatographically well separated with base line resolution and high sensitivity. The peak height and therefore sensitivity was increased with increasing the carbon number of samples. Fig. 5 also illustrates the separation of some inorganic anions. Inorganic anions tend to be more highly retained as observed in Table 1. To reduce and improve analysis times, the stronger eluent anion was required. Therefore, a mobile phase consisting of 10% methanol-water, 0.2mM MTB⁺ and 0.5mM Na₂SO₄ was used to obtain the chroma-

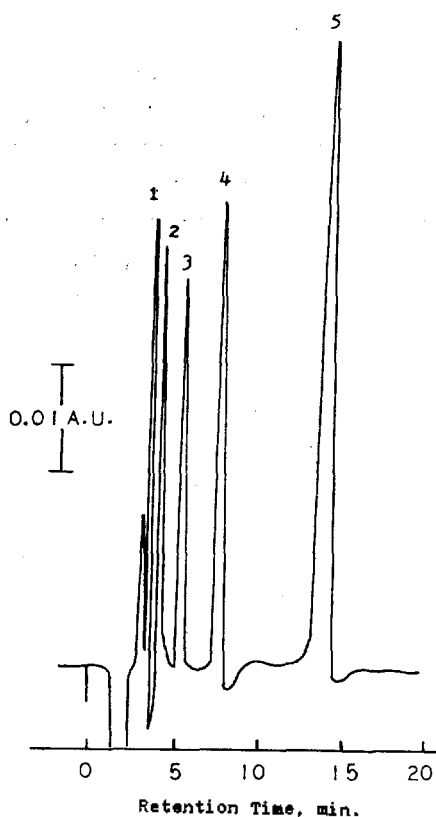


Fig. 4. Separation of organic anions. Mobile phase: 10% (V/V) MeOH-H₂O containing 0.1 mM MTB⁺ and 0.1mM NaCl. 1. Acetic acid (15μg) 2. Propionic acid (12μg) 3. Butyric acid (10μg) 4. Valeric acid (5μg) 5. Caproic acid (5μg)

togram in Fig. 5. Under these conditions, a mixture of Cl⁻, Br⁻, SCN⁻, and ClO₄⁻, was able to be separated with good resolution, but they showed a little tailing phenomena.

The detection limit was determined at detection wavelength (665nm) in order to compare the sensitivity. The detection limit was obtained from the peak height equivalent to twice the noise level of detector. The data in Table 2 show that even non-absorbing analytes could be detected by photometric detector using MTB⁺ cationic dye of high molar absorptivity in visible range. The sensitivity obtained with this method is comparable to other HPLC detector (e. g.

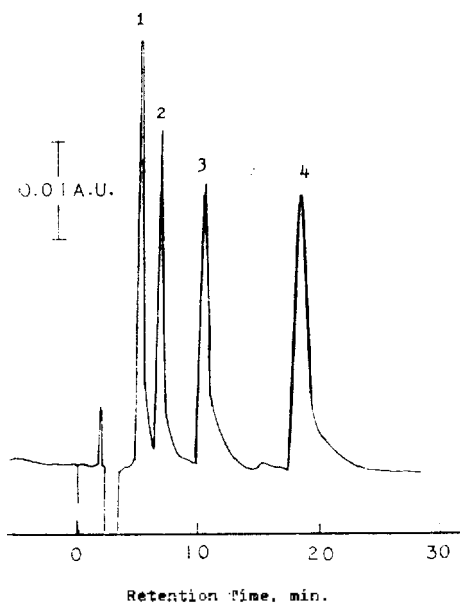


Fig. 5. Separation of inorganic anions. Mobile phase: 10% (V/V) MeOH-H₂O containing 0.2 mM MTB⁺ and 0.5 mM Na₂SO₄. Injection: 10 μg. 1. Cl⁻ 2. Br⁻ 3. SCN⁻ 4. ClO₄⁻

Table 2. Detection limits for a number anions

| Anions | Limit of Detection (μg) |
|-------------|-------------------------|
| | Eluent A |
| Acetic | 0.46 0.90* |
| Propionic | 0.44 |
| Butyric | 0.37 |
| Valeric | 0.25 |
| Caproic | 0.13 |
| | Eluent B |
| Chloride | 0.27 0.15* |
| Bromide | 0.38 0.26* |
| Thiocyanate | 0.54 |
| Perchlorate | 0.60 0.20* |

Eluent A: 0.1 mM MTB⁺, 0.1 mM NaCl and 10% methanol. Eluent B: 0.2 mM MTB⁺, 0.5 mM Na₂SO₄ and 10% methanol. *Conductivity Detection.

conductivity detector).

In conclusion, this technique provides that even a compound of non-chromopore could be detected and enhanced their sensitivity with photometric detector by the assistance of dye.

The present data is consistent with the retention mechanism of ion-interaction model for reverse phase ion pair chromatography.

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