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Forced-Flow Chromatography of Alkaline Earths Using Spectrofluorometric Detector

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After magnesium (or calcium), strontium and barium ions are separated with forced-flow chromatography using the eluent having a metallofuorochromic reagent, Calcein, and 0.1 N KOH, the alkaline earth is detected sensitively by a spectrofluorometric method. By this method, 2 ng of magnesium, 5 ng of calcium, 20 ng of strontium and 50 ng of barium can be detected, and the linear variations of the emission intensity with alkaline earth concentrations are obtained in the range of 0 to 1 μg.

KEY WORDS : Forced-flow chromatography/ Calcein/
Fluorometric detector/ Alkaline earths/

INTRODUCTION

3, 6-Dihydroxy-2,7-bis [N, N′-di(carboxymethyl)-aminomethyl]-fluoran (Calcein) has been used as a metallofluorochromic indicator for EDTA titration of calcium[1-3]. The reagent does not fluoresce in strong alkaline solution, but an intense fluorescence appears in the presence of alkaline earths, especially calcium. However, little attempt has been made to study the spectrofluorometry of alkaline earths using Calcein. Each fluorescence spectrum of Calcein-alkaline earth chelates has a maximum peak at around 535 nm, and this makes the selective fluorometric determination of alkaline earths difficult.

An ion exchange method has been applied to separate inorganic ions for many years, and recent development of a high pressure pump and an ion exchange resin has made the high speed separation of these ions possible. Usually, by coulometry,[4] radioactivity measurements,[5] spectrophotometry[6-8] and flame photometry,[9] eluted metal ions can be detected.

Previously, authors obtained the rapid separation and sensitive quantitation of such alkaline earth ions as Mg, Ca, Sr and Ba by using forced-flow chromatography equipped with spectrophotometer as a detection device.[10] In this paper authors dealt with the continuous and sensitive detection of the alkaline earth ions by using the eluent containing a sensitive metallofluorochromic reagent, Calcein.

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EXPERIMENTAL

Apparatus and Reagents

A Yanagimoto liquid chromatograph, Model L-1013 fitted up with a glass column (2 mm i. d. × 220mm or × 330 mm in length) was used, and the column was filled with Hitachi custom ion exchange resin 2611. Non-fluorescent quartz flow cell (16 μl in vol., 2 mm in dia. × 5 mm in length) was attached to Jasco spectrofluorometer, Model FP-550, and this was used a detector of the chromatograph. The flow cell device was made of polyfluoroethylene (Teflon) and connected with the liquid chromatograph by a Teflon tube. Excitation and emission spectra of Calcein and its alkaline earth chelates were measured with a Jasco spectrofluorometer, Model FP-550 with 1. 0 cm cell. A schematic diagram of liquid chromatograph is shown in Fig. 1.

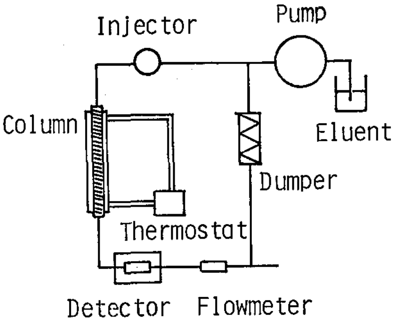

Fig. 1. A schematic diagram of liquid chromatograph.

Stock solutions of magnesium, calcium, strontium and barium were prepared by dissolving their chlorides with 10^{-4} N hydrochloric acid. Calcein, which was purchased from the Dojindo Co. Ltd., Research Laboratories, was purified by recrystalization from ethanol-aqueous solution to remove such impurities as fluorecein. A stock solution of calcium was prepared from the purified reagent and stored in a polyethylene bottle having the shade to intercept the light. A potassium hydroxy solution (0. 1 N) was prepared by diluting 30% solution (Merck Superpur Reagent) with deionizedredistilled water. As the eluent were used 0. 025-0. 2 N KOH and (1-4) $\times 10^{-5}$ M Calcein solutions.

Procedure

After the eluent enough to obtain a stable base line flowed through the column, samples of metal ions (1-10 μl) were injected into the column with a Hamilton microsyringe. The excitation wave length was set at 495 nm, and the fluorescence intensity was measured at 530 nm.

RESULTS AND DISCUSSION

Excitation and Emission Spectra

Calcein alone scarcely fluoresces at pH less than 4 and more than 12, while Calcein alkaline earth chelates fluoresce intensely as the chelates form at pH more than 12.

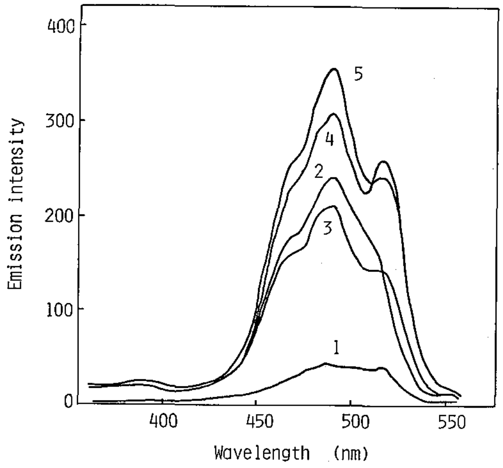

Fig. 2. Excitation spectra of Calcein and Calcein-alkaline earth chelates.
Calcein ; 4×10^{-5} M,
alkaline earth ion ; 2×10^{-5}M,
KOH ; 0. 1 M, emission wavelength ; 550 nm,
1 ; Calcein alone, 2 ; Mg, 3 ; Ca, 4 ; Sr, 5 ; Ba.

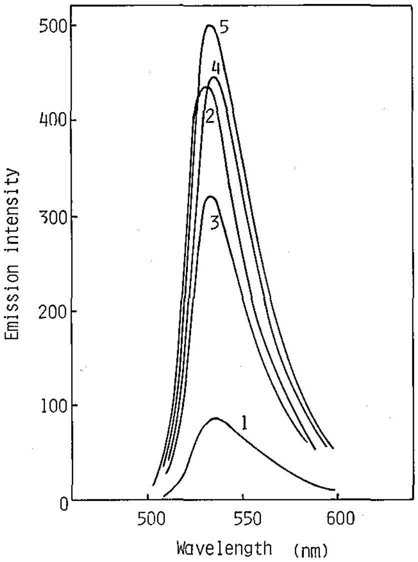

Fig. 3. Emission spectra of Calcein and Calcein-alkaline earth complexes.
Calcein ; 4×10^{-5} M,
alkaline earth ion ; 2×10^{-5} M,
KOH ; 0. 1M,
excitation wavelength ; 495nm (slit 5nm)
1 ; Calcein alone, 2 ; Mg, 3 ; Ca, 4 ; Sr
5 ; Ba.

Figure 2 shows the excitation spectra of Calcein and Calcein-alkaline earth chelates in 0. 1 N KOH solution (λ_{em}=550 nm). Since the excitation maximum of the spectra exists around at 495 nm as shown in Fig. 2, emission spectra were measured at 495 nm throughout the experiment.

Comparison of the fluorescence of Calcein alone and in the presence of alkaline earths is shown in Fig. 3. Emission maximum exists between 525 and 535 nm. Consequently, it seems suitable to make the measurement of fluorescence at 530 nm, where there exists the mean emission maximum of the chelates.

Fluorescence as a Function of Potassium Hydroxide Concentration

The decrease in fluorescence of Calcein in alkaline range depends upon the dissociation of the two imino nitrogens. It becomes negligible at pH 13 and above. Therefore, the influence of KOH concentration on the emisston intensity was examined. This is illustrated in Fig. 4, which shows that the emission intensity of Calcein chelates has a maximum value at about 0. 1 N KOH. The curves obtained with alkaline earth ions are similar in shape to one another; there exists the fluorescence decrement in each curve above 0. 1 N KOH. Sodium ions and chelating reagents such as EDTA as well as a large amount of potassium ions (~1 M) decrease the emission intensity.

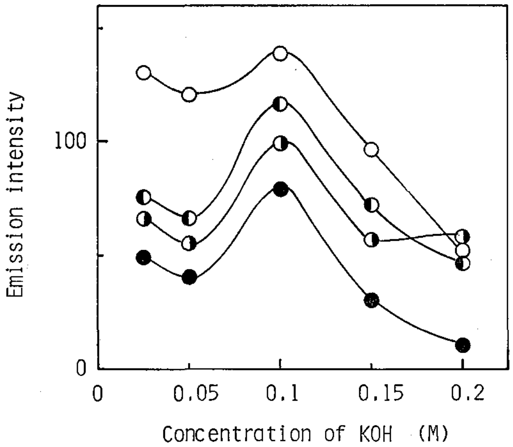

Fig. 4. Effect of KOH concentration on emission intensity.
Calcein; 4×10^{-5} M, alkaline earths; 2×10^{-5} M,
excitation wavelength; 495nm (slit 10nm),
emission wavelength; 530 nm (slit 5 nm),
○; Mg, ●; Ca, ◑; Sr, ◐; Ba.

Effect of KOH Concentration on Retention Time

The relationship between the potassium hydroxide concentration and the retention time is illustrated for Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} in Fig. 5. The curves obtained with Mg^{2+} and Ca^{2+} are considerably flatter than those with Sr^{2+} and Ba^{2+}. The separation factor between Mg^{2+} and Ca^{2+} is scarcely affected by the KOH concentration. The addition of less than 0.06 N KOH makes the separation time of Sr^{2+} and Ba^{2+} longer and the reagent emission higher. Subsequent experiments were carried out at 0.1 N KOH.

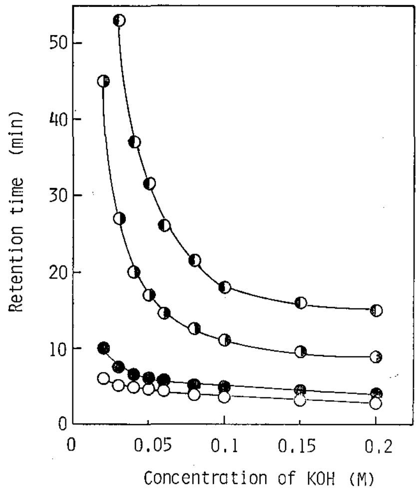

Fig. 5. Effect of KOH concentration on retention time
Calcein; 2×10^{-5} M, column; 22cm×2mm,
flow rate; 0.42 ml/min, temp.; 25°C, dead time; 2 min,
○; Mg, ●; Ca, ◑; Sr, ◐; Ba.

Effect of Calcein Concentration and Column Temperature

Figure 6 illustrates the changes in the retention time of alkaline earths as a function of Calcein concentration. The decreasing order of stability of the alkaline earth chelates with Calcein is as follows: Mg>Ca>Sr>Ba,[11] which is the same as that found in the retention time. A decrement of the Calcein amount results in insufficient formation of Calcein alkaline earth chelates. As shown in Fig. 6, the Calcein amount scarcely affects the retention time of Mg^{2+} and Ca^{2+}. This may be caused by the high stability of Calcein with Mg^{2+} and Ca^{2+}.

The effect of the column temperature on retention time is shown in Fig. 7. As the temperature of the column rises, the retention time of Sr^{2+} and Ba^{2+} increases considerably, but that of Mg^{2+} and Ca^{2+} is almost constant.

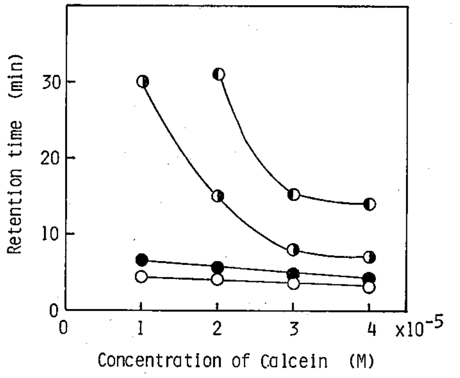

Fig. 6. Effect of Calcein concentration on retention time
KOH; 0.1 M, flow rate; 0.42 ml/min, column; 33cm×2mm, temp.; 25°C, dead time; 2.5 min.

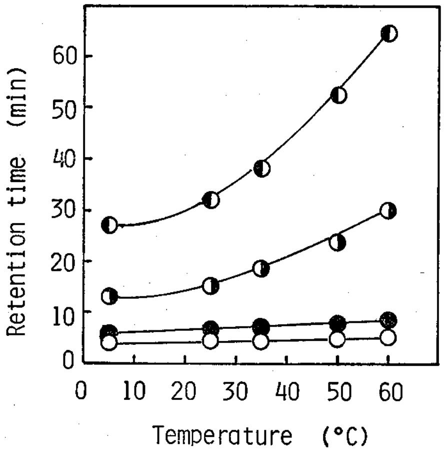

Fig. 7. Effect of column temperature on retention time
Calcein; 2×10^{-5} M, KOH; 0.1M, flow rate; 0.42 ml/min, column; 33 cm×2 mm, dead time; 2.5 min.

Chromatogram and Calibration Curves

Figure 8 indicates a typical example of the chromatogram of Mg^{2+}, Sr^{2+} and Ba^{2+},

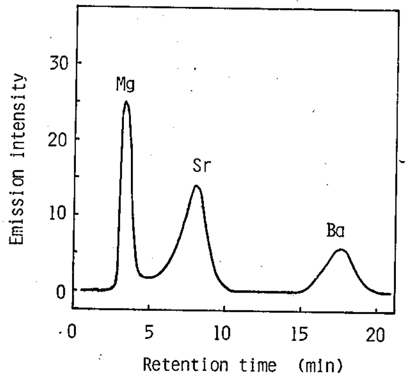

Fig. 8. Typical chromatogram of alkaline earth ions.
Calcein; 4×10^{-5} M, KOH; 0.1M, flow rate; 0.42 ml/min, Mg; 0.1μg, Sr; 0.5μg, Ba; 0.5μg.

where 4×10^{-5} M Calcein and 0. 1 N KOH is used as eluent. Three metals were separated within 20 min. It is difficult to separate Ca^{2+} from Mg^{2+} even by using a low concentration of Calcein and a high column temperature.

The relationship between the peak height and the amount of metal ions is shown in Figs. 9 and 10. Linear relations are obtained in the range of 0. 01 to 0. 5 μg for Mg^{2+} and Ca^{2+}, 0. 1 to 1. 0 μg for Sr^{2+} and Ba^{2+}. Two nanograms of Mg^{2+}, 5 ng of Ca^{2+}, 20 ng of Sr^{2+} and 50 ng of Ba^{2+} can be detected, respectively.

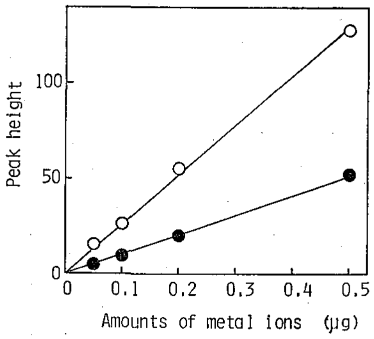

Fig. 9. Calibration curves
Calcein; 4×10^{-5} M, column; 33 cm, KOH; 0. 1M, temp.; 25°C, flow rate; 0. 42 ml/min,
○; Mg, ●; Ca.

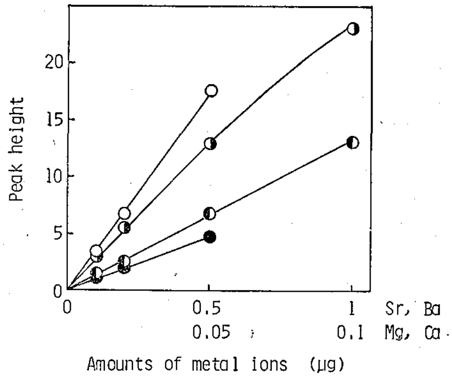

Fig. 10, Calibration curves.
Calcein; 4×10^{-5} M, KOH; 0. 1M, column; 33cm, flow rate; 0. 42 ml/min, temp.; 25°C,
○; Mg ●; Ca, ◐; Sr, ○; Ba.

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