Reversed-Phase High-Performance Liquid-Chromatographic Determination Systems Specific to Ultratrace Hard Metal Ions with Tridentate Schiff Bases and Pyridylhydrazones

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Two Schiff-base ligands derived from salicylaldehyde and two 2-pyridylhydrazones were examined for the ultratrace determination of metal ions by reversed-phase high-performance liquid chromatography (HPLC). Although these ligands have been thought to be rather unattractive in inorganic analysis, their renewal in the HPLC methodology was demonstrated in this work. In these ligand systems, certain hard metal ions such as Al(III), Ga(III), V(V), Fe(III), and Co(III) ions, were effectively separated on octadecyl- or cyanopropyl-bonded silica packing materials. A highly sensitive fluorimetric detection of Al(III) ion was achievable with *N*-salicylidene-2-hydroxyaniline having an O,N,O donor set; although the contamination problem actually limited the detection capability, the calculated detection limit for Al(III) ion was down to 0.1 nM (3 ppt) on a 3 σ blank basis. The chromatography of chelates with 4-*N*,*N*-diethylaminosalicylaldehyde-2-pyridylhydrazone seems to be attractive for actual analysis; Al(III), Ga(III), V(V), Fe(III), and Co(III) ions at the sub-ppb to ppb levels have been photometrically detected with a relative standard deviation of less than 5%. The fluorimetric detection using 4-hydroxysalicylaldehyde-2-pyridylhydrazone was very effective to access trace levels of Al and Ga ions, giving detection limits of 3.0 nM and 1.3 nM, respectively ($\mathrm{M} = \mathrm{mol\ dm^{-3}}$).

The high-performance liquid chromatography (HPLC) of metal chelate compounds is now one of the most important methodologies for trace-metal analysis.[1,2] The applications have been extended to a wide variety of real samples, especially those obtained in environmental and clinical fields, since HPLC methods often have an advantage over atomic spectroscopic means in terms of sensitivity, matrix toughness and cost-effectiveness.[3–8]

Our continuing study of ligand systems to be employed for the derivatization of certain metal ions has extracted a working principle for system designing, *kinetic differentiation mode* HPLC.[8–11] Unlike ordinary organic compounds possessing stable covalent bonds, the coordination bonds between a metal ion and ligating atoms in some metal chelates often cannot afford to resist the overwhelming force arising from the reversed-phase (RP) HPLC resolution between the ligand and its chelates. Under the Gaussian peak approximation,[12] a rough calculation using eq. 1) suggests that the ligand concentration ($C_{\Delta z}$) at a point being apart at $\Delta z = 6\sigma$ from its peak is just as low as $1.5 \times 10^{-8}\ C_{\mathrm{peak}}$, where σ and C_{peak} are the standard deviation of the peak measured in min and the ligand concentration at its peak:

$$C_{\Delta z} = C_{\mathrm{peak}} \exp\left[-\Delta z^2/2\,\sigma^2\right]. \qquad (1)$$

Conversely, because a ligand system can control the kinetic nature of certain metal chelates in the RPHPLC separation processes, this potentially produces unique analytical selectivity for metal ions.

In this report, the HPLC behavior of metal chelates of several Schiff bases and hydrazones (see Fig. 1) is described in connection with kinetic differentiation. In our continuing studies, a thorough examination of azomethin-type ligands has been provoked by the success of the HPLC determinations of metals with benzoylhydrazone reagents.[3,7] In the family of these ligands, the analytical usefulness of the four ligands listed in Fig. 1 seems to be highlighted and compatible with the ease of preparation. Those ligands having nitrogen and oxygen donor functions are suitable for hard metal ions, such as Al(III), Ga(III), V(V), Fe(III), and Co(III). These ligands of quite common class seem to have had rather poor analytical merits in terms of sensitivity and selectivity in conventional spectrometric methods.[13–16] In addition, Schiff bases are often apt to undergo decomposition in water, which actually restricted their analytical use. The HPLC approach is promising to overcome the above-mentioned drawbacks; the actual sensitivity can be significantly enhanced with modern absorbance detectors (e.g. a 0.001 absorbance unit full-scale is available), and chromatographic separation eliminates any problems associated with mutual interference, spectral overlapping and ligand instability. The RP-HPLC–photometry and –fluorimetry schemes developed in this work endow these ligands with a practical usefulness for the determination of ultratrace hard metal ions, such as Al(III), Ga(III), and Fe(III) ions, leading to a renewal of this class of ligand in analytical chemistry.

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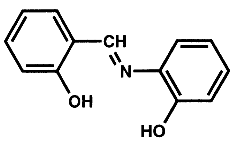
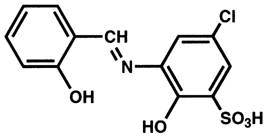
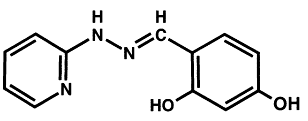


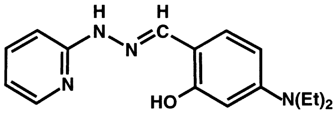
I *N*-salicylidene-2-hydroxyaniline, SHA



II 5-chloro-2-hydroxy-*N*-salicylideneaniline-3-sulfonic acid, CHSA



III 4-hydroxysalicylaldehyde-2-pyridylhydrazone, HSP



IV 4-*N*,*N*-diethylaminosalicylaldehyde-2-pyridylhydrazone, DASP

Fig. 1. Structure of ligands employed in this work.

Experimental

Apparatus: Absorption and fluorescence spectra were recorded on a Hitachi model U-3200 recording spectrophotometer and a Hitachi F-2000 spectrofluorimeter, respectively. A Horiba M-8S pH meter was used. Two HPLC systems were employed; a TWINCLE pump unit with a UVIDEC 100-III absorbance detector and an FP-110 fluorescence detector from JASCO, as well as an LC-5A pump with an SPD-2A absorbance detector from Shimadzu. The injector used was a Rheodyne 7125 with a 100 µL loop.

Reagents and Solutions: The ligands shown in Fig. 1 were prepared by ordinary condensation between aldehydes and amines or hydrazines in a boiling ethanol solvent.[13,17] The crude products were recrystallized twice from a hot ethanol solution. The Schiff bases were dissolved in *N*,*N*-dimethylformamide (DMF) to give a concentration of 10 mM ($M = mol\ dm^{-3}$). The hydrazones were used as aqueous ethanol or DMF solutions. Aqueous solutions of hydrazones containing 4 wt% of polyethyleneglycol mono(4-nonylphenylether with 20 oxyethylene units (PONPE 20) were also employed. Stock metal-ion solutions (Al(III), Co(II), Cu(II), Fe(III), Ga(III), In(III), Ni(II), and Zn(II), each 0.01 M) were prepared from the chlorides in a 0.01 M HCl solution. Aqueous solutions of V(V) and Mo(VI) ions were prepared by dissolving NH_4VO_3 and $Na_2MoO_4 \cdot 2H_2O$, respectively. The pH buffer solutions (1.0 M) used for off-line chelation were hydrochloric acid–sodium acetate at pH 5.1 and tris(hydroxymethyl)aminomethane (Tris)–hydrochloric acid at pH 7.6 for the Schiff base and the hydrazone systems, respectively. All other reagents and solvents used were of analytical reagent-grade or HPLC-grade.

Procedure: In order to avoid any serious aluminium contamination, polyethylene and Teflon wares were exclusively employed. Complexation with Schiff Bases; to a solution containing metal ions, 5 mL of the Schiff base ligand solution and 1 mL of the acetate buffer solution (pH 5.1) were added. The mixture was diluted to about 40 mL with water, followed by heating for 30 min at 50 °C on a water bath. After cooling, the solution was made up to 50 mL and an aliquot (ca. 2 mL) was taken to inject through a 100 µL loop. Complexation with hydrazones; the ligand solution (0.5 mL) and the Tris-HCl buffer solution (pH 7.6; 5 mL) were used for the off-line complexation protocol in a manner similar to the Schiff-base systems. The HPLC conditions are summarized in Table 1.

Results and Discussion

Complexation and Spectral Properties of Chelates. Schiff-Base Systems. The ligand I, SHA gives a sufficiently stable chelate with Al(III) ion in the pH range over 4.5 to 6.0 in

Table 1. HPLC Conditions Used for the Separation of Metal Chelates of Schiff Bases and Hydrazones

	Column	Eluent[a)] (flow rate 1.0 mL min^{-1})	Detection[b)]
Schiff bases System			
I SHA	LiChrosorb RP-18 (Cica-Merck)	30.0 wt% THF; 6.2 mmolal TBABr; 0.1 mmolal Na_2H_2EDTA; pH 3.5 (10 mmolal chloroacetate)	sp; 420 nm fl; ex 405 nm, em 505 nm
II CHSA	µ-Bondapak CN (Waters, Millipore)	25.0 wt% THF; 16 mmolal TBABr; 0.1 mmolal Na_2H_2EDTA; pH 3.5 (10 mmolal chloroacetate)	sp; 420 nm fl; ex 411 nm, em 510 nm
Hydrazone system			
III HSP	LiChrosorb RP-18 (Cica-Merck)	33.0 wt% AcCN; 1.6 mmolal TBABr; 0.1 mmolal Na_2H_2EDTA; pH 7.5 (5.0 mmolal Tris-HCl)	sp; 385 nm
		20.0 wt% AcCN; 50 µmolal TBABr; 0.1 mmolal Na_2H_2EDTA; pH 5.1 (5.0 mmolal acetate buffer)	fl; ex 390 nm, em 445 nm
IV DASP	LiChrosorb RP-18 (Cica-Merck)	50.0 wt% AcCN; 3.1 mmolal TBABr; 0.1 mmolal Na_2H_2EDTA; pH 7.5 (5.0 mmolal Tris-HCl)	sp; 412 nm

a) THF, tetrahydrofuran; TBABr, tetrabutylammonium bromide; EDTA; ethylendiamine-*N*,*N*,*N*′,*N*′-tetraacetate; AcCN, acetonitrile; molal, mol kg^{-1}. b) sp, spectrophotometry; fl , fluorimetry; ex, excitation; em, emission.

4 v/v% aqueous DMF solutions. However, a large excess amount of I of 200 µM against the ion at 5 µM is necessary for a full development of fluorescence. This suggests that the parallel hydrolytic decomposition of I most likely reduces its effective concentration available for complexation. Such a situation is also the case for the II (CHSA) system. It is stressed that, as described later, this instability of ligands in solutions is no longer crucial for HPLC applications. The uncorrected fluorescence spectra of the SHA chelates of Al and Ga ions have maxima at 405 nm for excitation and at 505 nm for emission, respectively. These spectra are in good agreement with those reported by Morisige.[13] The fluorescence intensity of the Al chelate is 10-times greater than that of the Ga chelate. Almost similar spectral properties were seen in the II chelates but, compared with the SHA system, the emission was enhanced by factors of 2 and 7 for Al and Ga, respectively. In slightly acidic solutions, those chelates of I and II have a stoichiometry of 1 : 1.[13]

Hydrazone Systems. The off-line complexation of hydrazone ligands III and IV was made in Tris-hydrochloric acid buffer solutions at pH 7.6 containing 0.04% of PONPE 20. The absorption spectra of some metal chelates of ligands III and IV are shown in Fig. 2 and 3, respectively. Ligands III and IV have absorption maxima at 350 nm and 400 nm, respectively, which seriously overlap with those of the chelates. This fact suggests the restricted analytical merits of these hydrazone ligands in conventional spectrophotometry; however, the HPLC-coupled scheme relaxes this limitation because the ligand and its chelates are readily base-line resolved. The sufficiently great molar absorptivities of the chelates are listed in Table 2; the nanomolar detection of these chelates at a 0.001 absorbance unit full-scale range is suggested by these ε values.

The uncorrected fluorescence emission spectra of the III chelates of Al and Ga ions are shown in Fig. 3. The emission from the chelates is strongly facilitated in slightly acidic solutions at pH 3 and 5 for Ga and Al ions, respectively. The emissive chelates have a stoichiometry of 1 : 1 confirmed by molar ratio plots, while the 1 : 2 chelates which predominate in neutral solutions are very weakly fluorescent. A similar fluorescence behavior was found for the 2,2′-dihydroxyazobenzene chelates.[18]

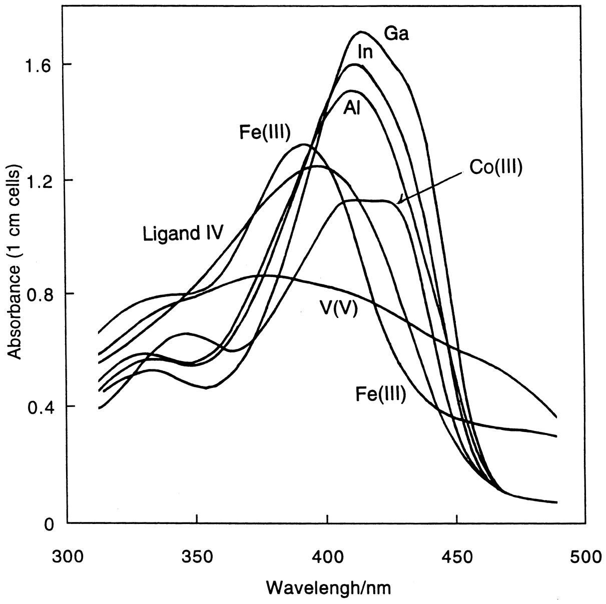


Fig. 3. Absorption spectra of hydrazone ligand chelates of IV in slightly acidic aqueous DMF (10 v/v) solution at pH 5.10.

Spectra are uncorrected for those of excess ligands. $C_{\mathrm{metal}} = 20.0$ µM, $C_{\mathrm{ligand}} = 40$ µM, acetate buffer 10 mM.

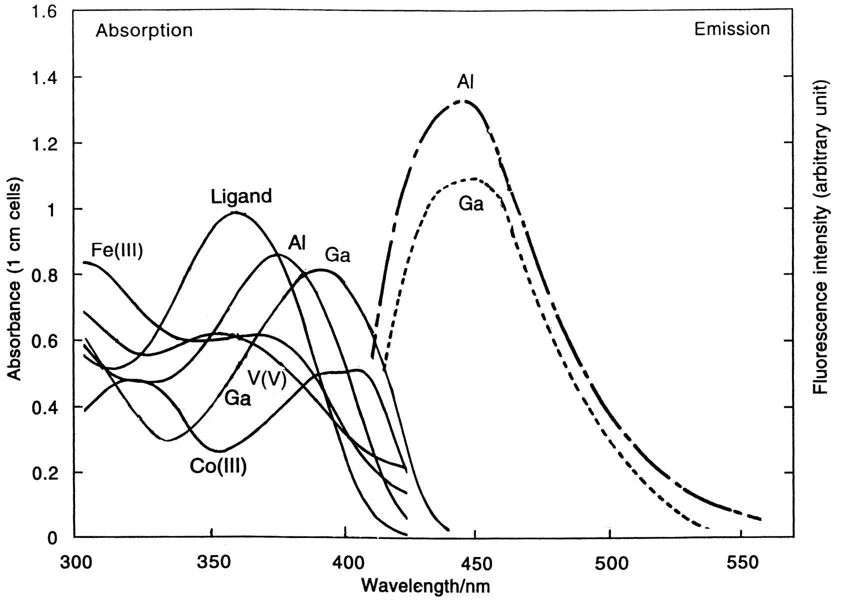


Fig. 2. Absorption and fluorescence emission spectra of hydrazone ligand chelates of III in aqueous ethanol (10 v/v) solution at pH 5.10.

Absorption spectra are uncorrected for those of excess ligands. Absorption (solid lines): $C_{\mathrm{metal}} = 20.0$ µM, $C_{\mathrm{ligand}} = 40$ µM, acetate buffer 10 mM. Emission (broken lines): $C_{\mathrm{ligand}} = 20$ µM, $C_{\mathrm{Al}} = 8.0$ µM, $C_{\mathrm{Ga}} = 16$ µM. Excitation at 374 nm.

Table 2. Absorption Maxima and Molar Absorptivity of Chelates with Hydrazone Ligand III and IV[a),b)]

Metal ion	λ_{max}/nm	ε/(M^{-1} cm^{-1})
	Ligand III	
Al	360	25000
Ga	380	43000
Co	380	22000
Fe	365	15000
	Ligand IV	
Al	410	43000
Ga	410	55000
Co	440	30000
Fe	380	36000
V	370	12500

a) At pH 5.1 in aqueous ethanol (v/v) solutions. b) All data are corrected for those of excess ligands.

Chromatography, Sensitivity, and Selectivity. Schiff-Base Systems. Typical chromatograms are shown in Fig. 4. The chromatographic conditions are listed in Table 1. Positively charged Al–I chelate having the 1 : 1 composition interacts to some extent with residual silanolate groups on an octadecylsilanized silica packing, thus giving a tailing peak profile, as shown in Fig. 4. Even acidifying the eluent to pH 3.1 and adding tetrabutylammonium bromide (TBABr) up to 10 mM in the eluent could not sufficiently minimize this peak tailing. By contrast, the peak symmetry is improved in the reagent II system on a packing having a relatively polar surface, such as a µ-Bondapak–CN, because the Al–II chelate is possibly neutral. The Schiff-base systems potentially give a very sensitive fluorimetric method for Al ion; in fact, with reagent I, the calculated detection limit is as low as 1.0×10^{-10} M (2.7 ppt, Al), though the actual one of 10 nM (300 ppt) is fixed by the relatively high Al contamination (10 nM level) from water, solutions and laboratory dust. The former detection limit is smaller than that of the HPLC method with salicylaldehydebenzoylhydrazone as 1.5 nM (40 ppt);[3] even the latter one is still in a range similar to that obtained by ICP-MS, typically 300 ppt.[19,20]

It should be noted that the chromatographic separations shown in Fig. 4 were achieved in the presence of equimolar amounts of 9 metal ions of Co(II), Cu(II), Fe(III), Ga(III), In(III), Mo(VI), Ni(II), V(V), Zn(II) towards 2.0 µM of Al(III) ion. Of those cations, only several gave the resolved peaks, but caused no actual interferences with the detecting Al ion in the 10 nM range, even if they were present at µM concentrations. Added divalent cobalt ion was oxidized to the trivalent state in the chelate by dissolved dioxygen. Also, when working with reagent II tedious contamination control for Al at the very low level was not attempted in this work; thus, the potentially lowered detection limit accessible with this system was not demonstrated. It is emphasized that this high sensitivity most likely arises from chromatographic peak intensity measurements with a high S/N ratio, because of no actual contribution from the free ligand on the chelate band. In solution fluorimetry, a deterioration of the ligand solution is a crucial problem which causes serious fluctuations of the background signal; however, the HPLC alternative enables one to approach the ultratrace quantitation of Al ion in the sub-ppb range using this unstable ligand.

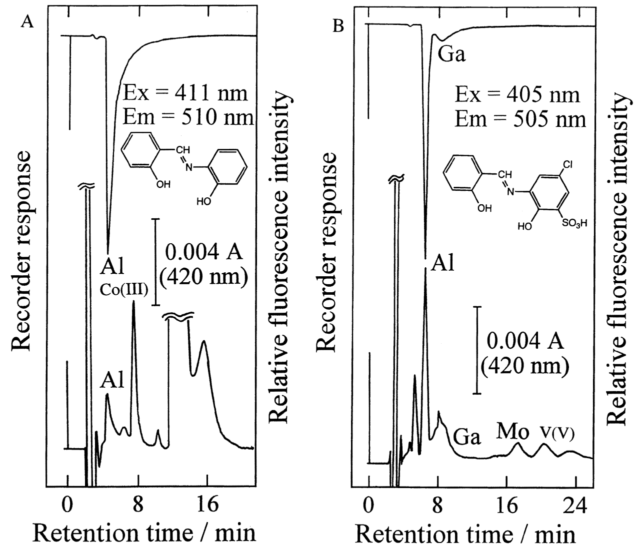


Fig. 4. Chromatograms for metal–Schiff base ligand systems of I (A) and II (B).
Sample: C_{metal} = 2.0 µM each of Al(III), Co(II), Cu(II), Fe(III), Ga(III), In(III), Mo(VI), Ni(II), V(V), Zn(II) ions, C_{ligand} = 1.0 mM, acetate buffer 10 mM at pH 5.10 in 10 v/v DMF solution. The HPLC conditions are given in Table 1.

Pyridylhydrazone Systems. A highlighted feature of these ligands is absorbance detectability towards some hard metal ions, such as V(V), Co(III), Ga(III), Al(III), and Fe(III) in the elution order. These chelate peaks are excellently base-line resolved, as shown in chromatograms A and B in Fig. 5. Ligand IV is more preferable than III for the simultaneous detection of the above-mentioned metal ions in terms of greater molar absorptivities of the chelates. Additionally, comparing Fig. 5 B) with A) with respect to Al, Ga and Fe, it is noted that the peak widths of the more slowly eluted chelates of IV at 16 to 22 min, are still smaller than those of the rapidly eluted ones of III at 8 to 12 min. This higher resolution of the IV chelates in the HPLC system endows the high practicability of IV in absorbance detection. Since the trivalent metal ion chelates of IV are most likely cationic, the elution is retarded by electrostatic interactions with an anionic surface of the ODS stationary phase packing. In contrast to the ligand I system, the addition of TBABr at 2 mmolal (molal = mol kg^{-1}) in the eluent is effective for shortening the retention times of the chelates without an undesirable reduction of the peak resolution, while giving no influence on the retention of neutral ligand IV.

With spectrophotometric detection at a 0.01 absorbance unit full-scale range (412 nm), the detection limits for Al(III) and Ga(III) ions are 7.0 nM (0.19 ppb) and 5.0 nM (0.35 ppb), respectively. The precision of this method with IV in terms of the relative standard deviation is acceptable as 4.50% and 5.16% for 5 replicate analyses for Al and Ga, respectively, at 0.50 µM. It should be stressed that the clear-cut resolution of Al and Ga ions achieved in this work is highly valuable be-

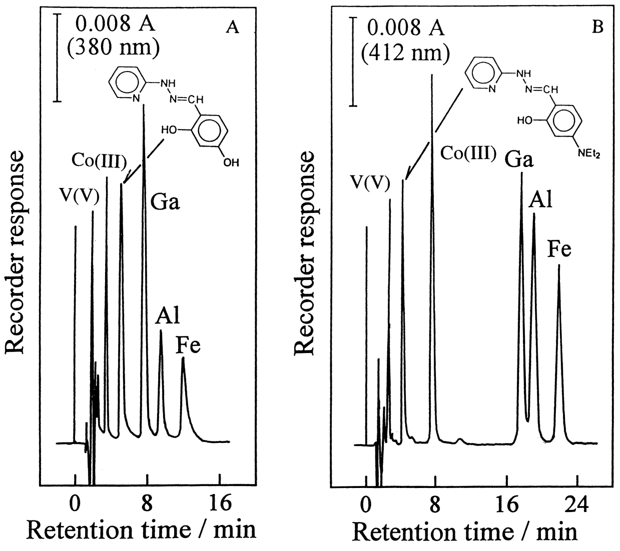


Fig. 5. Chromatograms of metal chelates of hydrazone ligand III (A) and IV (B) with the absorbance detection. C_{ligand} = 0.10 mM, Tris-HCl buffer 10 mM at pH 7.6. The HPLC conditions are given in Table 1. (A): C_{metal} = 1.00 μM each of Al(III), Co(II), Cu(II), Fe(III), In(III), Mo(VI), Ni(II), V(V), Zn(II) ions, C_{Ga} = 2.00 μM, (B): C_{metal} = 1.00 μM.

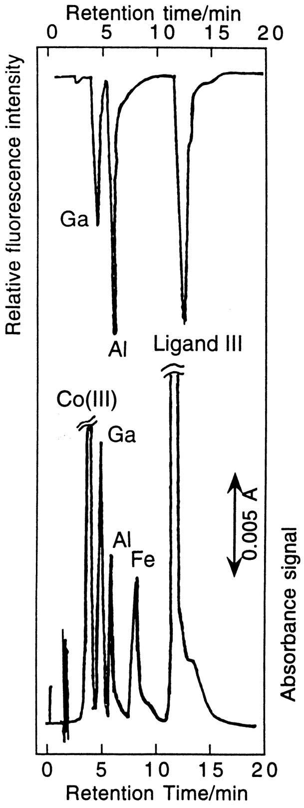


Fig. 6. Fluorimetric detection of Al and Ga chelates of hydrazone ligand III with a slightly acidic mobile phase. Sample: C_{metal} = 0.50 μM each of Al(III), Co(II), Cu(II), Fe(III), Ga(III), In(III), Mo(VI), Ni(II), V(V), Zn(II) ions, C_{ligand} = 40 μM, Tris-HCl buffer 10 mM at pH 7.6. The HPLC conditions for the fluorimetric detection are given in Table 1.

cause the existing HPLC methods are most often not compatible with simultaneous determinations of these cations.[3] In most cases, one could not afford to detect Al ion with sacrificing Ga ion and *vice versa*.

In contrast, ligand III is amenable to the fluorimetric detection of Al(III) and Ga(III) ions, as shown in Fig. 6. Since the strongly emissive species are the 1:1 chelates for both cations, HPLC separation is performed using a slightly acidic eluent. It has been found that even if the off-line complexation pH is adjusted at 7.6, where the 1:2 chelate is predominant, the fluorimetrically detected species is doubtless the 1:1 chelate when eluted with a slightly acidic mobile phase. The 1:1 chelate outlives the 1:2 one during elution; the latter is decomposed in

Table 3. Influence of Diverse Ions to the HPLC-Fluorimetric Detection Systems of Al and Ga Ions with Ligand III

	For Al ion[a)]		For Ga ion[a)]	
Ion[b)]	Molar ratio	Relative error/%	Molar ratio	Relative error/%
Na	1000	−0.6		2.2
K	1000	1.2		−2.7
Mg	1000	−2.4		−1.6
Ca	1000	3.0		−0.9
Fe(III)	100	−3.3		−4.4
Mn(II)	100	−1.8		0.9
Al	—	—	100	−0.8
Ga	100	−2.5	—	—
Co(II)	50	−1.0		−4.7
V(V)[c)]	50	−2.2		−3.5
Ni	50	2.1		−2.7
Zn	50	−0.3		2.9
Cu(II)	50	1.9		−2.2
B(III)[d)]	50	2.6		4.6

a) Added at 1.00×10^{-7} M, b) Added as chlorides, c) Added as ammonium metavanadate, d) Added as boric acid.

an acidic solution to give the kinetically inert 1:1 chelate. At pH 5.1, the quantitative formation of the 1:1 chelate is not accessible, unless this pH is approached from an alkaline side. This HPLC scheme is very beneficial to make a compromise between the different pH conditions relevant to the quantitative formation of the chelate (pH 7.6) and to sensitive fluorimetric detection (pH 5.1). In this case, the addition of TBABr at a low concentration, 50 μmolal, to an eluent with a decreased acetonitrile content of 20.0 wt% was effective to move the ligand peak behind the chelate ones. The fluorimetric detection limits for Al and Ga ions were 80 ppt (3.0 nM) and 90 ppt (1.3 nM), respectively, on a 3σ blank basis. Also, these detection limits were eventually restricted by the contamination levels. The influences from diverse ions in terms of a 5% toleration limit for Al and Ga ions are given in Table 3. The metal ions examined actually gave no interference in the molar ratio ranges over 50 towards 0.10 μM of Al and Ga ions.

Particularly, the fluorimetric detection system for Al ion is practicable in a clinical scene where Al ion is known to be fatal for renal-failure patients, even at a trace level.[21] Also, the detection of trace Ga in biological tissues and fluid is a pressing need because of the increasing use of radioactive isotopes, such as ^{67}Ga and ^{68}Ga, in diagnostic nuclear medicine.[22,23] In addition, the anthropogenic emission of Ga in the near future is suspected. Thus, in this connection, the HPLC method proposed here is promising for quantifying Ga ion in environmental samples, such as rain water, dry fall-out, and airborne particulates.[24–26]

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