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Enantiomeric purity determination of acetyl-L-carnitine by NMR with chiral lanthanide shift reagents

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Abstract

Enantiomer signal separation of acetyl-carnitine chloride was obtained on a 500 MHz Nuclear Magnetic Resonance (^{1}H NMR) analysis by fast diastereomeric interaction with chiral shift reagents such as chiral lanthanide-camphorato or chiral samarium-pdta shift reagents. Effects of the kinds of chiral shift reagents and the molar ratio of chiral shift reagent to acetyl-carnitine chloride on enantiomer signal separation were investigated and evaluated. Optimization of the experimental conditions provided two significant split signals for the enantiomers, leading to the successful quantitative analysis. Distinguishment of 0.5% of the minor enantiomer (D-form) in acetyl-L-carnitine chloride was found to be possible by ^{1}H NMR with tris[3-(heptafluoropropylhydroxymethylene)-D-camphorato] and praseodymium derivative, (Pr[hfc]$_3$), as chiral shift reagents.

Keywords: Enantiomer signal separation; Acetyl-L-carnitine; Nuclear magnetic resonance spectrometry; Chiral lanthanide shift reagents

1. Introduction

The on going trends in the pharmaceutical industry, many racemic drugs are remanufactured in a single enantiomer, because the antipodes of chiral drugs are regarded as one of the impurities from the viewpoint of quality control [1,2]. During the past few decades, several analytical methods, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), nuclear magnetic resonance (NMR) [3–12], etc. have been developed and successfully applied for the enantiomeric purity determination. Among them, NMR is a still useful method, although most of the enantiomer separation of chiral drugs has been performed by HPLC or CE.

In the NMR method, chiral derivatizing reagents (CDRs) or chiral shift reagents (CSRs) are employed for the enantiomer signal separation. (*R*) or (*S*)-2-methoxy-2-(trifluoromethyl)phenylacetic acid (MTPA) [7], which was introduced by Mosher, and (*R*)-(−)-2,2,2-trifluoro-1-(9-anthryl)ethanol [8] have been widely used as CSRs. Camphor-based chiral lanthanide shift reagents (CLSRs) are also useful for the determination of enantiomers [9]. (*S*)-2-methoxy-2-(1-naphthyl)propionic acid was developed and employed for determination of absolute configurations of alcohol [11]. In our previous work [12], we reported the capability of (+)-(*R*)-18-crown-6-tetracarboxylic acid (18C6H$_4$) as a CSR. The NMR method, especially CSR method, has advantages such as simplicity, rapid analysis, etc.

Acetyl-L-carnitine chloride (L-AC) has stimulatory actions of learning behavior from an ethopharmacology viewpoint [13]. It is subsequently expected to be useful as a cerebal metabolic [14]. On the other hand, the D-enantiomer does not exhibit these effects [15]. Therefore, an active enantiomer, L-form, has been developed as a drug in Italy. There are some reports concerning the HPLC determination of the D-enantiomer contained in L-AC [16–20]. In the NMR method, Voeffray et al. [21] reported enantiomeric purity of L-AC employing tris[3-(heptafluoropropylhydroxymethylene)-D-camphorato] europium(III) derivative ([Eu(hfc)$_3$]) as a

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CSR. As little as 0.5% of D-AC could be discerned, however, effects of the kinds of CSRs and its concentration on the enantiomer signal separation were not studied at that time. Furthermore, 0.5% of D-AC signal overlapped with L-AC signal in the report, therefore, previous report did not give us any information concerning quantitative analysis.

In this work, we systematically investigated the signal separation of DL-AC by NMR with various CSRs. Effects of the kinds and molar ratio of CSRs to DL-AC on the enantiomer signal separation were investigated. Optimization of experimental conditions provided two significant split signals for the quantitative use. Finally, a possibility of this method as an optical purity testing method of L-AC was also investigated.

2. Materials and methods

2.1. NMR equipment

^{1}H NMR spectra were taken in CD_3OD on Brucker DRX-500 FT-NMR spectrometer (Rheinstetten, Germany) operating at 500 MHz in the ^{2}H lock mode. Chemical shifts were reported in parts per million (ppm) relative to tetramethylsilane (TMS). Spinning tubes of 5 mm i.d. containing 500 μL of a solution were employed.

Experimental data was obtained in the following conditions:

Typical acquisition parameters: SWH (sweep width) of 8000 Hz; temperature of 27 °C; digital resolution of 0.1834 Hz/Point.
Typical processing parameters: WDW (window type) of EM (exponential multiplication); SSB (sine bell shift) of 0; LB (line broadening) of 0.30 Hz; GB (gaussian broadening) of 0.

2.2. Materials

The following eight kinds of CSRs were purchased from the commercial sources. Tris[3-(trifluoromethylhydroxymethylene)-D-camphorato], europium(III) derivative (Eu[tfc]$_3$), tris[3-(trifluoromethylhydroxymethylene)-D-camphorato], praseodymium derivative (Pr[tfc]$_3$), tris[3-(heptafluoropropylhydroxymethylene)-D-camphorato], praseodymium derivative (Pr[hfc]$_3$), and europium tris(d,d-dicampholylmethanate) (Eu[dcm]$_3$) were purchased from ACROS (Geel, Belgium). Tris[3-(trifluoromethylhydroxymethylene)-D-camphorato], ytterbium derivative (Yb[tfc]$_3$), tris[3-(heptafluoropropylhydroxymethylene)-D-camphorato], europium(III) derivative (Eu[hfc]$_3$) and tris[3-(heptafluoropropylhydroxymethylene)-D-camphorato], ytterbium(III) derivative (Yb[hfc]$_3$) were purchased from Aldrich Chemicals (WI, USA). Sodium [(*R*)-1,2-diaminopropane-*N*,*N*,*N*′,*N*′-tetraacetato]samarate(III) hydrate (Sm-(*R*)-pdta) was purchased from Tokyo Kasei (Tokyo, Japan). TMS, CD_3OD, D_2O and CD_3Cl were purchased from ISOTEC Inc. (OH, USA). Several batches of L-AC tested and DL-AC were obtained from Aldrich Chemicals.

2.3. Enantiomeric purity testing of L-AC

2.3.1. General procedure

Spinning tubes of 5 mm i.d. containing 500 μl of a solution were employed. The 2 mM (for 500 μl) of the testing compound (DL-AC or L-AC) was taken in the tube, and a Pr(hfc)$_3$ solution (10 mM, 500 μl) was added. Then, 5 μl of a 1% TMS solution used as the internal standard (IS) solution were added.

2.3.2. Determination of enantiomeric purity

Weigh accurately about 240 μg of L-AC (test sample), add 500 μl of a Pr(hfc)$_3$ stock solution, and add exactly 5 μl of the IS solution, shake vigorously for about 1 min, and use this solution as the sample solution. Separately, weigh accurately about 240 μg of DL-AC, add 500 μl of Pr(hfc)$_3$ stock solution, and add exactly 5 μl of the IS solution, shake vigorously for about 1 min, and use this solution as the standard solution. Perform the test with the sample solution and the standard solution as described above, and calculate the ratios, Q_T and Q_S, of the peak intensity of D-AC to that of the internal standard (TMS):

$$\text{D-AC content(\%)} = \frac{\text{amount (μg) of DL-AC (standard)}}{\text{amount (μg) of L-AC (test sample)}} \times \frac{Q_T}{Q_S} \times 50$$

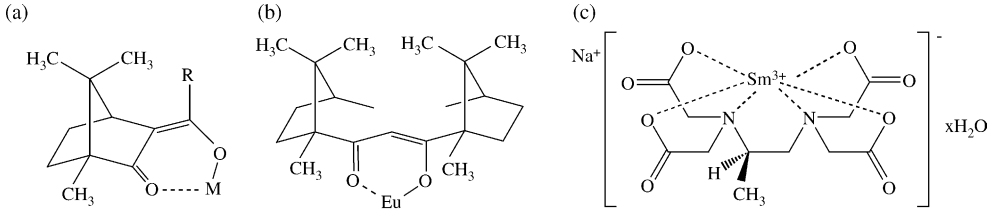
Pr(hfc)$_3$ stock solution: 147.8 mg of Pr(hfc)$_3$ in 10 ml of CD_3OD.

3. Results and discussion

3.1. NMR studies of chiral lanthanide-camphorato shift reagents (CLSRs)

Chiral discriminaton capability of Eu[hfc]$_3$, Yb[hfc]$_3$, Pr[hfc]$_3$, Eu[tfc]$_3$, Yb[tfc]$_3$ and Pr[tfc]$_3$ was investigated for enantiomer signal separation of DL-AC. Structures of CLSRs used are shown in Fig. 1(a) and (b). Two sets of N–CH_3 protons of DL-AC appeared in the ^{1}H NMR spectra in the presence of CLSRs (Eu[hfc]$_3$, Yb[hfc]$_3$ and Pr[hfc]$_3$). Each spectrum from the individual diastereomeric interaction complex that has formed, were shown in Fig. 2(b), (c) and (d). The N–CH_3 (δ 3.2 ppm, Fig. 2(a)) protons of the enantiomers of DL-AC were significantly separated into the two sets of resonance in the presence of CLSRs. Therefore, it seems that the N–CH_3 protons can be utilized to investigate the trend of the shift change in each enantiomer, and to measure the enantiomeric purity of the chiral compound.

The effect of the concentration of CLSRs (from 2 to 20 mM) on the change of chemical shift ($\Delta\delta$) and the



	M	R
Eu[tfc]$_3$	Eu	CF_3
Yb[tfc]$_3$	Yb	CF_3
Pr[tfc]$_3$	Pr	CF_3
Eu[hfc]$_3$	Eu	C_3F_7
Yb[hfc]$_3$	Yb	C_3F_7
Pr[hfc]$_3$	Pr	C_3F_7

Fig. 1. Structures of chiral shift lanthanide reagents investigated.

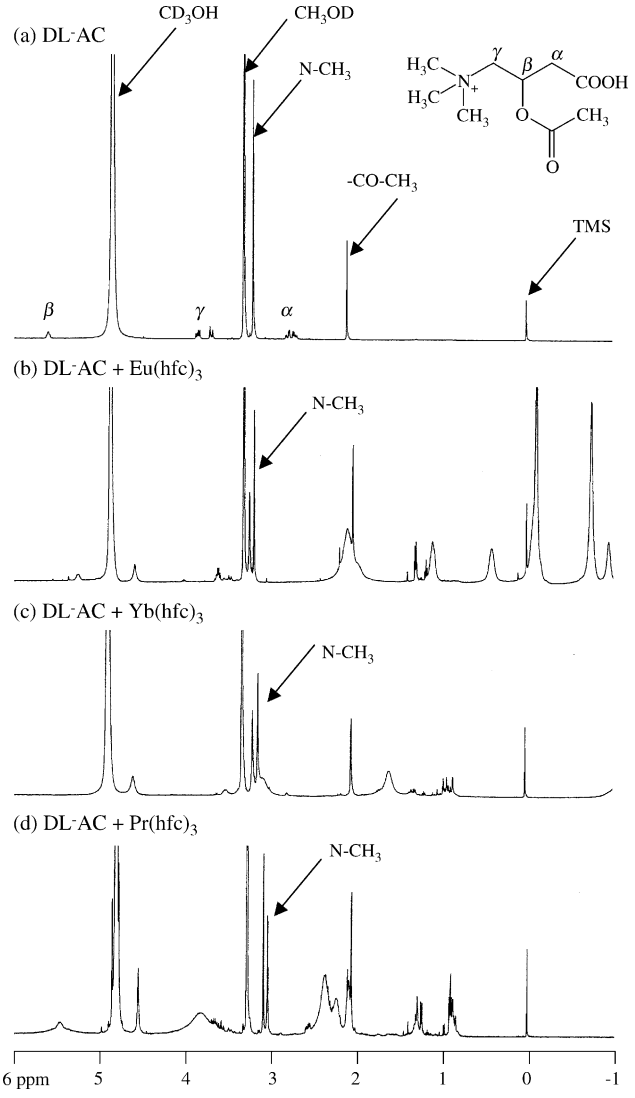


Fig. 2. ^{1}H NMR spectra of DL-AC (2 mM) and CLSRs (Eu[hfc]$_3$, Yb[hfc]$_3$ and Pr[hfc]$_3$, 5 mM each) mixtures in CD_3OD.

induced shift ($\Delta\Delta\delta$) between the diastereomeric complexes of DL-AC (2 mM) was investigated. The well separated singlet signal at $\delta = 3.2$ ppm (N–CH_3 protons) was evaluated. The results obtained from europium, ytterbium, and praseodymium derivatives, are summarized in Table 1. Both

Table 1
Shift of the methyl protons signals of mixtures of DL-AC (2 mM) with various molar ratio of CSRs

CSRs	Molar ratio	L-AC		D-AC		
		δ	$\Delta\delta$	δ	$\Delta\delta$	$\Delta\Delta\delta$
Eu(hfc)$_3$	1	3.189	−0.007	3.206	0.010	0.017
	2.5	3.196	0.000	3.248	0.052	0.052
	5	3.234	0.038	3.361	0.165	0.127
	10	3.302	0.106	3.529	0.333	0.227
Eu(tfc)$_3$	1	3.246	0.050	3.246	0.050	0.000
	2.5	3.313	0.117	3.313	0.117	0.000
	5	3.548	0.352	3.497	0.301	0.051
	10	3.910	0.714	3.787	0.591	0.123
Yb(hfc)$_3$	1	3.176	−0.020	3.155	−0.041	0.021
	2.5	3.207	0.011	3.144	−0.052	0.063
	5	–		–		
	10	–		–		
Yb(tfc)$_3$	1	3.316	0.120	3.316	0.120	0.000
	2.5	3.632	0.436	3.528	0.332	0.104
	5	–		–		
	10	–		–		
Pr(hfc)$_3$	1	3.183	−0.013	3.166	−0.030	0.017
	2.5	3.101	−0.095	3.051	−0.145	0.050
	5	2.946	−0.250	2.839	−0.357	0.107
	10	2.659	−0.537	2.476	−0.720	0.183
Pr(tfc)$_3$	1	3.136	−0.060	3.136	−0.060	0.000
	2.5	3.020	−0.176	3.038	−0.158	0.018
	5	2.852	−0.344	2.886	−0.310	0.034
	10	2.387	−0.809	2.621	−0.575	0.234
Sm-(*R*)-pdta	1	3.201	0.005	3.201	0.005	0.000
	2.5	3.219	0.023	3.234	0.038	0.015
	5	3.251	0.055	3.307	0.111	0.056
	10	–		–		

All chemical shifts reported in ppm relative to TMS at 27 °C. –: not assigned.

$\Delta\delta$ and $\Delta\Delta\delta$ values increased with an increase of the molar ratio.

The effects of three kinds of lanthanide in CLSRs (europium, ytterbium, and praseodymium derivatives) on enantiomer signal separation of DL-AC were compared. The signal for the N–CH_3 protons of the DL-AC was shifted to a low field by the europium derivatives (Eu[hfc]$_3$ and Eu[tfc]$_3$, see Table 1), whereas this signal was shifted to a high field by the praseodymium derivatives (Pr[hfc]$_3$ and Pr[tfc]$_3$, see Table 1). In general, these europium derivatives are commonly known as downfield reagents, while the corresponding praseodymium derivatives are employed as upfield reagents [3–5]. On the ytterbium derivatives, the signal for the N–CH_3 protons of the DL-AC was shifted to a low field as the same as europium derivatives in use of the Yb[tfc]$_3$, on the other hand, this signal was not significantly shifted by Yb[hfc]$_3$ (see Table 1).

The effect of two kinds of camphorate part in CLSRs, i.e., 3-(heptafluoropropylhydroxymethylene)-D-camphorato (hfc) and 3-(trifluoromethylhydroxymethylene)-D-camphorato (tfc), on the chemical shift ($\Delta\delta$ and $\Delta\Delta\delta$) was investigated in Eu[hfc]$_3$, Pr[hfc]$_3$, Eu[tfc]$_3$, Yb[tfc]$_3$ and Pr[tfc]$_3$. Yb[hfc]$_3$ was not evaluated in this section, because the signal for the N–CH_3 protons of the DL-AC was not significantly shifted by Yb[hfc]$_3$. The signal for the N–CH_3 protons of the D-AC was shifted greater than that of the L-AC by hfc, whereas the signal for the N–CH_3 protons of the L-AC was shifted to a greater extent compared with that of the D-AC by tfc. These trends are the effective information. The target signal (N–CH_3 protons of the D-AC) could be evaluated out of other signals (especially, N–CH_3 protons of the L-AC). By monitoring the change of the signal for the N–CH_3 protons of the D-AC, suitable conditions for the quantitative determination seem to be as follows: 5.0 molar equivalents (10 mM) of Pr[hfc]$_3$ to 2 mM of each substrate.

Eu[dcm]$_3$ (see Fig. 1(b)) was also employed for the investigation of the enantiomer signal separation of DL-AC. Eu[dcm]$_3$ is well soluble in deuterated chloroform, but is not soluble in deuterated water and methanol. On the other hand, DL-AC is soluble in deuterated water and methanol, but is not soluble in deuterated chloroform. Therefore, Eu[dcm]$_3$ was not applicable for this study.

3.2. NMR studies of chiral samarium-pdta reagents

Sodium [(*R*)-1,2-diaminopropane-*N*,*N*,*N*′,*N*′-tetraacetato]samarate(III) hydrate (Sm-(*R*)-pdta) is known to be able to resolve the enantiomer signals of α-amino acids on a NMR apparatus with less signal broadening than the corresponding europium(III) complexes [10]. The structure of Sm-(*R*)-pdta is shown in Fig. 1(c). Sm-(*R*)-pdta was investigated for the enantiomer signal separation of DL-AC. From the comparison of the ^{1}H NMR spectra of DL-AC in the presence and in the absence of Sm-(*R*)-pdta, it was found that two split sets of N–CH_3 proton signal of DL-AC revealed through the addition of Sm-(*R*)-pdta. These peaks observed in Fig. 3,

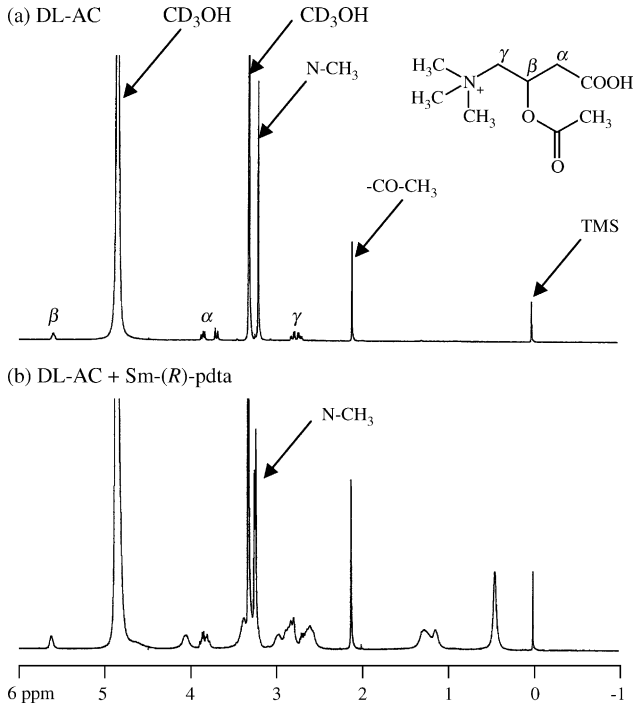


Fig. 3. ^{1}H NMR spectra of DL-AC (2 mM) and Sm-(*R*)-pdta (5 mM) mixtures in CD_3OD.

are a simple superposition of each spectrum from the individual diastereomeric interaction complex that has formed. The samarium derivative was also a downfield reagent, as the europium derivatives.

The effect of the concentration of Sm-(*R*)-pdta (from 2 to 20 mM) on the change of chemical shift ($\Delta\delta$ obs) of DL-AC (2 mM) was investigated. The well split singlet signal at $\delta = 3.2$ ppm (N–CH_3 protons) was evaluated. The results are summarized in Table 1. Sm-(*R*)-pdta afforded the shift of N–CH_3 protons of DL-AC. The signal for the N–CH_3 protons of the L-AC was shifted to a greater extent than that of the D-AC by Sm-(*R*)-pdta. Both $\Delta\delta$ and $\Delta\Delta\delta$ values increased with an increase of the molar ratio. In general, the enantiomer signal of the substrate obtained from samarium complexes is less signal broadening than that of the corresponding europium(III) complexes. However, in this case, the shape of the signal for the N–CH_3 protons of the DL-AC in the presence of Sm-(*R*)-pdta was similar with that in the europium derivatives.

3.3. Validation and application to the enantiomeric purity determination of L-AC (drug)

Enantiomeric purity testing of L-AC was investigated by employing NMR with CLSRs. It is important to investigate the molar ratio of chiral shift reagent to the substrate on the enantiomer signal separation. This is because, the signal for the N–CH_3 protons of the DL-AC is shifted with an increase of the molar ratio, and the CLSRs signal may overlap with the signal of the substrate. From the observation mentioned

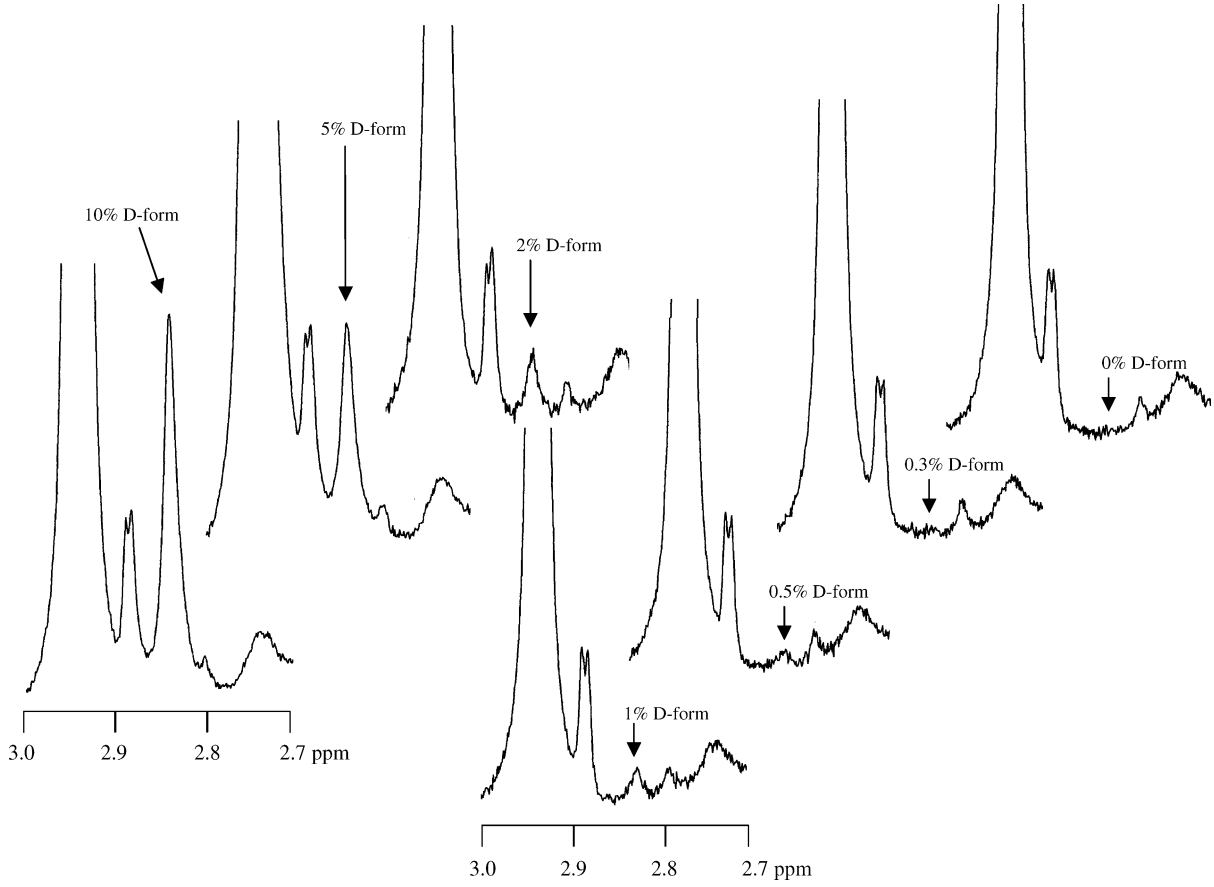


Fig. 4. Linearity and detection limit of D-form in L-AC.

above, suitable conditions for the quantitative determination of L-AC were fixed as follows: 5.0 molar equivalents of $Pr[hfc]_3$ (10 mM) to the substrate (2 mM).

Some validation data such as linearity and limit of detection (LOD) were obtained for the method. Linearity was investigated through the signal intensity ratio to TMS of D-form in the L-form standard. D-form content of the various sample solutions (prepared by mixing D-form in the range from 0.5% to 10.0% with the L-form standard) were examined. The relationship between the found values (y) and the theoretical values (x) was found to be straight ($y = 0.990x + 0.379$) (correlation coefficient = 0.991); good linearity was confirmed in the method (Table 2 and Fig. 4). The LOD of D-form was investigated by the visual (signal to noise) evaluation, and 0.5% D-form in L-AC could be determined. Through an increase of the concentration of an analyte, around 0.1% of LOD probably will be obtained.

Table 2
Linearity of D-AC added to L-AC

Theoretical values (%)	Found values (%)
0	0.000
0.5	0.599
1.0	1.142
2.0	3.004
5.0	5.985
10.0	9.854

Enantiomeric purity testing of three lots of L-AC from the commercial sources, was performed by the method. D-AC was not detected in all batches tested (i.e., not more than 0.5%). These results were the same with another analytical data (HPLC method by chiral stationary phases [22]).

4. Conclusion

Enantiomer signal separation capability of several NMR chiral shift reagents was investigated for DL-AC. The $N–CH_3$ protons of DL-AC were shifted selectively by adding chiral lanthanide-camphorato or chiral samarium-pdta shift reagents. The $N–CH_3$ proton signals of DL-AC were observed in the low field by using europium and samarium. On the other hand, the $N–CH_3$ proton signals in DL-AC were observed in the high field by using praseodymium. This behavior can be usable as a quality control method of the enantiomer purity of L-AC. The suggested approach proves advantages in regard to (a) avoidance of potential racemization because of no diastereomeric derivatization; (b) ease of obtaining chiral shift reagents from the commercial sources; (c) the easy

performance of quality testing within a short time. The NMR method with $Pr(hfc)_3$ as a chiral lanthanide shift reagent was successfully applied to determine the enantiomer purity of L-AC in the acceptable criteria (not more than 0.5%). However, for further application such as a new drug application to the authorities, a full validation data according to ICH guidelines will be required.

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