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Journal of Chromatography A, 727 (1996) 55–59

JOURNAL OF CHROMATOGRAPHY A

Novel approach to the measurement of enantiomeric purity by high-performance liquid chromatography using a polarized photometric detector equipped with a split cell

Atsushi Yamamoto[a,*], Akinobu Matsunaga[a], Kazuichi Hayakawa[b], Motoichi Miyazaki[b], Masayuki Nishimura[c]

[a] *Toyama Institute of Health, 17-1 Nakataikoyama, Kosugi-machi, Toyama 939-03, Japan*
[b] *Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa 920, Japan*
[c] *Shimadzu Corporation, 1 Nishinokyo-kuwabara-cho, Nakagyo-ku, Kyoto 604, Japan*

First received 19 July 1995; revised manuscript received 9 October 1995; accepted 9 October 1995

Abstract

A novel method for the high-performance liquid chromatographic measurement of enantiomeric purity by using only a polarized photometric detector (PPD) equipped with a split-type flow cell was developed. When the effluent flow-rate between the sample and reference sides of the split cell is disturbed, the basic property of the PPD as a photometric detector leads to two factors, absorptivity and optical activity, at the same time. D,L-Lactic acid in fermented milk was determined by applying the method based on this phenomenon. Using a copper(II)–hexanesulfonic acid mobile phase, lactic acid was separated from other carboxylic and amino acids on a reversed-phase column. The values measured by this method were in agreement with enzymatic results.

Keywords: Detectors, LC; Enantiomeric purity; Lactic acid

1. Introduction

A polarized photometric detector (PPD) is a non-modulated polarimetric detection system which measures the optical rotation of optically active compounds as a change in absorbance [1,2]. The development of a split cell for the PPD, in which the column effluent is simultaneously passed through both the sample and reference sides, enables one to determine the optical rotation of compounds at their absorption bands [3]. A PPD equipped with a split cell is superior in cost, performance and sensitivity to a polarimetric detector based on the Faraday effect, so the former may replace the latter.

For the application of the polarimetric detector in HPLC, the measurement of enantiomeric purity without chromatographic separation of enantiomers has been studied, where combined use with another detector is required [4,5]. Fundamentally, the PPD is a photometric detector, where the normal, single flow cell gives a signal with which the optical rotation and the absorbance of a compound are indistinguishable at the

* Corresponding author.

0021-9673/96/$15.00
SSDI 0021-9673(95)01154-4

absorption band. A split cell is able to cancel the change in absorbance, and gives a signal depending solely on the optical rotation. If one makes the effluent flow-rate between the sample and reference cells turbulent, however, it is thought that this unbalanced cell gives a signal superposed by two factors, i.e., the total amount of enantiomers present and their ratio. It might follow from this that a PPD with a split cell would enable one to measure enantiomeric purity without combined use with another detector, and the practical value of this detector would hence be improved.

The aim of this work was to establish a new theory for the determination of enantiomers using only a PPD with a split cell and to examine its applicability, with the determination of D,L-lactic acid in fermented milk as an example.

2. Theoretical

If the concentration of an analyte flowing into the flow cell follows the function $f(x)$, as indicated in Fig. 1, its amount in the sample cell, [S], for an arbitrary time t is given by

$$[\mathrm{S}] = \int_{t-c}^{t} f(x)\,\mathrm{d}x$$

where c represents the time required for the effluent to pass through the flow cell. Although both the sample and reference cells have the same volume, the velocity of the effluent flowing into each of them is controllable. When the ratio of this velocity is F (ref./sam.), then the concentration of the analyte flowing into the reference cell follows $Ff(x)$. The amount of the analyte introduced into the reference cell over a period c from time t is $F\int_{t-c}^{t} f(x)\,\mathrm{d}x$. If the analyte is not diffused in the flow cell, however, $F\int_{t-c-(c-cF)/F}^{t-c} f(x)\,\mathrm{d}x$ of the analyte (the unshaded portion at the reference cell in Fig. 2) remains in it when $F<1$. Consequently, the amount of the analyte in the reference cell, [R], is given by

$$[\mathrm{R}] = F\int_{t-c/F}^{t} f(x)\,\mathrm{d}x$$

The intensity of a light transmitted through the cell x, I_x, is given by

$$I_x = 10^{-\varepsilon[x]l}$$

where ε and l are the molar absorptivity of the analyte and cell length, respectively. The absorbance, Abs, from a detector equipped with a split cell can be expressed as

$$Abs = \log I_r/I_s = \varepsilon l([\mathrm{S}] - [\mathrm{R}]) \qquad (1)$$

When the width of the analyte elution band is much larger than the cell volume, [S] and [R] can be geometrically obtained as

$$[\mathrm{S}] = f(t)c - f'(t)c^2/2$$

$$[\mathrm{R}] = f(t)c - f'(t)c^2/2F$$

Substitution of these into Eq. 1 gives

$$Abs = \varepsilon l c^2(1-F)f'(x)/2F \qquad (2)$$

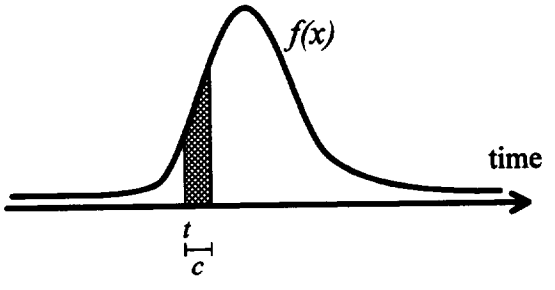

Fig. 1. Elution profile of an analyte.

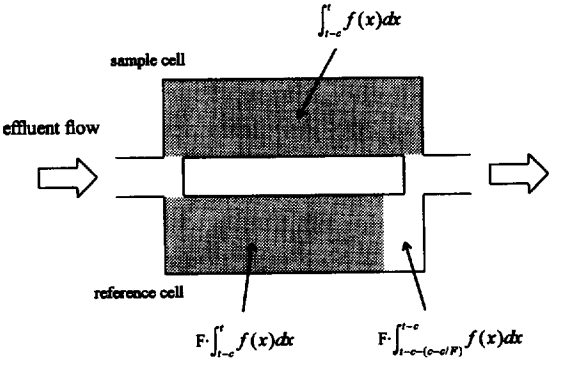

Fig. 2. Migration of the analyte zone in the split cell over a period of time c.

Eq. 2 implies that the peak shape obtained from the split cell to which the balance of the effluent flow is disturbed becomes a first-order differential curve of the shape obtained from the normal cell, when an analyte has no optical rotation at the absorption band. It is convenient to define a wave-like signal in terms of the deflection to the upside to the baseline, a, and that to the downside, b.

Next, we consider a case where the analyte has optical rotation. In a PPD, the angle of rotation, β, caused by the analyte in the flow cell, where two polarizers are placed on either side with a phase angle α, brings about a change in absorbance by $2\beta \log e \tan \alpha$ [2]. This change in absorbance, $\Delta\varepsilon$, is riding on the inherent absorbance, ε, of the analyte. In the case when $\alpha_s = -\alpha_r$, the total change in absorbance for each cell is expressed as

$$\varepsilon_s = \varepsilon + \Delta\varepsilon$$

$$\varepsilon_r = \varepsilon - \Delta\varepsilon$$

Similarly, substitution into Eq. 1 gives

$$Abs = \varepsilon l([S] - [R]) + \Delta\varepsilon l([S] - [R])$$
$$= \varepsilon l c^2(1 - F)f'(x)/2F + 2\Delta\varepsilon l c f(x) \quad (3)$$

As sketched in Fig. 3, the amplitude of the peak, $a + b$, is derived from the first term in Eq. 3 and is dependent on the amount of the analyte. The ratio of deflections, a/b, is dependent on the second term, i.e., the optical rotation. Consequently, the measurement of enantiomeric purity is possible by using only one detector without chromatographic separation of the enantiomers.

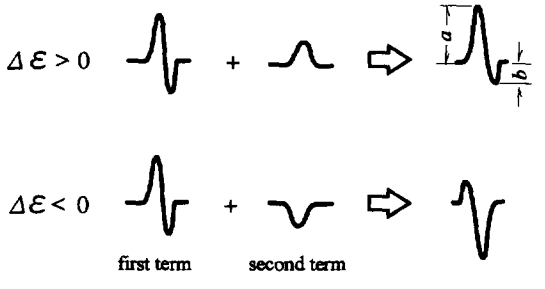

Fig. 3. Schematic formation models of the wave-like signal derived from Eq. 3.

3. Experimental

The LC system consisted of a Shimadzu (Kyoto, Japan) LC-10AD pump, a Rheodyne (Cotati, CA, USA) Model 7125 injector, a Tosoh (Tokyo, Japan) CO-8011 column oven and a Shimadzu SPD-10AV detector. The prototype split cell assembly was inserted into this detector, on which linear polarizers (Type HN32, 0.01 in. thick) obtained from Polaroid (Norwood, MA, USA) were placed. The phase angles of the two polarizers on the transmitting light side against that on the incident light side were set at ±0.9 rad (see Fig. 1 in Ref. [3] for the set-up of the split cell). The balance of the effluent flow to the split cell was disturbed by driving a small wedge made of Teflon into the outlet of the reference cell. The detection wavelength was 560 nm.

The chromatographic separation was performed on a 25 cm × 4.6 mm I.D. reversed-phase column (Capcell Pak C_{18}; Shiseido, Tokyo, Japan) maintained at 40°C. A mobile phase containing 5 m*M* sodium hexanesulfonate, 1 m*M* copper(II) acetate and 5 m*M* acetic acid (pH 4.3) was delivered at 0.8 ml/min.

All reagents were of guaranteed grade. Sodium hexanesulfonate and lithium D-lactate were purchased from Aldrich Japan (Tokyo, Japan), lithium L-lactate from Nacalai Tesque (Kyoto, Japan) and acetic acid and copper(II) acetate from Wako (Osaka, Japan). The mobile phase was filtered through a 0.45-μm membrane filter.

Five commercially available lactic acid beverages were analysed. The only pretreatment of the sample was dilution, followed by ultrafiltration through a Tosoh Ultracent-10 disposable cartridge. A 20-μl volume of the ultrafiltrate was injected onto the HPLC system.

Test-Combinations (Boehringer Mannheim,

Tokyo, Japan) were used for the enzymatic determination of D- and L-lactic acid.

4. Results and discussion

Although lactic acid shows no absorption in the visible region and its optical rotation is small, copper(II) provides a coloured complex with lactic acid showing an absorption spectrum with a maximum around 770 nm. The optical rotatory dispersion curve of this complex shows the Cotton effect in concert with the coloration [6]. We thought that the proposed detector might provide a first-order differential curve for lactic acid at this absorption band, which contains two factors, the total amount and the enantiomeric ratio.

Lu et al. [7] reported a chromatographic system in which α-hydroxycarboxylic acid–copper(II) complexes are retained and separated on a reversed-phase column. Examination of the mobile-phase composition made it clear that the carboxylic acids, such as tartaric or malic acid, eluted before lactate and amino acids eluted after lactate when hexanesulfonic acid was used as a ligand at ca. pH 4.3. Typical chromatograms of authentic lactic acid under these conditions are shown in Fig. 4. The proposed detector gave peak shapes following Eq. 3, i.e., the first-order differential curve for D-lactate is pushed upwards because it is dextrorotatory and that for L-isomer downwards because it is levorotatory. The detection wavelength was chosen as 560 nm, where the first and second terms in Eq. 3 are well balanced. It is difficult to measure $a + b$ with high accuracy at shorter wavelengths because the contribution of the second term becomes large. In contrast the difference in a/b becomes small at longer wavelengths. Essentially, the detection is performed at a wavelength where the specific rotation of the analyte is largest, and the peak shape should be adjusted by the balance of the effluent flow, F. The lactic acid–copper(II) complex showed approximately constant optical rotation in the

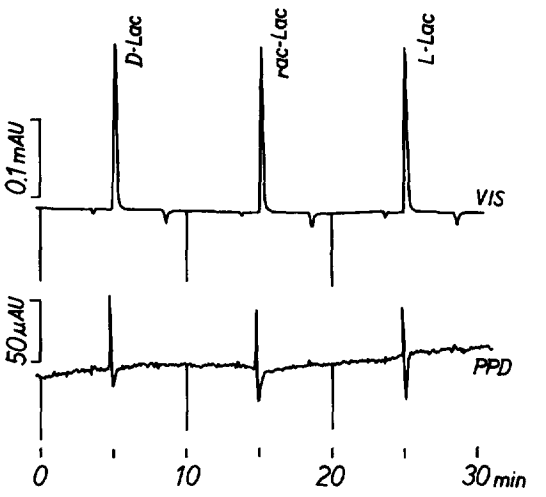

Fig. 4. Chromatograms obtained by using a PPD equipped with a split cell to which the balance of effluent flow was broken. Sample amounts injected were 200 nmol of lactic acid.

range 500–700 nm, but the peak shape was adjusted by the wavelength in this work.

Next, we evaluated the accuracy of two factors, absorbance and optical rotation, in the first-order differential curve. Various enantiomer ratios and concentrations of lactic acids were injected and a and b were measured. The enantiomeric purity did not affect the peak amplitude, $a + b$. When the amount injected was small, however, the measured value of $a + b$ varied widely. It is desirable to inject as large an amount as possible for the determination of enantiomeric purity with high accuracy by the proposed method. Plots of peak amplitude versus amount of lactate injected showed linearity up to 200 nmol under these conditions.

The effect of the enantiomeric purity on the peak division ratio with the total amounts of enantiomers injected kept constant at 200 nmol is shown in Fig. 5. The ordinate represents the ratio of a to $a + b$. The good linear relationship suggests that the enantiomeric purity of lactic acid can be calculated from a chromatogram produced by a PPD with a split cell.

The method established was applied to real samples. Fig. 6 shows the chromatograms of lactic acid beverages pretreated only by dilution

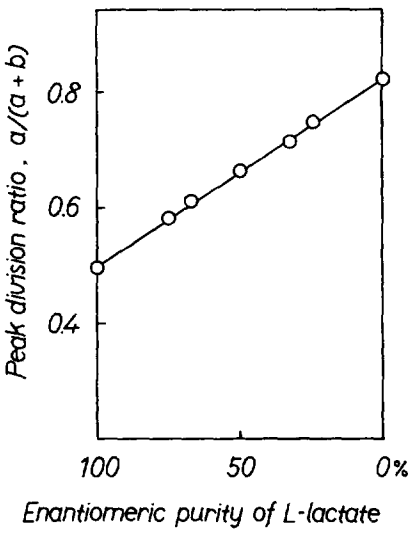

Fig. 5. Peak division ratio versus composition of enantiomer.

and ultrafiltration. The wave-like signals around 5 min after injection are lactic acid, from which the measurement of enantiomeric purity was feasible without interference from impurities. D,L-Lactate contents in these samples were also determined by the enzymatic method. The results obtained by both the proposed and enzymatic methods agreed well: Fig. 6A gives

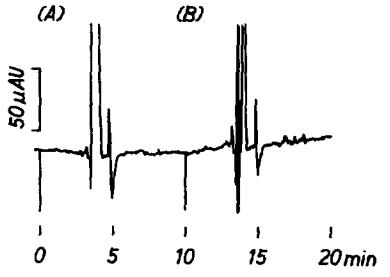

Fig. 6. PPD chromatograms of lactic acid beverages.

calculated values of 57 m*M* for L-lactate and 2 m*M* for D-isomer, whereas L- = 56.0 m*M* and D- = 3.7 m*M* were obtained by the enzymatic method. Similarly, L- = 28 m*M* and D- = 9 m*M* were obtained from Fig. 6B and L- = 28.4 m*M*, and D- = 6.7 m*M* by the enzymatic method, and three other samples gave similar results. The D-isomers in these samples might result from fermentation, because many kinds of lactic acid bacteria take part in the production of fermented milk and some bacteria produce optically inactive, racemic lactic acids.

The present method can determine the enantiomeric purity of a lactate using a simple system, which requires no physical separation of enantiomers and no combined use with other detectors. This system is suitable for the routine detection of D-lactate in fermented milk.

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