**Abstract**

In order to explore the possibility of determining whether clenbuterol (CLB) was ingested unintentionally via meat products contaminated with CLB or taken intentionally for doping purposes by athletes, we conducted an *in vitro* study assuming *in vivo* chiral conversion of CLB. Enzymatic reaction using swine liver tissue or chemical reaction in artificial gastric juice was performed to clarify the chiral conversion of CLB enantiomers. LC/UV measurement revealed no chiral conversion in the enzymatic reaction and temperature-dependent chiral conversion in the artificial gastric juice where CLB finally racemized. From the calculated reaction rate, activation energy ($E_a$), and activation entropy ($\Delta S$) in the chiral conversion reaction of $R$-CLB in artificial gastric juice, we expected that this chiral conversion would proceed slowly because $E_a$ was relatively high. After heating at 38°C for 2 h, only approximately 1% of CLB underwent chiral conversion. Therefore, when humans ingest meat products contaminated with CLB having a different enantiomeric ratio, chiral conversion hardly progresses in the stomach and such ingestion would have very little effect on the enantiomeric excess of CLB excreted in urine. This suggests that measuring urinary CLB enantiomeric ratio would reveal whether CLB was ingested unintentionally via CLB-contaminated meat products or taken intentionally.

**Keywords:** Clenbuterol; Sports doping; Veterinary drug; LC/UV

**1. Introduction**

Clenbuterol (CLB) has been used mainly as a selective adrenergic $\beta_2$ receptor agonist for the treatment of respiratory diseases. It has also been approved for use as a veterinary drug because of its tracheal smooth muscle relaxing action [1] and uterine relaxing action [2]. CLB has been employed as a feed additive for fattening livestock because it increases muscle growth [3]. If CLB-treated livestock is shipped without a sufficient drug withdrawal period, CLB may remain in livestock organs [4-6]. Food poisoning incidents due to residual CLB have occurred overseas [7,8] and there are concerns about damage to human health. On the other hand, CLB is also illegally used as a sports doping drug for increasing muscle mass. The World Anti-Doping Organization (WADA) has designated CLB as a prohibited substance for sports doping. However, CLB is also used for fattening livestock, so even if CLB is detected in an athlete's urine during a doping test, it is difficult to distinguish between unintentional ingestion via meat products contaminated with CLB and intentional intake for doping purposes.

CLB has one chiral center in its molecule and a pair of enantiomers (Fig. 1). CLB as a general pharmaceutical is marketed as a racemate, which is a 1:1 mixture of $R$-(–)-CLB and $S$-(+)-CLB.

**Fig. 1.** Chemical structures of clenbuterol enantiomers.

However, although the CLB enantiomers have the same physical and chemical properties, they have different effects on the living body due to the difference in optical rotation. The $\beta_2$ receptor stimulating action, which is the main action
of CLB, is due to R-CLB, and S-CLB has no such action [9]. On the other hand, S-CLB has β1 receptor blocking action, blood pressure lowering action, glucocorticoid rising action, and blood sugar rising action [10]. Furthermore, these enantiomers differ in bioavailability. Although pharmaceutical CLB is a racemate, it is presumed that the enantiomeric ratio of CLB is altered in livestock tissue. In fact, S-CLB more easily accumulates in animal tissue than R-CLB [11,12]. It was reported that S/R enantiomer ratio in swine liver, lung, and broiler liver each was approximately 3 [11], while the S/R enantiomer ratios in rat lung, liver, kidney, and muscle were 3.7, 4.8, 5.9, and 3.3, respectively [12]. Therefore, when humans ingest CLB via contaminated meat products, the enantiomeric ratio of renally eliminated CLB is expected to differ depending on the residual composition in the meat products [13].

It has been reported that patients who received therapeutic doses of CLB showed slightly predominant urinary excretion of the S(+)-enantiomer relative to the R(−)-enantiomer [14]. These phenomena offer the possibility of identifying whether CLB was ingested unintentionally via meat products contaminated with CLB or taken intentionally. It is expected that the enantiomeric ratio of CLB excreted in urine will differ between humans who unintentionally ingest CLB via contaminated meat products and those who intentionally ingest pharmaceutical racemic CLB [15-18]. Therefore, when humans ingest meat products contaminated with CLB having a different enantiomeric ratio, compared with taking racemic CLB for doping purposes, it is presumed that the enantiomeric ratio of CLB excreted in urine would be different as well.

We speculated that orally ingested CLB via contaminated meat products would undergo chiral conversion in the human body when it comes in contact with gastric juice in stomach or is affected by enzymatic reactions in liver. The following example is cited as one of the reasons for our speculation. Thalidomide has a pair of enantiomers like CLB and undergoes chiral conversion and racemization in the living body relatively quickly even when only the R-form is administered [19,20]. Therefore, in this study, in order to explore the possibility of discriminating whether CLB was ingested unintentionally via meat products contaminated with CLB or taken intentionally in the doping test on the basis of the difference in bioavailability of CLB enantiomers, an in vitro study assuming in vivo chiral conversion of CLB was performed. We investigated whether or not orally ingested CLB enantiomers undergo chiral conversion toward racemization. Swine liver tissue was used for the enzymatic reactions, because the S/R enantiomer ratio of CLB in swine liver was higher than that in other organs as described above. On the other hand, artificial gastric juice was used for assuming chemical reactions in stomach.

2. Experimental

2.1. Materials and reagents

R-(−)-CLB (chemical purity: 98%; ee: 95.2%) and S-(+)-CLB (chemical purity: 98%; ee: 99.4%) were purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Racemic CLB (RS-form; residual veterinary drug testing grade) was purchased from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan). Acetonitrile and methanol (both HPLC grade); hydrochloric acid (precision analysis grade); and metaphosphoric acid, potassium carbonate, sodium hydroxide, aqueous ammonium hydroxide, ethyl acetate, and n-hexane (all special grade) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Sodium chloride and formic acid (both special grade) were from Kanto Chemical Co., Inc. (Tokyo, Japan). Water was purified with a Milli-Q Gradient A10 system equipped with an EDS-PAK® polisher (Merck Millipore Ltd., Bedford, MA, USA). All other chemicals were of special grade. Swine liver was purchased from a local retail shop.

2.2. Preparation of standard solutions and artificial gastric juice

Racemic CLB standard stock solution (100 μg/mL) was prepared by dissolving 5.0 mg of CLB in 50 mL of purified water. Working solutions were prepared by diluting the stock solution with purified water to the appropriate concentrations (1, 2, 5, 10, 20, 50, and 100 μg/mL). Each CLB enantiomer (R-form and S-form) standard stock solution (100 μg/mL) was prepared with purified water in a similar manner. All standard stock solutions and working solutions were stored at 4°C until use.

2.3. LC/UV apparatus and operating conditions

A PU610C-10 pump system (GL Sciences, Inc., Tokyo, Japan) equipped with an SPD-10A (Shimadzu Corporation, Kyoto, Japan) was used. LC separation was performed with a Lux Cellulose-3 (250 × 4.6 mm i.d., 5 μm; Phenomenex, Torrance, CA, USA). Column temperature was maintained at room temperature (24°C). The mobile phase was a mixture of 10 mmol/L ammonium formate formate aqueous solution (pH 10.3) and methanol in the ratio of 37.5:62.5 (v/v), and was delivered in the isocratic elution mode at the flow rate of 0.5 mL/min. The UV detection wavelength was set at 264 nm. A 10 μL aliquot of the sample was injected into the system.

2.4. Chiral conversion of CLB by enzymatic reaction

As organ sample, 4.0 g of commercially available swine liver tissue was used. To this, 35 mL of physiological saline was added. The mixture was homogenized under ice-cooling and then diluted to 40 mL with physiological saline. A 10 mL aliquot of this solution was weighed and 1.0 mL of CLB (R-form and S-form) standard stock solution
(100 µg/mL) was added. The resultant solution was incubated for 24 h in a thermostatic bath set at 38°C. Thereafter, 55 mL of 2% aqueous metaphosphoric acid solution was added as the deproteinization reagent and the resultant solution was homogenized and filtered by suction filtration. To the filtrate were added 10 mL of 2 M aqueous sodium hydroxide solution, 10 g of potassium carbonate, and 10 g of sodium chloride. Next, 25 mL of ethyl acetate and 25 mL of n-hexane were added, and liquid-liquid partition extraction was performed. The organic phase was collected and 25 mL each of ethyl acetate and n-hexane was again added to the aqueous phase. The organic phase was collected after liquid-liquid partition extraction. The organic phase was combined and evaporated to dryness at 40°C in vacuo, and the remaining solvent was purged off by nitrogen gas blowing. The residue was redissolved in 2 mL of purified water to obtain a sample solution for LC/UV measurement.

2.5. Chiral conversion of CLB in artificial gastric juice

To 900 µL of artificial gastric juice was added 100 µL of each CLB enantiomer (R-form and S-form) standard stock solution (100 µg/mL). Then, the solutions were stored in a thermostatic bath at 38°C, 70°C, and 93°C each, and the time course of chiral conversion was monitored by LC/UV measurement. As control experiment, 100 µL of CLB standard solution (100 µg/mL, acetonitrile solution) was added to 900 µL of purified water and the solution was stored at 38°C, 70°C, and 93°C, and then measured in a manner similar to that described above.

3. Results and discussion

3.1. Enantiomeric analysis of CLB in meat sample

First, an enantiomeric determination method of CLB in swine liver tissue by LC/UV measurement was studied. As previously reported methods for the residual analysis of CLB in meat products, a solid-phase extraction (SPE) method with molecularly imprinted polymer (MIP) for sample pretreatment [21] and LC/MS/MS or GC/MS using stable isotope dilution methods [22,23] have been employed. However, because these methods are intended for the trace analysis of residual compounds, highly effective clean-up operations are required. In this study, we used relatively high concentrations of CLB to examine in vitro the presence or absence of chiral conversion, and employed only simple liquid-liquid partition extraction without purification by SPE with MIP. We used an LC/UV combination because the measurement does not require trace analysis and is minimally susceptible to sample matrix effects.

Commercially available chiral columns, namely, TCI Chiral MB-S, CHIRALPAK AD-RH, CYCLOBOND I 2000RN, CHIRALCEL OA, and Lux Cellulose-3, were examined for LC separation on a chiral column. Good enantioselective separation of R- and S-CLB was achieved (Rs = 3.1) when Lux Cellulose-3 with optically active maleimide polymer was used as the stationary phase. In contrast, R- and S-CLB were not separated in the other columns. As regards the mobile phase, 10 mM ammonium formate (pH 10.3)/methanol (37.5:62.5) was employed according to the already existing report [24].

3.2. Method validation

To confirm the validity of the developed analytical method, the limit of detection (LOD), the limit of quantification (LOQ), and the linearity were calculated using the standard solution. The LOD (S/N = 3) and the LOQ (S/N > 10) of CLB were 0.1 µg/mL and 0.5 µg/mL, respectively. The calibration curve showed good linearity (r = 0.998) in the range of 0.5–100 µg/mL.

Accuracy, intra-day, and inter-day precision tests were conducted using commercially available swine liver tissue. Racemic CLB standard solution was added to swine liver tissue and CLB concentration was adjusted to the low concentration level (10 µg/g) and the high concentration level (100 µg/g). Two replicate determinations at each concentration were carried out for five days each. Statistical analyses were performed using one-way analysis of variance. Accuracy (average recovery) at the low concentration level was 95.7% and that at the high concentration level was 91.0%. Intra-day and inter-day precision values at the low concentration level were 9.8% and 11.5%, respectively. On the other hand, those at the high concentration level were 6.7% and 11.3%, respectively. The representative chromatograms obtained at the high concentration level are shown in Fig. 2.

![Fig. 2. LC/UV chromatograms of CLB. (a) Racemic CLB standard solution (100 µg/mL), (b) blank (swine liver), and (c) swine liver sample to which CLB standard at 100 µg/g was added.](image-url)
3.3. Chiral conversion by enzymatic reaction using swine liver tissue

To investigate the biochemical effect on the chiral conversion of CLB by enzymes in swine liver tissue, enzymatic reactions were performed in vitro. To commercially available swine liver tissue that was pretreated according to Experimental Method 2-4 was added 100 µg/mL of each CLB enantiomer standard solution, and LC/UV measurement was conducted. The production of the corresponding enantiomer was not observed. Therefore, even if meat products contaminated with CLB having a difference in enantiomeric ratio were ingested, it was speculated that there would be little possibility of chiral conversion due to metabolic processes in the liver.

3.4. Chiral conversion of CLB by chemical reaction

3.4.1. Chiral conversion of CLB by heating

We examined whether chiral conversion occurred when an aqueous solution was heated without the addition of artificial gastric juice. Specifically, 0.5 mL of each CLB enantiomer standard solution at 10 µg/mL was incubated in a thermostatic bath set at 38°C and 70°C. After 72 h, we measured each CLB and found no production of the corresponding enantiomer. Therefore, it was confirmed that the chiral conversion of CLB does not occur by heating.

3.4.2. Chiral conversion of CLB in artificial gastric juice

Artificial gastric juice was added to each CLB enantiomer standard solution at 10 µg/mL and the time course of the chiral conversion was measured during incubation at 38°C, 70°C, and 93°C. We found that both R- and S-forms produced their corresponding enantiomers. Therefore, CLB underwent chiral conversion when artificial gastric juice was added to each CLB enantiomer standard solution and the solution was heated.

Fig. 3 shows the time courses of chiral conversion when artificial gastric juice was added to R-CLB and the solution was incubated at 38°C. Figure 4 shows the time courses of the chiral conversion rate at each temperature. This chiral conversion reaction was temperature-dependent and plateaued in the racemized state. The time required for CLB racemization at each temperature (38°C, 70°C, and 93°C) was approximately 1,000 h, 25 h, and 2–3 h, respectively. Based on these results, the assumed in vivo chiral conversion rate was examined. As human stomach temperature is 37–38°C and ingested food stays in the stomach for approximately 2 h, if chiral conversion does occur in the stomach, it is estimated that only approximately 1% of CLB would undergo chiral conversion.

3.4.3. Calculation of physicochemical parameters in chiral conversion of CLB in artificial gastric juice

The above-mentioned results indicated that the initial stage of the chiral conversion of CLB is a pseudo first-order reaction, as shown in Fig. 4. Then, in order to confirm the chiral conversion of R-CLB in artificial gastric juice, physicochemical parameters, such as reaction rate, activation energy (Ea), and activation entropy (ΔS), were calculated using the following formulas. When an Arrhenius plot (Fig. 5) of the chiral conversion of R-CLB was created and its physicochemical parameters were determined by using the equations shown below, Ea was 82.4 kJ/mol and ΔS was −101.5 J/K·mol.

\[ \ln k = - \frac{E_a}{RT} + \ln A \]

\[ \Delta S = (\ln A - \ln \frac{K_B T}{h})R \]

k: Reaction rate constant, A: Frequency factor, R: Gas constant (8.314 J/K·mol), T: Absolute temperature, \( K_B \): Boltzmann constant (1.381 × 10⁻²³ J/K), h: Planck constant (6.6261 × 10⁻³⁴ m²·kg/s)
3.4. Comparison with chiral conversion of thalidomide under in vitro conditions

Thalidomide, a representative drug that has enantiomers like CLB, was marketed and widely used as a hypnotic analgesic in the 1950s, but was later found to cause teratogenicity in early-term pregnant women [25]. Dr. Lenz issued a warning in November 1961 and sales in Japan were suspended in 1962. In 1979, it was found that only R-thalidomide exerts a hypnotic effect and only the S-form causes teratogenicity. However, it was reported in 1994 that administration of only the R-isomer resulted in relatively rapid chiral conversion and racemization in the animal body [19,20]. Furthermore, it was reported that the half-life ($T_{2/3}$) of thalidomide (time when $R:S = 2:1$) as an enantiomer in vitro under the conditions of 37°C at pH 7.4 was 566 min [26].

Therefore, the half-life for the chiral conversion of CLB was compared with that of thalidomide. The time when $R:S = 2:1$ in the conversion of R-CLB into S-CLB was calculated using the following equation.

$$T_{2/3} = \frac{\ln 1.5}{k}$$

$k$: Reaction rate constant

The results were 225 h (13,500 min) at 38°C, 11.25 h (675 min) at 70°C, and 112.5 min at 93°C.

4. Conclusions

CLB has been used improperly for fattening livestock and as a sports doping drug. Although CLB is designated as a prohibited substance, it is difficult to determine by a
doping test whether an athlete intended to take it for doping purposes or unintentionally ingested it via CLB-contaminated meat products. Therefore, it is important to develop a method for identifying whether CLB was ingested unintentionally via meat products contaminated with CLB or taken intentionally.

Chiral conversion did not occur in the enzymatic reaction using swine liver tissue, whereas temperature-dependent chiral conversion was observed in the chemical reaction using artificial gastric juice, which plateaued when the racemic state was attained. Furthermore, from the calculated physicochemical parameters, it was expected that this chiral conversion reaction would proceed slowly because $E_a$ was relatively high. In fact, it was found that only approximately 1% of CLB underwent chiral conversion after heating at 38°C for 2 h. In addition, as the half-life for the chiral conversion of CLB was markedly long, it was considered that when humans ingest CLB-contaminated meat products per os, chiral conversion in the stomach would not progress easily and the enantiomeric ratio of CLB in urine would be little affected.

From the above results, we expect that when CLB (in contaminated meat products or as a pharmaceutical) having a difference in enantiomeric ratio is ingested, a difference in the enantiomeric ratio of CLB would be detected in human urine as well.

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Conflict of interest
There are no financial or other relations that could lead to a conflict of interest.

Ethical approval
This article does not contain any studies with human participants or animals performed by any of the authors.

References
[1] Erichsen, D. F.; Aavid, A. D.; Schultz, R. H.; Kennedy, T.; J. Equine Vet. J. 1994, 26, 331-336.
[2] Menard, L.; Can. Vet. J. 1994, 35, 289-292.
[3] Reeds, P. J.; Hay, S.; Dorwood, P. M.; Palmer, R. M.; Br. J. Nutr. 1986, 56, 249-258.
[4] Pleadin, J.; Vulic, A.; Persi, N.; Vahcic, N.; Meat Sci. 2010, 83, 733-737.
[5] Pena, B. S.; Uribe, A.; Cordova, I. A.; Michel, A. M.; Res. J. Biol. Sci. 2008, 3, 1444-1445.
[6] Estrada-Montoya, M. C.; Gonzalez-Cordova, A. F.; Torrescano, G.; Camou, J. P.; Vallejo-Cordoba, B.; Cienc. Tecnol. Aliment. 2008, 6, 130-136.
[7] Barbosa, J.; Cruz, C.; Martins, J.; Silva, J. M.; Neves, C.; Alves, C.; Ramos, F.; Da Silveira, M. I.; Food Addit. Contam. 2005, 22, 563-566.
[8] Salleras, I.; Dominguez, A.; Mata, E.; Taberner, J. L.; Moro, I.; Salva, P.; Public Health Rep. 1995, 110, 338-342.
[9] Deutsch, D. A. V.; Abukhalaf, I. K.; Wineski, L. E.; Aboul-enein, H. Y.; Pitts, S. A.; Parks, B. A.; Oster, R. A.; Paulsen, D. F.; Potter, D. E.; Chirality 2000, 12, 637-648.
[10] Culmsee, C.; Junker, V.; Thal, S.; Kremers, W.; Maier, S.; Schneider, H. J.; Plesnila, N.; Kriegstein, J. Eur. J. Pharm. 2007, 575, 57-65.
[11] Smith, D. J. J. Agric. Food Chem. 2000, 48, 6036-6043.
[12] Hirosawa, J.; Ishikawa, M.; Ogino, M.; Ito, H.; Hirano, Y.; Yamada, H.; Asahi, M.; Kotaki, H.; Sai, Y.; Miyamoto, K.; Biopharm. Drug Dispos. 2014, 35, 207-217.
[13] Parr, M. K.; Blokland, M. H.; Liebetrau, F.; Schmidt, A. H.; Meijer, T.; Stanic, M.; Kwiatkowska, D.; Waraksa, E.; Sterk, S. S.; Food Addit. Contam. Part A. 2017, 34, 525-535.
[14] Gausepohl, C.; Blaschke, G. J. Chromatogr. B 1998, 713, 443-446.
[15] Dave, M.; Sauer, M. J.; Fallon, R. J. Analyst 1998, 123, 2697-2699.
[16] Yamamoto, I.; Iwata, K.; Nakashima, M. J. Pharmacol. 1985, 8, 385-391.
[17] Zalko, D.; Debraywer, L.; Bories, G.; Tulliez, J.; Drug Metab. Dispos. 1998, 26, 891-899.
[18] Soma, L. R.; Uboh, C. E.; Guan, F.; Moate, P.; Luo, Y.; Teleis, D.; Li, R.; Birks, E. K.; Rudy, J. A.; Tsang, D. S. J. Vet. Pharmacol. Ther. 2004, 27, 71-77.
[19] Tommy, E.; Sven, B.; Bodil, R.; Asa, F.; Peter, H.; Chirality 1998, 10, 223-228.
[20] Silas, W. S. Toxicol. Sci. 2009, 110, 4-30.
[21] Du, W.; Lei, C.; Zhang, C.; Bai, G.; Zhou, H.; Sun, M.; Fu, Q.; Chang, C. J. Pharm. Biomed. Anal. 2014, 91, 160-168.
[22] Wang, X.; Guo, T.; Wang, S.; Yuan, J.; Zhao, R. J. Anal. Toxicol. 2015, 39, 213-218.
[23] Jiang, Y.; Ni. J. Vet. Pharmacol. 2015, 38, 418-425.
[24] Valasco-Bejarano, B.; Bautista, J.; Noguez, M. O.; Camacho, E.; Rodriguez, M. E.; Rodriguez, L.; Drug Test Anal. 2017, 9, 1738-1743.
[25] Reist, M.; Carrupt, P. A.; Francotte, E.; Testa, B.; Chem. Res. Toxicol. 1998, 11, 1521-1528.
[26] Nishimura, K.; Hashimoto, Y.; Iwasaki, S.; Chem. Pharm. Bull. 1994, 42, 1157-1159.