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Enantioselective analysis of glufosinate using precolumn derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate and reversed-phase liquid chromatography

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Abstract

We have developed a new analytical method to quantify the DL-homoalanine-4-yl(methyl)phosphinate (DL-GLUF) enantiomers in biological specimens using a reversed-phase high-performance liquid chromatography system with a fluorescence detection system. The derivatization of DL-GLUF enantiomers with (+)-1-(9-fluorenyl)ethyl chloroformate was carried out under mild conditions (40 °C for 30 min) without inducing racemization. The lower limit of quantitation was 0.01 μg/ml for both D-GLUF and L-GLUF, and the detection limit was 5 ng/ml. When DL-GLUF enantiomers were added to serum to produce concentrations between 0.1 and 100 μg/ml, the mean recovery rate was at least 93.8%. The recovery rate from urine was also satisfactory.

Keywords: Enantiomer separation; Derivatization, LC; Glufosinate

1. Introduction

The liquid herbicide Basta®, which contains glufosinate (DL-homoalanine-4-yl(methyl)phosphinate; DL-GLUF), is a nonspecific phosphorus-containing amino acid-type herbicide registered for use as an agricultural chemical in 1984 [1]. Since then, its manufacture in Japan has increased as a result of the 1987 ban on the production and shipping of the highly toxic preparation paraquat, and there has been a concomitant increase in the number of cases of poisoning due to DL-GLUF ingestion [2–4].

In cases of DL-GLUF poisoning, after a latent period of 4–60 h, symptoms such as coma, apnea, and generalized convulsions appear. The ingestion of 100 ml or more of Basta is reported to result in serious illness [2–7]. One reason for the occurrence of such clinical symptoms is that DL-GLUF poisoning is thought to exert conflicting effects on the central nervous system, both inhibitory and stimulat-

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ory, but the details of the mechanism of action and the toxicokinetics are not clearly understood [8]. Various methods have already been reported for the analysis of DL-GLUF and other structurally related glyphosates [9], and applied to the analysis of DL-GLUF in human specimens [10,11]. In our studies on the toxic effects and toxicokinetics of DL-GLUF, we were intrigued by the racemic nature of DL-GLUF. The stereoisomers of racemic drugs often differ in their pharmacological activity and their determination in biological systems is important, as is the assessment of their optical purity [12]. To the best of our knowledge, there have been no reports on the analysis of the enantiomers of DL-GLUF in biological specimens. Therefore our first task was to determine the technique for the enantioselective analysis of DL-GLUF enantiomers in biological specimens. So far, two methods have been reported for enantioselective analysis using a standard DL-GLUF. One of those methods is the liquid chromatography–mass spectrometry (LC–MS) method [13], in which the DL-GLUF enantiomers are separated directly on a chiral column and examined by chemical ionization mass spectrometry at atmospheric pressure. The other is gas chromatography–mass spectrometry (GC–MS) analysis [14], in which the DL-GLUF enantiomers are separated on a chiral column after derivatization with trifluoroacetic anhydride and trimethyl orthoacetate. However, both of these methods require a special type of chiral column.

In the present investigation, we first derivatized enantiomers in biological specimens into diastereomers using (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) and then separated them and determined their amounts using a common reversed-phase high-performance liquid chromatography (HPLC) system. Specifically, the method was used to analyze serum obtained from a patient who had ingested Basta.

2. Experimental

2.1. Chemicals and solutions

DL-GLUF was purchased from Wako (Osaka, Japan). Before use, it was confirmed by GC–MS analysis [14] that this standard substance consisted of the D form and the L form in equal proportions. L-AMPB (L-GLUF) was donated by Meiji Seika (Tokyo, Japan), and a mixed standard solution of 17 amino acid enantiomers (0.1 *M*) and 18 m*M* acetone solution of FLEC was purchased from Sigma (St Louis, MO, USA). The other reagents were for HPLC use or of special grade.

2.2. Biological specimen collection

The standard human serum specimens used for D-GLUF and L-GLUF recovery were purchased from Sigma. The urine specimens used to determine D-GLUF and L-GLUF recovery rates were from healthy adult volunteers (five men and five women) who gave prior written informed consent for study participation.

2.3. Sample preparation

Four hundred microliters of 0.1 *M* borate buffer solution, pH 8.5, was added to a 100-μl serum sample or to a 100-μl urine sample previously diluted 1:10 with 0.1 *M* borate buffer solution (pH 8.5), and then acetone 1 ml was added. After stirring, the resulting mixture was centrifuged at 2500 *g* for 5 min, and 50 μl of the resulting serum or urine mixture was removed from the supernatant and used as a sample for derivatization.

2.4. Derivatization

To 50 μl of each supernatant, 200 μl of 0.1 *M* borate buffer solution, pH 8.5, was added, followed by 50 μl of 18 m*M* FLEC-acetone solution, and the resulting mixture was agitated. The mixture was stored for 30 min at 40 °C, and derivatization was performed. Ethyl acetate (500 μl) was added, the mixture was shaken for 3 min, and excess FLEC (residual FLEC) was extracted in the ethyl acetate phase. Then 2 μl of the aqueous phase was applied to the HPLC column.

2.5. HPLC conditions

The HPLC instrument (SCL10AVP; Shimadzu, Kyoto, Japan) was adjusted so that the mobile phase

of 10 m*M* ammonium acetate (pH 5.0)/acetonitrile at a ratio of 77:23 had a flow-rate of 0.8 ml/min. Infused samples were separated on an Inertsil ODS 2 column (4.6 mm×150 mm, 5-μm particle size; GL Sciences Inc., Tokyo, Japan) at 40 °C. Fluorescence detection (model RF-10AXL, Shimadzu) was carried out at an excitation wavelength of 260 nm and emission wavelength of 305 nm. Quantitation was carried out using the absolute calibration curve method using analysis software (Class VP; Shimadzu).

2.6. LC–MS conditions

LC–MS structural analysis of the FLEC derivative of DL-GLUF enantiomers was performed under the following conditions: HPLC, Alliance 2690 (Waters, Milford, MA, USA); MS, platform LCZ (Micromass, Manchester, UK); ionization method, electrospray ionization (ESI) method; corona voltage, 3 kV; source temperature, 130 °C; probe temperature, 450 °C; and cone voltage, 20 V (positive and negative). The mobile phase composition, flow-rate, column type and temperature were identical to the HPLC conditions specified above.

2.7. Recovery test

Ten microliters of DL-GLUF standard solution was added to 1 ml of standard serum, and the mixture was adjusted so that the concentrations of both D-GLUF and L-GLUF were 0.1, 1.0, 10.0 and 100.0 μg/ml. Then both D-GLUF and L-GLUF at concentrations of 1, 10, 100, and 1000 μg/ml were added to urine samples collected from healthy adult volunteers. These concentrations reflect those seen in DL-GLUF poisoning patients [8]. After preparation and derivatization were carried out, the absolute calibration curve method was used to determine the DL-GLUF enantiomer concentration, and the DL-GLUF enantiomer values for blank samples of serum and urine to which DL-GLUF had not been added were subtracted. DL-GLUF standard solution was derivatized, and the recovery rate was calculated relative to the measured control values (serum, $n=5$; urine, $n=10$).

2.8. Clinical application

The method described here was employed for the analysis of the serum DL-GLUF level and the gastric lavage fluid level from a 69-year-old female patient with DL-GLUF poisoning who had ingested approximately 100 ml of Basta fluid (DL-GLUF content, 18.5 g) in a suicide attempt. Gastric lavage was performed promptly, and the lavage fluid was analyzed. On admission to hospital, the patient was lucid, but 14 h after poison ingestion, spontaneous respiration suddenly ceased, and consciousness became impaired. She was therefore managed with artificial ventilation. Thereafter she improved steadily, and on the fourth day of hospitalization was weaned from the respirator. Hemodialysis and blood adsorption procedures were not carried out.

Serum samples were obtained 4, 8, 14, 24, and 48 h after poison ingestion and subjected to immediate analysis. The third sample collection (at 14 h) corresponded with the cessation of spontaneous respiration.

The toxicokinetic parameters of DL-GLUF enantiomers were calculated using the following two formulae based on the blood concentrations of the enantiomers and the time since ingestion:

1. In the one-compartment model:

   $$C = C^0 \exp(-kt)$$

   where C represents the concentration of GLUF in the systemic circulation at time t, C^0 is the intercept on the y-axis for the exponential segment of the curve in the equation; and constant k is the first-order rate constant for the elimination phase. The area under the concentration–time curve (AUC) and the elimination half-life ($t_{1/2}$) were calculated as $\mathrm{AUC}=C^0/k$ and $t_{1/2}=\ln 2/k$, respectively.
2. In the two-compartment model:

   $$C = A \exp(-\alpha t) + B \exp(-\beta t)$$

   where A and B are the intercepts on the y-axis for the exponential segment of the curve in the equation, and constants α and β are the hybrid first-order rate contents for the distribution phase and the elimination phase, respectively. The

AUC, the distribution half-life ($t_{1/2\alpha}$), and the elimination half-life ($t_{1/2\beta}$) were calculated as AUC$=C^0/k$, $t_{1/2\alpha}=\ln 2/\alpha$, and $t_{1/2\beta}=\ln 2/\beta$, respectively.

3. Results and discussion

3.1. Derivatization of DL-GLUF enantiomers

The reaction dynamics of the derivatizations carried out at 25 and at 40 °C with FLEC in a standard solution containing 100 μg/ml of DL-GLUF enantiomers are shown graphically in Fig. 1. At both temperatures, the reaction dynamics of D-GLUF and L-GLUF were similar. Derivatization reactions carried out at 40 °C produced the highest derivative yields at 30 min, and the relative standard deviation (RSD) of its area was 0.8% ($n=5$). Moreover, DL-GLUF derivatives were stable for 48 h at room temperature. At 25 °C, the reactions required 90 min, and the RSD was 9.8%, thus showing some scattering of results. The derivatization reaction dynamics of DL-GLUF enantiomers yielded results identical to those obtained in experiments in which DL-GLUF was added to actual serum samples. On the basis of these results, we set the derivatization conditions at 40 °C and 30 min.

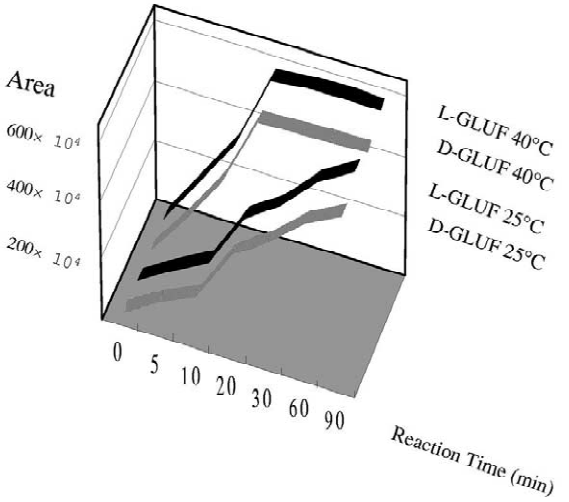

Fig. 1. Peak areas for DL-GLUF enantiomers as a function of the derivatization reaction.

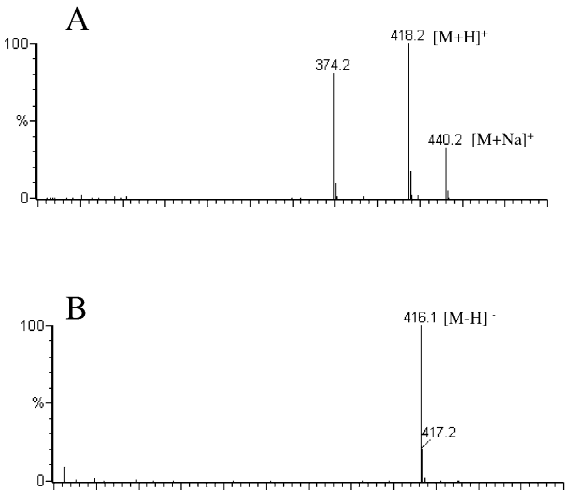

Fig. 2. ESI-mass spectra of FLEC derivatives of DL-GLUF. (A) Positive ion mode. (B) Negative ion mode. DL-GLUF enantiomers showed the same mass spectra.

Next, the molecular masses of the DL-GLUF enantiomer derivatives were determined by LC–MS analysis (ESI method). Fig. 2 shows ESI mass spectra of FLEC-derivatized DL-GLUF. The ESI mass spectra of D-GLUF and L-GLUF derivatized with FLEC were the same, and m/z 418.2 $[M+H]^+$ and m/z 416.1 $[M\text{-}H]^-$ base ions were obtained in the positive and negative mode, respectively. Therefore the molecular masses of the derivatives were thought to be between 416 and 418. From these results, it was concluded that diastereomers were formed through single-molecule amide binding of FLEC to the DL-GLUF amino acid bases (Fig. 3). In addition, this precolumn enantioselective derivatiza-

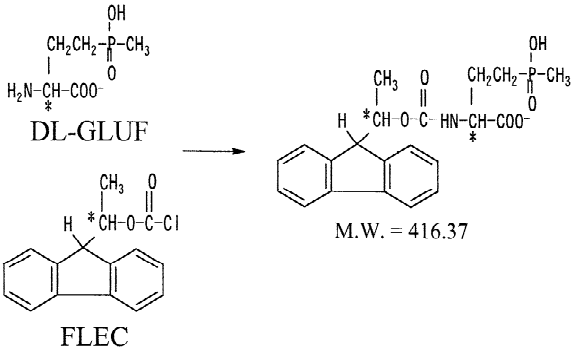

Fig. 3. Structure of derivatized DL-GLUF. *Chiral center.

tion technique can be employed for LC–MS analysis.

Analysis of a standard solution of L-GLUF confirmed that racemization from the L form to the D form did not occur during the derivatization reaction using FLEC at 40 °C within a 60-min period. In addition, analysis of the standard DL-GLUF solution, which had been confirmed to contain a D form to L form ratio of 1:1, demonstrated that racemization from the D form to the L form did not occur since the ratio of the areas in chromatograms of D-GLUF derivatized with FLEC and of L-GLUF derivatized with FLEC was also 1:1.

Regarding the relationship between the derivatization reaction and the reagent pH, when 0.1 *M* borate buffer was adjusted to pH 8, pH 8.5, and pH 9 with 0.1 *M* hydrochloric acid, the areas obtained in the analysis showed no changes attributable to the differences in pH, and therefore the pH was set at 8.5.

In relation to the concentration of the FLEC-acetone solution, when 100 μg/ml of DL-GLUF standard solution was derivatized, the RSD of the area at a concentration of 1.8 m*M* was 1% or less. However, when DL-GLUF was added before derivatization to normal human serum and specimens of urine obtained from 10 healthy individuals and was diluted 1:10 to lower the RSD to 2% or less, a FLEC-acetone concentration of 9 m*M* was required. Therefore, a concentration of 18 m*M* was used.

When the derivatization reaction is complete, the excess reagent and the hydrolysis byproduct are removed from the aqueous phase by ethyl acetate extraction, and the sample is then ready for separation.

3.2. HPLC analysis

Fig. 4 shows chromatograms of fluorescence detection of standard DL-GLUF solution derivatized with FLEC and separated on an Inertsil ODS column. The retention factor (k' value) of the FLEC-derivatized D-GLUF peak was 4.68, while that of the FLEC-derivatized L-GLUF peak was 5.21, as confirmed by analysis of FLEC derivatization of L-GLUF standard solution. Peak 3 in Fig. 4 is that of the unreacted FLEC.

For the calibration curve of D-GLUF, the straight line $y = 1\,097\,787x + 2\,036\,935$ was obtained for D-GLUF concentrations between 0.01 and 100 μg/ml, with a correlation coefficient of $r = 0.997$. The same straight line, $y = 1\,227\,342x + 1\,736\,113$, was derived for the calibration curve of L-GLUF, but with a correlation coefficient of $r = 0.998$. The lower limit of quantitation was 0.01 μg/ml and the detection limit was 0.005 μg/ml with the signal-to-noise ratio set at 5.

It has been reported that FLEC converts amino acid enantiomers to diastereoisomeric derivatives under alkaline conditions [12]. A standard solution containing a mixture of 17 amino acids was adjusted so that each amino acid was present at a concentration of 2 m*M* and then analyzed for FLEC derivatization. The peaks of FLEC-derivatized DL-

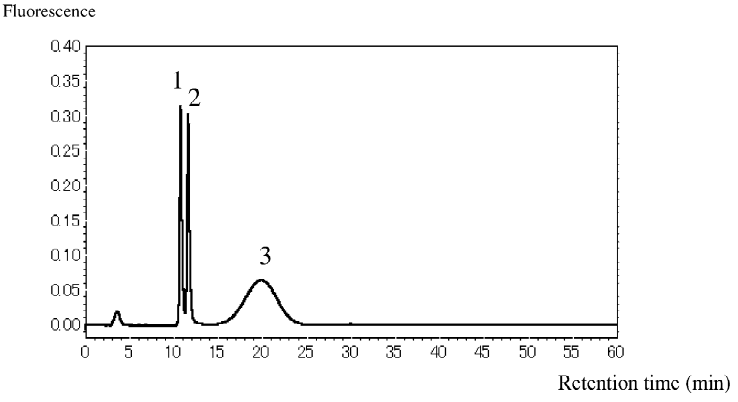

Fig. 4. Separation of the DL-GLUF enantiomer. The derivatization was carried out on DL-GLUF standard with the FLEC reagent. 1, D-GLUF (2.66 ng); 2, L-GLUF (2.66 ng); 3, FLEC.

Table 1
Recovery of DL-GLUF enantiomers from biological specimens

Added[a]	Serum specimen				Urine specimen			
	D-GLUF		L-GLUF		D-GLUF		L-GLUF	
	Recovery[b]	RSD (%)	Recovery[b]	RSD (%)	Recovery[c]	RSD (%)	Recovery[c]	RSD (%)
1000	–	–	–	–	100.2±0.8	0.8	99.7±1.0	1.0
100	98.6±11	1.1	98.7±1.2	1.2	99.6±1.1	1.1	98.4±1.1	1.1
10	96.6±1.5	1.6	97.3±1.6	1.6	98.5±1.5	1.5	97.9±1.2	1.2
1	95.3±1.8	1.9	96.7±1.8	1.9	97.3±2.0	2.1	97.5±1.9	1.9
0.1	93.8±3.5	3.7	95.1±3.4	3.6	–	–	–	–

[a] Amounts are expressed as μg/ml of specimen.
[b] Values are means (SD), $n=5$.
[c] Values are means (SD), $n=10$.

GLUF enantiomers were confirmed to be clearly separated from the peaks of the amino acid components.

3.3. Recovery of GLUF from clinical specimens

The recovery rates of DL-GLUF after addition to standard human serum samples are presented in Table 1. The recovery rates from serum were at least 93.8%. In the case of urine specimens to which DL-GLUF enantiomers were added at concentrations of 1.0, 10.0, 100.0, and 1000.0 μg/ml, the recovery rates were at least 97.3%.

3.4. Clinical application

D-GLUF (778.5 mg/ml) and L-GLUF (760.8 mg/ml) were detected in 3000 ml of gastric lavage fluid 4 h after the patient had ingested 100 ml of Basta fluid. A typical chromatogram of a serum sample that was collected 8 h after Basta fluid ingestion and subjected to FLEC derivatization is presented in Fig. 5, which demonstrates that this analytical method permits an excellent separation of DL-GLUF enantiomers from other serum components.

Fig. 6 plots the time after Basta fluid ingestion against the serum concentrations of DL-GLUF en-

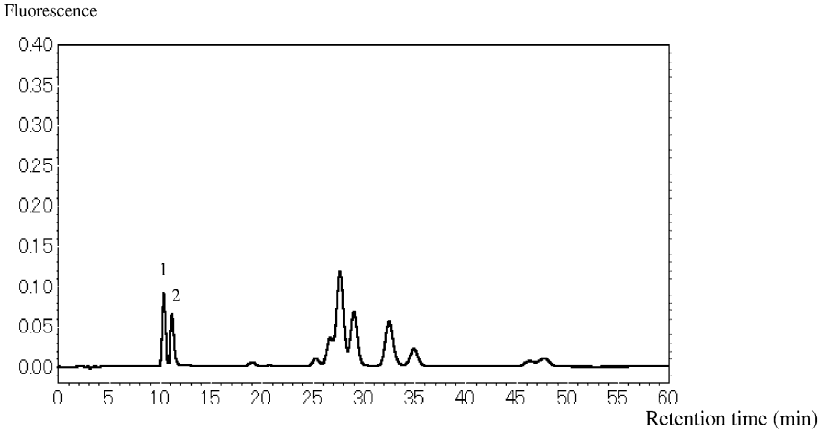

Fig. 5. Typical chromatogram of FLEC derivative of the serum from a patient with acute DL-GLUF poisoning. 1, D-GLUF (0.66 ng); 2, L-GLUF (0.49 ng).

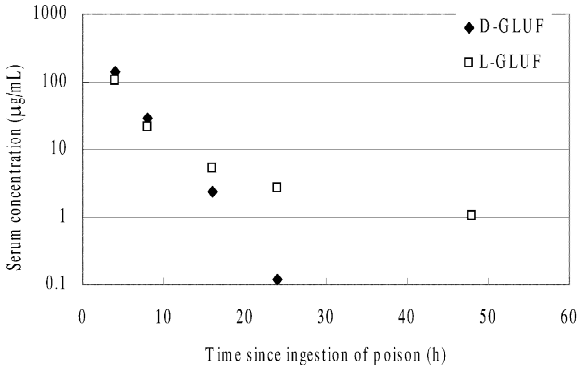

Fig. 6. Changes in serum concentration of DL-GLUF enantiomers in a patient who ingested Basta fluid. The patient swallowed 100 ml of Basta fluid with the intention of committing suicide, and was brought to the hospital 4 h later. On arrival, she was lucid, and 778.5 μg/ml of D-GLUF and 760.8 μg/ml of L-GLUF were found in the gastric lavage fluid. Fourteen hours after the ingestion, spontaneous respiration suddenly failed, consciousness became impaired, and artificial ventilation was administered. Blood dialysis and adsorption were not performed, but on the fourth day of the patient's illness, extubation was carried out, and thereafter, she recovered steadily.

antiomers. D-GLUF was measured for 24 h, and by 48 h the level fell below the detectable limit, exhibiting a one-compartment model of elimination dynamics. However, it was possible to carry out L-GLUF measurements for 48 h, and the elimination dynamics followed a two-compartment model. The toxicokinetic parameters of D-GLUF and L-GLUF are given in Table 2. The AUC for D-GLUF was 1592.8 h μg/ml, and that for L-GLUF was 1278.3 h μg/ml. Although the values were similar, the elimination half-life of L-GLUF was clearly longer than that of D-GLUF, suggesting that the two GLUF enantiomers have different rates of transfer to the tissues.

Table 2
The toxicokinetic parameters for DL-GLUF enantiomers in this patient

	D-GLUF	L-GLUF
Area under the concentration–time curve (AUC) (h μg/ml)	1592.8	1278.3
Elimination half-life ($t_{1/2}$; h)	2.00	–
Distribution half-life ($t_{1/2\alpha}$; h)	–	3.61
Elimination half-life ($t_{1/2\beta}$; h)	–	17.33

D-GLUF showed the elimination dynamics of a one-compartment model, and L-GLUF, those of a two-compartment model.

The symptoms of DL-GLUF intoxication, including delayed-onset impairment of consciousness and inhibition of respiration, are also caused by bialaphos preparations that generate L-GLUF by decomposition [15]. It is our hypothesis that the main cause of the central nervous symptoms resulting from DL-GLUF poisoning is L-GLUF. However, to confirm this hypothesis, the following would be necessary:

1. clarification of the toxicokinetic parameters of DL-GLUF by analyzing the concentrations of DL-GLUF enantiomers in the serum and urine of multiple DL-GLUF poisoning victims; and
2. determination of whether D-GLUF and L-GLUF play equal or differing roles in delayed-onset central nervous symptoms in animal experiments.

4. Conclusions

Since the first report of chromatographic separation of optical isomers [12], numerous methods have been introduced. The methods can be divided into three groups: (1) direct separation on a chiral column; (2) separation on an achiral column with chiral mobile phases; and (3) separation of diastereomers formed by precolumn derivatization with chiral reagents. The techniques previously used to analyze DL-GLUF enantiomers were LC–MS [13] and GC–MS [14], which both use chiral columns. We avoided the use of a chiral column or a chiral mobile phase and devised a method to analyze DL-GLUF enantiomers in clinical specimens using a common analytical system. Our HPLC analytical technique has the following useful features:

1. Derivatization is achieved rapidly under mild conditions (40 °C for 30 min).
2. No racemization between DL-GLUF enantiomers occurs during the process of derivatization.
3. The FLEC-derivatized DL-GLUF enantiomers are stable for a long time (48 h) at room temperature, and therefore multiple specimens can be derivatized in a single session.

4. The lower quantitative limit of the calibration curve is 0.01 μg/ml, calculated as the GLUF concentration in a specimen.
5. When D- and L-GLUF enantiomers are added to serum samples to produce concentrations between 0.1 and 100 μg/ml of each, the mean recovery rate is at least 93.8%. After their addition to urine at concentrations between 1.0 and 1000 μg/ml each, the mean recovery rate is at least 97.3%.

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