Fluorous Derivatization Method for Selective Analysis of Curcumin with Liquid Chromatography-Tandem Mass Spectrometry

Shimba KAWASUE, Yohei SAKAGUCHI, Ena YANO, Tadashi HAYAMA, Reiko KOGA, Hideyuki YOSHIDA, Hitoshi NOHTA*

Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Johnan, Fukuoka 814-0180, Japan.

1. Introduction
Curcumin, which is a yellow pigment used as a food coloring, is containing in the rhizome of the turmeric (Curcuma longa) of the ginger family. Curcumin is known to show antioxidative, anti-inflammatory, and antitumor actions with inhibition of NF-κB activity [1]. Furthermore, it has been reported that curcumin suppresses the aggregation of β-amyloid, and it is thought to may be helpful as effective therapy of Alzheimer's disease [1-3]. Although determination of curcumin in biological samples provides useful information for study of its effectiveness to certain diseases, the related studies, such as blood pharmacokinetics, are limited with poor bioavailability of curcumin due to poor water solubility, low absorption, and rapid elimination in physiological conditions [4-10]. Therefore, for systemically study of in vivo kinetics of curcumin, highly sensitive analytical methods are required. Liquid chromatography (LC) with mass spectrometry (MS) or tandem MS (MS/MS) is excellent in sensitivity and selectivity, and some reports demonstrated that it is suitable for analyzing trace amounts of curcumin in biological samples [11-18].

In this study, for realizing more sensitive and selective determination of curcumin in biological samples, we have developed a fluorous derivatization method for analysis of curcumin with LC-MS/MS. The fluorous means a specific affinity of perfluoroalkyl groups. The perfluoroalkylated compounds are selectively retained on the perfluoroalkyl-modified stationary phase LC column via fluorous affinity, whereas the non-perfluoroalkylated compounds are early eluted from the fluorous-phase LC column without retention [19-22]. Therefore, this fluorous derivatization method can be selectively analyzed the obtained perfluoroalkyl-derivatives without interference from non-perfluoroalkyl components, such as biological matrix, especially to phospholipids (which cause matrix effects in LC-MS analysis). Furthermore, the hydrophobicity of perfluoroalkyl derivative is amenable to ESI-MS because hydrophobic ions preferentially locate to the electrospray droplet surface layer
and enter the gas phase more readily when the solvent evaporates. For utilizing this strategy, curcumin was derivatized with $^{1}H,^{1}H,^{2}H,^{2}H$-perfluoro-1-decanethiol (PFDT) via Michael addition reaction (Fig. 1). The obtained fluorous derivative was detected with high sensitivity by negative electrospray ionization-MS/MS following selective retention to fluorous stationary phase LC column. After the conditions for derivatization, fluorous LC separation, and MS/MS detection were optimized using standard of curcumin, the method was applied to the analysis of spiked human serum sample.

2. Experimental
2.1. Reagents and materials
Curcumin and 2,2,2-trifluoroethanol (TFE) were purchased from Tokyo Chemical Industry (Tokyo, Japan). PFDT was from Sigma-Aldrich (St. Louis, MO, USA). Potassium hydroxide (KOH) was obtained from Kishida Chemical (Osaka, Japan). Ultrapure water, purified using a Milli-Q Gradient-A10 system (Merck KGaA, Darmstadt, Germany), was used to prepare all aqueous solutions. All other organic solvents and reagents from Fujifilm Wako Pure Chemical (Osaka, Japan) were of LC-grade and used as received.

2.2. Derivatization reaction
To 30 μL of sample solution, 30 μL of 0.6 M PFDT in TFE as derivatization reagent and 10 μL of 10 mM KOH were added. The reaction mixture was stood at room temperature for 30 min. After this, 5 μL of 0.1% formic acid in acetonitrile was added. The resulting solution (10 μL) was injected into LC-MS/MS.

2.3. Instrumentation and conditions
LC analysis was performed on a Nexera HPLC system (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a system controller (CBM-20A), binary-solvent delivery system (LC-30AD), an auto sampler (SIL-30AC), and a column heater oven (CTO-20A). LabSolutions LCMS software was used to control the instruments and process the data. A Fluofix-II 120E (150 × 2.0 mm ID, particle size 5 μm, Fujifilm Wako Pure Chemical) was used for the separation. Solvent A (40 mM ammonium formate : acetonitrile : TFE = 40:40:20, v/v) and solvent B (40 mM ammonium formate : acetonitrile : TFE = 25:35:40, v/v) were used as the mobile phases for gradient elution. The column oven temperature was set at 40 °C. The injection volume was set at 10 μL. The total flow rates were 0.2 mL/min. The concentration of mobile phase B (%) was changed held at 0% from 0 to 5 min, changed linearly from 0 to 100% from 5 to 10 min, held at 100% from 10 to 20 min, and held at 0% from 20 to 30 min.

The mass spectrometer used in this study was an LCMS-8050 triple quadrupole (Shimadzu Scientific Instruments) equipped with an electrospray ionization (ESI) interface. The ESI-MS/MS was operated in the negative ionization mode in either precursor ion scan or selected reaction monitoring (SRM) modes for fluorous-derivatized curcumin. The MS/MS conditions were set as follows: ion spray voltage, 5.0 kV; desolvation line temperature, 150 °C; heat block temperature, 500 °C; drying gas, 5.0 L/min; nebulizer gas, 3.0 L/min; and collision-induced dissociation (CID) gas, 450 kPa. The precursor ion (Q1), product ion (Q3), and collision energy (CE) in the SRM transition for the fluorous-derivatized curcumin were m/z 847.1, m/z 367.1, and 17.0 eV, respectively. For comparison, underivatized curcumin was analyzed under same LC conditions although the MS/MS was operated in the positive ionization mode and its SRM transition was set at m/z 369.4 (Q1), m/z 285.0 (Q3), and -35.0 eV (CE).

2.4. Human serum sample
Human serum sample (male; type AB; product number H4522; Lot No. SLBB2994V) was obtained from Sigma-Aldrich. To 20 μL of human serum, 180 μL of acetonitrile was added. After shaking for 3 min at room temperature, the mixture was centrifuged at 17,500 × g for 10 min. The resulting supernatant (30 μL) was subjected to derivatization with PFDT using the procedure outlined above.

2.5. Validation study
We investigated a validation study to assess the linearity of calibration curve, sensitivity, precision, and...
accuracy of the method in terms of the recovery of curcumin from spiked human serum sample. The standards for calibration curves were prepared in the range of 2, 5, 10, 20, and 50 nmol/L (corresponding to 0.74, 1.8, 3.7, 7.4, and 18.4 ng/mL). The spiked human serum samples for calibration curves were prepared in the range of 7.4, 18, 37, 74, 184, 368, and 442 ng/mL serum. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the human serum concentrations at which the ratio of signal to noise was 3 and 10, respectively. Precisions were determined using replicate analysis \((n = 6)\) of both standard (2, 10, and 50 nmol/L) and spiked human serum (7.4, 37, and 184 ng/mL serum) samples at three concentrations. The accuracy for analysis of human serum sample was also calculated at three concentrations of 7.4, 37, and 184 ng/mL serum using the following formula:

\[
\text{Accuracy (\%)} = \frac{\text{peak area obtained from spiked serum sample}}{\text{peak area obtained from standard sample}} \times 100
\]

These were repeatedly measured six times during the day and calculated using the obtained peak area.

3. Results and discussion

3.1. Derivatization reaction

In this study, PFDT was used as the fluorous reagent for derivatization of curcumin. In the presence of a strong base (KOH), PFDT was introduced to \(\beta\)-position of \(\alpha,\beta\)-unsaturated carbonyl structure of curcumin via Michael addition reaction. For optimization of this reaction conditions, the concentrations of reagents, reaction solvent, and reaction time in the present derivatization reaction were examined. Investigation of the solvent for PFDT was examined using methanol, acetonitrile, tetrahydrofuran, and TFE. Among them, TFE accelerated the solubility of PFDT and the derivatization reaction for curcumin. Consequently, the following optimization studies were performed using TFE as the solvent for PFDT. The effect of concentration of PFDT (0, 0.2, 0.4, 0.6, 0.8, and 1 M) on the derivatization was examined. As a result, the peak area became plateau at a concentration of over 0.4 M. Therefore, the optimal concentration of PFDT was set as 0.6 M in this study. Furthermore, by varying the concentration of KOH (0, 5, 10, 25, and 100 mM), the peak area was maximized at 10 mM. The examination of reaction time at room temperature was performed at 0, 5, 10, 20, 30, 40, 50, and 60 min. As a result, the peak area became plateau after 20 min. Therefore, the reaction time of this derivatization reaction was set as 30 min.

3.2. LC-MS/MS analysis conditions

Fluorous-derivatized curcumin could be detected as \(m/z\) 847.1 ([M – H]–) in precursor ion scan mode (Fig. 2A). This deprotonated ion was set as the precursor ion of fluorous-derivatized curcumin to optimize SRM mode measurement. As a result, the most intense product ion as \(m/z\) 367.1 was obtained at CE 17.0 eV (Fig. 2B). Therefore, the optimum SRM conditions in this study were \(m/z\) 847.1 for the precursor ion, \(m/z\) 367.1 for the product ion, and 17.0 eV for CE in the ESI negative.

The fluorous-derivatized curcumin could be strongly retained on a fluorous LC column (Fig. 3B). In this study, TFE was used as the mobile phase because it can easily eluted the strongly retained fluorous compounds from fluorous LC column [21,22]. The retention time of fluorous-derivatized curcumin could be also controlled by adjusting the concentration of TFE in the mobile phase in gradient elution. In contrast, the underivatized curcumin was hardly retained on the column, and it was eluted within 2.5 min in spite of its moderate hydrophobicity (Fig. 3A). From these data, it consider that the fluorous-derivatized curcumin would be analyzed without interference from non-fluorous biological matrices, in the present method because it retained on the column with fluorophilicity not hydrophobicity.

3.3. Validation study

In order to evaluate the validity of the present method, the linearity of the calibration curve of the standard sample and the precision of intra-day were examined. The linearity of the...
calibration curve in the range of 2-50 nmol/L showed good linearity as \( r^2 = 0.9989 \). The precision of intra-day \( (n=6) \) showed good RSD of 5.9, 2.4 and 6.4% at concentrations of 2, 10, and 50 nmol/L, respectively. The LOD \((S/N = 3)\) and LOQ \((S/N = 10)\) were 1.8 ng/mL and 6.1 ng/mL, respectively.

In the previous study [23], human blood concentrations of curcumin administered were in the range of 30 – 120 ng/mL at 30 – 60 minutes after oral administration (1.5 mg of curcumin), and the present method had sufficient sensitivity to quantify them.

Furthermore, we investigated the feasibility of present method for application to human serum sample. The chromatogram of fluorous-derivatized curcumin in spiked human serum was shown in Fig.3.C. The calibration curve of spiked serum sample showed good linearity \((r^2 = 0.9998)\) in the range of 7.4 – 442 ng/mL serum. Utilizing the present method, trace amount of curcumin in spiked human serum sample could be detected with sufficient sensitivity. The intra-day precision \((n=6)\) showed RSD values of 7.9, 1.2 and 2.4% at concentrations of 7.4, 37 and 184 ng/mL serum, respectively. Accuracy was also 101.9%, 98.6% and 102.1% at concentrations of 7.4, 37 and 184 ng/mL serum, respectively. From these results, it was confirmed that this method enables sensitive as well as precise and accurate analysis of curcumin in biological sample.

### 4. Conclusion

We developed a method for selective analysis of curcumin by fluorous derivatization LC-MS/MS method. In this study, thiol-containing fluorous reagent, PFDT, was used for derivatization of curcumin via Michael addition reaction. The obtained fluorous-derivatized curcumin was successfully retained on the fluorous LC column and separated from non-fluorous component. The validations using the standard and spiked human serum samples of the present method showed good results, and this method was sufficient for sensitive, precise and accurate analysis for curcumin in human serum. The proposed method will be useful for the determination of curcumin in various biological samples related to pharmacokinetics studies. Further investigations are now in progress.

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