졸업논문청구논문

R-와 S- Etodolac의 거울상 이성질체의 (1R)-(-)-Menthyl Chloroformate의 유도체화를 통한 HPLC-FLD로 분리

Separation of R- and S- Etodolac Enantiomers by High Performance Liquid Chromatography – Florence Detector after Derivatization with (1R)-(-)-Menthyl Chloroformate

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2016

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1. 이 논문은 내가 직접 연구하고 작성한 것이다. ■
2. 인용한 모든 자료(책·논문·인터넷자료 등)의 인용표시를 바르게 하였다. ■
3. 인용한 자료의 표현이나 내용을 왜곡하지 않았다. ■
4. 정확한 출처제시 없이 다른 사람의 글이나 아이디어를 가져오지 않았다. ■
5. 논문 작성 중 도표나 데이터를 조작(위조 혹은 변조)하지 않았다. ■
6. 다른 친구와 같은 내용의 논문을 제출하지 않았다. ■

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A thesis submitted to the Gyeonggi Science Highschool in partial fulfillment of the requirements for the graduation. The study was conducted in accordance with Code of Research Ethics.[1)]

2016. 6. 19.

Approved by

Teacher Jeong, Najin

[Thesis Advisor]

[1)] Declaration of Ethical Conduct in Research**:** I, as a graduate student of GSHS, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidance of my thesis advisor.

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강 한 필

위 논문은 과학영재학교 경기과학고등학교 졸업논문으로
졸업논문심사위원회에서 심사 통과하였음.

2015년 6월 19일

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Abstract

Etodolac, one of nonsteroidal anti-inflammatory drugs(NSAIDs) found in R/S- form are shown to be have different pharmacodynamic and pharmacokinetic properties. R- contreats Leukemia while S- treats the symptoms of pain and inflammation. In this research, (1R)-(-)-Menthyl Chloroformate was used to derivate R/S- Etodolac which reacts with the Carboxyl group in pyridine catalyst. High Performance Liquid Chromatography - Florence Detector (HPLC-FLD) and Ultra Performance Liquid Chromatography - Mass Detector (UPLC-MS) with reverse phased columns are used to qualificate two enantiomers of Etodolac. Two peaks appears at 13.115 and 14.196 in HPLC-FLD. First peak is R-Etodolac derivatizated with (1R)-(-)-Menthyl Chloroformate while the other one is S-Etodolac. Resolution is over 1 in both standard and serum sample.

R-와 S- Etodolac의 거울상 이성질체의 (1R)-(-)-Menthyl Chloroformate의 유도체화를 통한 HPLC-FLD로 분리

초 록

비스테로이드성 진통제의 일종인 에토돌락의 두 광학 이성질체는 약역학과 약물통태학적으로 다른 성질을 가진다. R-의 형태는 백혈병 치료에 효과가 있고, S-의 형태는 진통소염 작용을 한다. 이 연구에서는, 피리딘 촉매 하에서 (1R)-(-)-멘틸 클로로포메이트를 R/S-에토돌락의 카르복실기와 피리딘 촉매하에서 유도체화시킨다. HPLC-FLD와 UPLC-MS에 역상 컬럼이 두 광학 이성질체를 분리하는데에 사용되었다. HPLC-FLD에서 13.115분과 14.196분에 두 봉우리가 발견되며, 첫번째 봉우리가 R-에토돌락이 (1R)-(-)-멘틸 클로로포메이트에 유도체화 된 것이고, 두번째 봉우리는 S-에토돌락이 유도체화 된 것이다. 표준과 소변 샘플에서 해상도 1 이상으로 정성할 수 있다.

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I Introduction

Etodolac, one of the nonsteroidal anti-inflamatory drugs (NSAIDs) is widely used for treating rheumatic and inflammatory diseases. This drug is used in the form of a racemic mixture but the pharmacodynamic and pharmacokinetic properties of the two enantimoers are different. R- contreats Leukemia[1] while S- treats symptoms like pain and inflammation. Menthyl Chloroformate was widely used as a derivating secondary amine, but it also reacts with the carboxyl group when the catalyst pyridine exists. High Performance Liquid Chromatography – Florence Detector (HPLC-FLD) and Ultra Performance Liquid Chromatography – Mass spectrometry (UPLC-MS) are powerful machines which can quantify and qualify products. In this research, HPLC-FLD was used to detect products made by the derivatization of (1R)-(-)-Menthyl Chloroformate to Etodolac. To check the two isomers' structure, UPLC-MS was used.

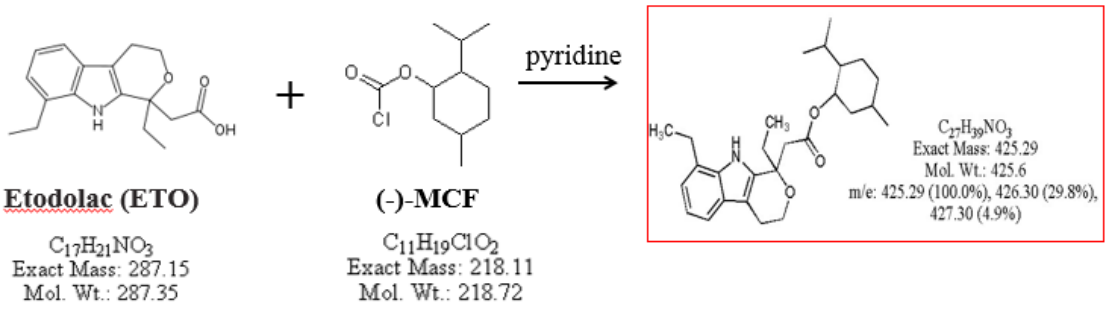

Figure 1: Etodolac, (-)-MCF and their products.

II Experimental

II.1 Chemicals and Reagents

R/S-Etodolac was purchased from Tokyo Chemical Industry (Tokyo, Japan). (1R)-(-)-Menthyl Chloroformate, Pyridine, L-Proline was purchased from Sigma-Aldrich Chemie (St. Louis, USA). Acetonitrile (ACN) and Methanol (MeOH) were purchased from Avantor Performance Materials, Inc (USA). NaCl was purchased from Merck KGaA (Darmstadt, Germany).

II.2 High-performance Liquid Chromatography - Florence Detector

High-performance liquid chromatography (HPLC) is widely used in analytic chemistry to separate, identify or quantify each component in a mixture. Gemini C18 Column was used at a flow rate of 1.0mL/min at 25°C used with 86.5% MeOH, 13.5% 10mM Acetic Acid. Florence Detector was used at a wavelength of λex= 235 nm; λ em= 345 nm.

Chemstation Software was used for system control and data processing.

II.3 Ultra-performance liquid chromatography – Mass Detector

Ultra-performance liquid chromatography – Mass Spectrometer (UPLC-MS) is widely used in analytic chemistry to separate, identify or quantify each component in a mixture through mass detection. A mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio. Mass spectra are used to determine the elemental or isotopic signature of a sample, find the masses of particles and of molecules, and to elucidate the chemical structures of molecules, such as peptides and other chemical compounds. UPLC BEH C18 Column (2.1 x 50mm, 1.7um) was used at a flow rate of 0.3mL/min, at 45°C. Elution conditons can be seen in [Table 1]. MassLynx Software was used for system control and data processing.

II.4 Protocol

The protocol for this experiment was as follows. 50uL 50mM etodolac soluted in ACN was added to 50uL urine. The solution was vortexed for 3 seconds, and then 900uL of ACN was added. The solution was then vortexed for 3 minutes, and then centrifuged for 5 minutes at 13500 rounds per minute. Next, 950uL of the upper layer of the solution was moved to another vial and evaporated using gentle nitorgen stream at 40 °C After, 160uL 100mM MCF soluted in ACN, 15uL

Table 1: Elution Condition of UPLC-MS.

Time(min)	Water(0.1% Formic Acid)	Methanol (0.1% Formic Acid)
0	20	80
9	10	90
11	0	100
13	0	100
15	20	80
17	20	80

pyridine, 25uL ACN were all added to the residue, and it was sonicated for 10 inutes. To finalize derivatization, 160uL 100mM proline soluted in distilled water was added, and then vortexed for 3 minutes. After being vortesed, it was placed at room temperature for 20 minutes. 28.7 mg of NaCl was added for Liquid-Liquid Extraction and the organic layer was filtered to complete to the optimization process.

II.5 In-vitro test

S-etodolac binds well to Albumin than R-etodolac.[4] Etodolac enantiomers were attempted to be identified using the stereoselective binding difference of etodolac to albumin. Protocol follows. Add 100uL 50ppm Etodolac. Evaporate in 40'C in gentle nitrogen stream. Add 100uL 50ppm Albumin in pH 7.4 phosphate buffer. Albumin purification was done to separate unbound Etodolac. We used Vivaspin 500 to separate protein. Protocol follows. In Vivaspin 500 add 100uL diluted water and centrifuge in 10000g for 5 minute. Add 100uL albumin and Etodolac mixture and centrifuge in 10000g for 3 minute. Filter 50uL and add 100 uL buffer and centrifuge in 10000g for 5minute. Repeat 4 times filtering 100uL and adding 100uL buffer and centrifuge in 100g for 5minute. Transfer concentrate and collect filtrate.

II.6 In-vivo test for rats

C57BL/6 mouse (male; 7 w) serum was used for Chrial determination of etodolac. The weight of mouse was 20g. Concentration of S-(+)-etodolac is lower than R-(-)-etodolac when injected 20mg/kg from 0 through 70 hour.[2] Protocol follows. Add 10mg etodolac to 50uL ethanol and vortex for 20s. Add 300uL Tween

80 and vortex for 1 minute and sonicate for 1 minute. After centrifuging 3 seconds, add saline to 10mL. Inject etodolac to mouse. 200uL was injected to C57 mouse when the weight was 20g.

III Results and Discussion

III.1 UPLC-MS Data

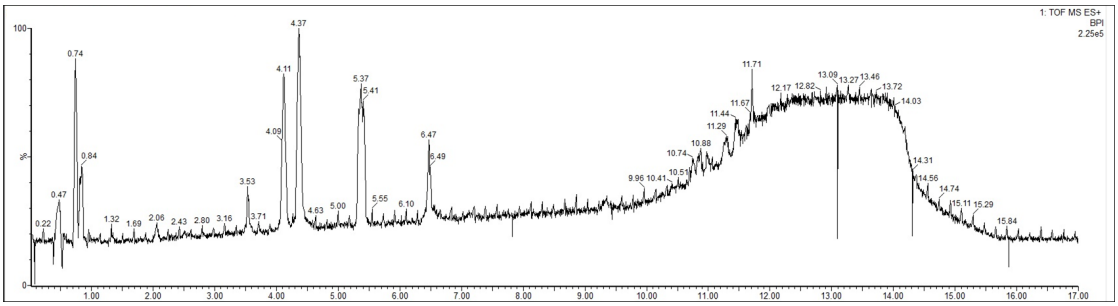

Figure 2: BPI chromatogram of Menthyl Chloroformate-derivatized Etodolac product

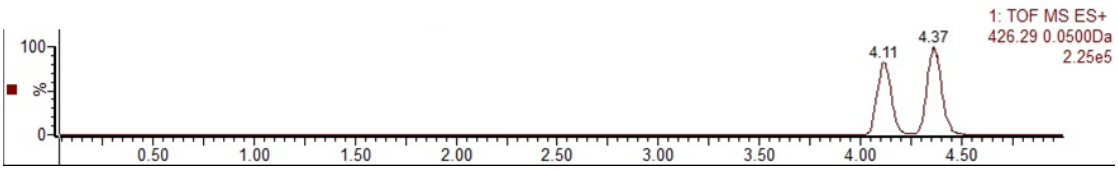

Figure 3: Extract ion chromatogram of Menthyl Chloroformate-derivatized Etodolac product, [M+H]=426.29

In [Figure 4], the mass fragment of the peak at 4.11 and 4.37 are equal. This means they are not geometric isomers, but they appear at different peaks. The mass at 172.1 and 287.15 shows that they have the structure of an etodolac.[3] So it can concluded that they are derivated etodolac.

III.2 HPLC-FLD Data

In [Figure 5], two peaks appear at 13.115 and 14.196. Two are enantiomers of etodolac derivatized by Menthyl Chlroforomate. The first peak will be referred to as Peak 1, and the second, Peak 2.

III.3 Protocol Optimization

The Pyridine volume and sonication time was optimized in this experiment. Peak 2 reacted faster than Peak 1. Therefore, the ratio of Peak 1 was used to verify that the reaction was completed.

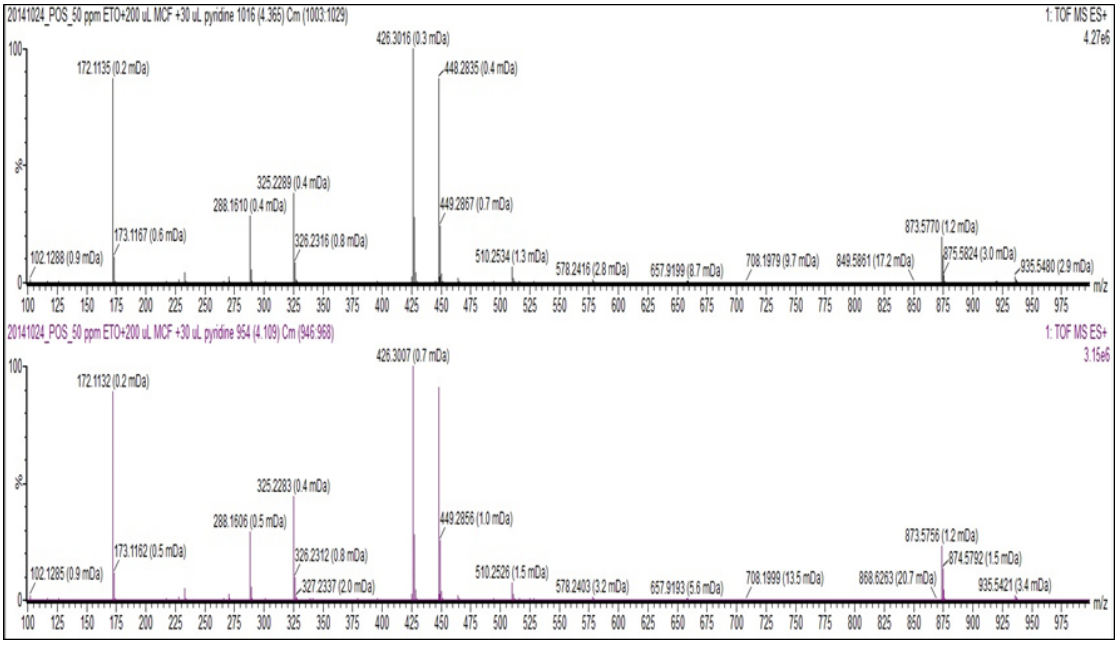

Figure 4: Mass fragment of peak at retention time 4.11 minute and 4.37 minute.

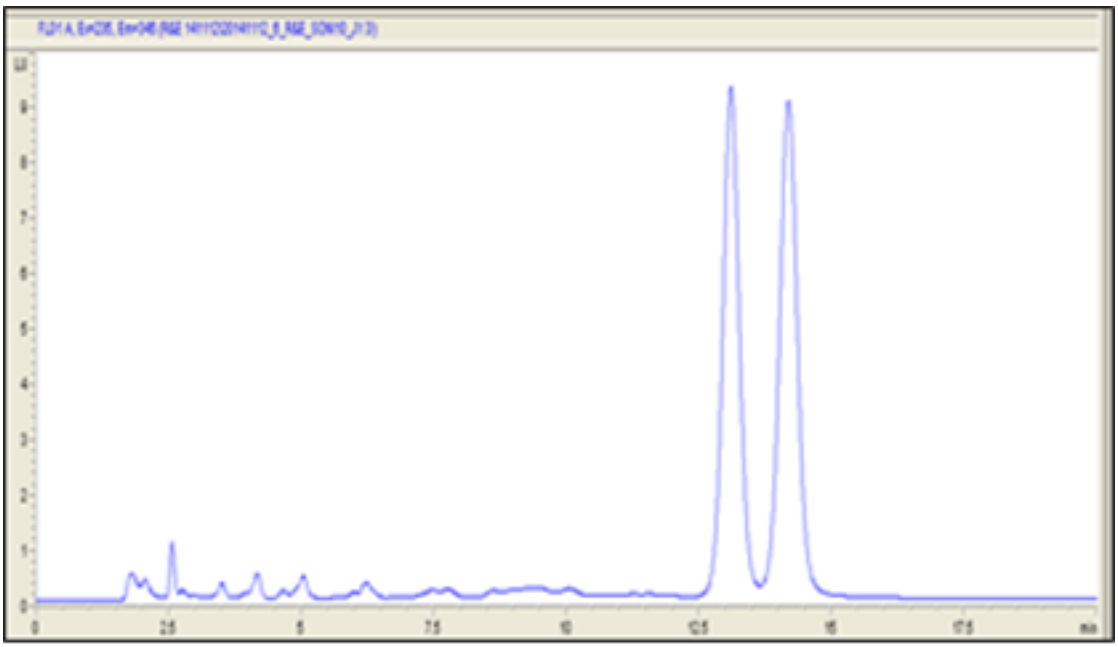

Figure 5: HPLC-FLD data of protocol

III.3.1 Pyridine Volume

In [Figure 6], 5uL and 25uL of pyridine volume were significant by t-test. Optimized value found to be the median of the pyridine volume, which was not

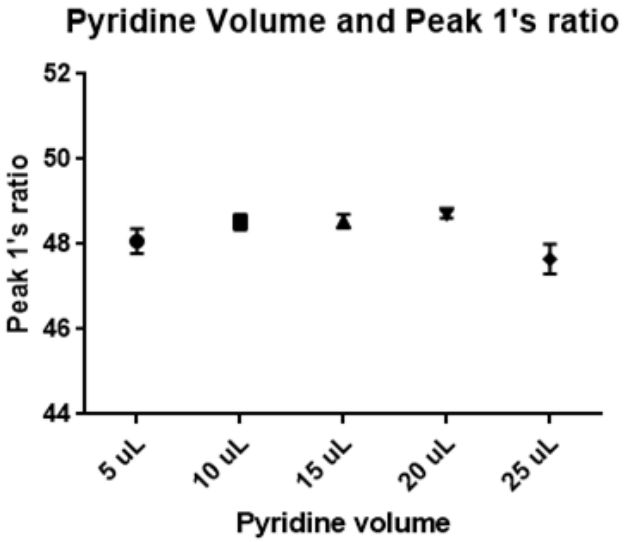

Figure 6: Pyridine Volume and Peak 1's ratio

significant, 15uL.

III.3.2 Sonication time

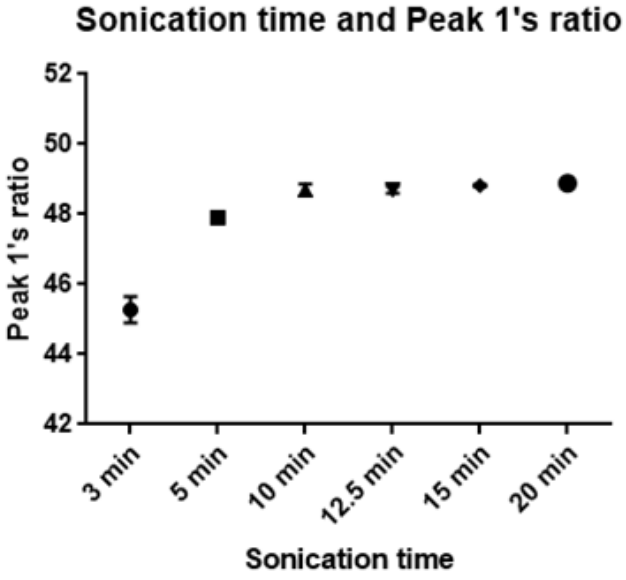

Figure 7: Sonication time and Peak 1's ratio

In [Figure 7], 3min and 5min of sonication time were significant by t-test. Optimized value found to be the first of the significant time, which was not significant, 10min.

III.4 In-vitro test

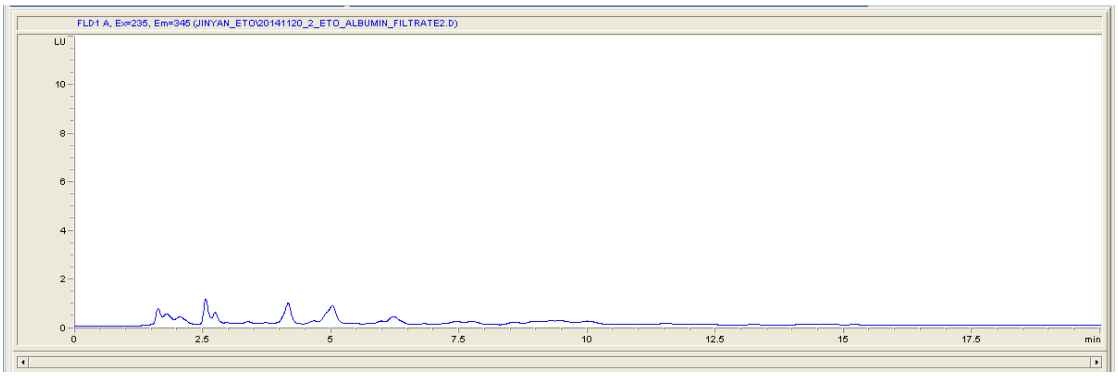

Figure 8: Chromatogram of Filtrate

The in vitro test failed beacuse both etodolac enantiomers were well binded with protein in a short time, so the results of in-vivo test were analyzed.

III.5 In-vivo test for rats

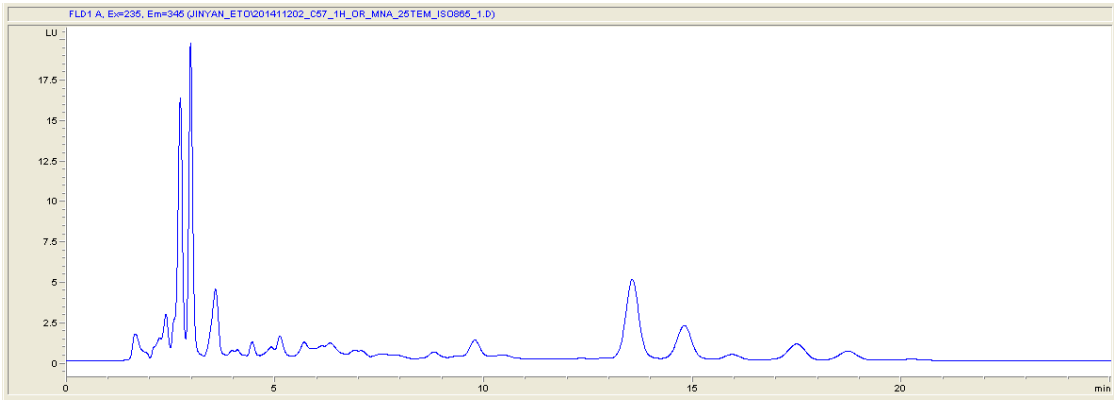

Figure 9: In vivo test result

In [Figure 9], 1 hour after injection, the peak area of Peak 1 is 0.4676, while the peak area of Peak 2 is 0.0694. S-etodolac binds faster[2] and Peak 2 has less peak area, which means that Peak 2 is S-etodolac and Peak 1 is R-etodolac.

References

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