Analysis of Ketoprofen and Mefenamic Acid by High-Performance Liquid Chromatography with Molecularly Imprinted Polymer as the Stationary Phase

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Abstract

A simple and sensitive high-performance liquid chromatographic method for simultaneous determination of ketoprofen and mefenamic acid in tablets has been developed. HPLC with UV detection (220 nm) was performed on an analytical column packed with molecularly imprinted polymer (MIP) as the stationary phase. The MIPs are prepared by bulk polymerisation followed by crushing and sieving to the desired particle size. In this paper, we selected ketoprofen, methacrylic acid, and ethylene glycoldimethacrylate as template, functional monomer, and crosslinker in the presence of chloroform as the solvent. The retention times of mefenamic acid and ketoprofen were approximately 5 and 20 min, respectively. In order to compare the chromatographic data from the stationary phase, separation factors (α) were given. The values of α were 4.36~4.39 and showed that the MIPs were able to recognize structurally subtle differences from the template molecule. The limits of detection for ketoprofen and mefenamic acid were found to be 0.029 and 0.038 (g/L), while the limits of quantitation were 0.097 and 0.127 (g/L), respectively. Our results showed good accuracy, indicating that a ketoprofen-selective polymer was suitable for ketoprofen and mefenamic acid separations. Therefore, the MIPs are certainly applied to commercial tablet analysis.

Introduction

Ketoprofen (2-(3-benzoylphenyl)-propionic acid (KET) (Figure 1A), is a derivative of propionic acid. It is a nonsteroidal anti-inflammatory drug (NSAID) and cyclooxygenase inhibitor, which also interferes with the bradykinin pathway and stabilizes lysosomal enzymes. Oral administration of KET is effective in treating fever, pain, and inflammation. As a group, NSAIDs are non-narcotic relievers of mild to moderate pain of many causes, including injury, menstrual cramps, arthritis, and other musculoskeletal conditions.

Several methods have been described for KET determination in pharmaceutical formulations and serum including UV spectrophotometry (1), high-performance liquid chromatography (HPLC) (2–8), HPLC with normal solid-phase extraction (9), and capillary electrophoresis (10–15). In addition, nuclear magnetic resonance (NMR) spectroscopy (16,17) and Fourier transform infrared spectrometry (FT-IR) (18) methods have also been used for the quantitative determination of KET. Nowadays, the speed and easiness of KET assay is more and more important. HPLC is an ideal technique for analysis because of its low cost, simplicity, and high speed. In this report, MIP was applied as the stationary phase in HPLC for the determination of KET and mefenamic acid (MEF) [*N*-(2,3-Xylyl) anthranilic acid (Figure 1B)]. A number of molecularly imprinted polymers were prepared for steroids, such as cholesterol (19) and various imprinted polymer-based sensors have been developed (20,21). An acrylonitrile–acrylic acid copolymer membrane imprinted was reported, in which uric acid was used as a template (22). Microsphere MIPs have subsequently been applied as antibody-binding mimics, they are non-porous so that recognition sites are considered to be limited to the surface (23). There is no doubt that polymers synthesised in the correct format for a particular application give a greatly enhanced performance. For example, nanospheres imprinted polymers made by core-shell emulsion polymerization allowed evaluation of MIPs for clinical uses (24,25). Therefore, it was thought that the proposed method would be a useful technique for the determination of KET and MEF. The procedure applied to imprinting the KET is outlined in Figure 2. In the first step, the template, functional monomer (MAA), and crosslinking monomer (EGDMA) are dissolved in chloroform, which is a poorly hydrogen-bonding solvent. The free radical polymerization is then initiated with an azo initiator, 2.2'-Azo-

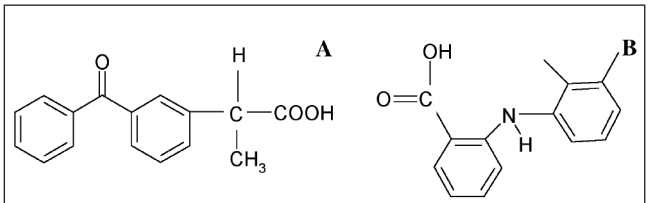

Figure 1. The structural formulas of KET (A) and MEF (B).

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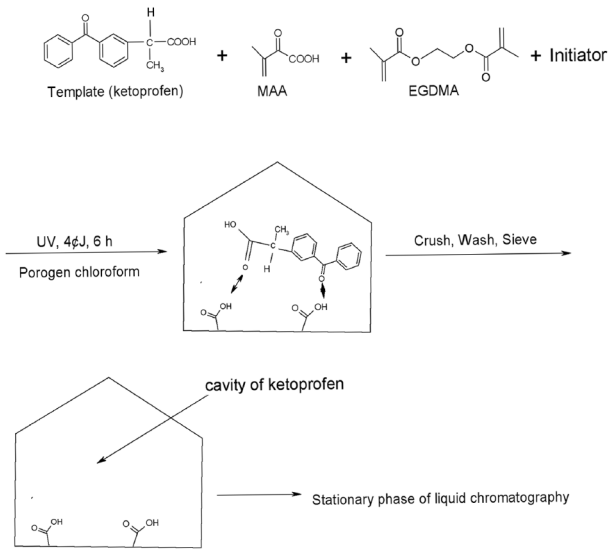

Figure 2. Molecular imprinting using KET as the template and MAA as the functional monomer.

bisisobutyronitrile (AIBN), by UV radiation below room temperature (4°C). Finally, the resultant polymer is crushed and sieved to a particle size, and would be suitable for chromatographic applications. Accordingly, the excellent retention time and separation factor (α) of the template with that of structurally related analogs indicated that the polymers are good to be utilized as stationary phases in liquid chromatography (26–30).

Molecular imprinting technology (MIT) is a simple, rapid, economical, specific, and sensitive method for KET and MEF measuring in tablets. In our study, these compounds were determined in less than 25 min under 3.0 mL/min flow rate. Besides, we resolved the detection limits of HPLC by applying the polymer. Therefore, separation of KET from excipients would be accomplished successfully, and accurate and precise quantification would be afforded.

Experimental

Reagents and materials

Freshly prepared double-distilled water from the Milli-Q system (Millipore, Bedford, MA) was used for HPLC. Acetonitrile, ethanol, methanol, acetone, phosphoric acid, and sodium phosphate were of HPLC-grade and were purchased from TEDIA (Fairfield, OH). KET and MEF were purchased from Sigma (St. Louis, MO). Ethylene glycol dimethacrylate (EGDMA, 98%) and methacrylic acid (MAA, 99%) were purchased from Merck (Darmstadt, Germany). EGDMA and MAA were distilled to remove the inhibitors prior to polymerization. Chloroform, acetic acid (GC grade), and 2.2'-azo-bisisobutyronitrile (AIBN) were purchased from TCI (Tokyo, Japan). All chemicals were of analytical reagent grade.

Analysis equipment

HPLC was performed with a JASCO PU-2080 (Tokyo, Japan) liquid chromatograph in conjunction with JASCO UV-2075 variable wavelength UV monitor and Rheodyne 7725 syringe loading sample (20 µL) injector. For data analysis, peak integration was performed using Peak ABC Chromatography Workstation Ver. 2.10 integrator. The apparatus provide assurance that all the UV-absorbing components are detected, if present in sufficient quantity. UV detection was performed at 220 nm. The mobile phase was a mixture of buffer and acetonitrile (3:2, v/v) and the flow rate was maintained at 3 mL/min.

Synthesis of MIP stationary phase

Imprinted and non-imprinted polymers were prepared by the method of bulk polymerization at a low temperature (31,32). Care was taken to ensure that the amount of functional monomer used for MIP synthesis as well as non-MIP was the same. The general procedure used MAA (3 mol %) as the functional monomer, EGDMA (95 mol %) as the cross-linker, KET (2 mol %) as the print molecule, AIBN as the initiator, and chloroform (10 mL) as the solvent. First, the solution was placed in an ultrasonic water bath until a clear solution was obtained. Then they were degassed and purged with dry nitrogen for 5 min, and the flask was sealed and placed under a UV-lamp (365 nm, 100 W) at 4°C for 6 h. Following polymerization, the chloroform was removed. The hard polymers were dried in a vacuum oven for 24 h at room temperature. Finally, polymers were ground to the required size using a laboratory mortar grinder. The 11–25 µm and 25–44 µm particle size fractions were collected.

Column packing

The particles were suspended in methanol (30 mL) by sonication for 3 min, placed in a slurry reservoir with an action reciprocating plunger pump, and they were then packed in a stainless steel column (150 × 4.6 mm i.d.) using an air-driven fluid pump. If the EGDMA content was too low, creating particles with insufficient hardness, it was not easy pack the column. From the experimental result, the suitable EGDMA content was approximately 95% of the total amount of monomers. The particle content in each column was approximately 3.32 g. Packing was carried out under a pressure of 300 bar with acetone (300 mL) as the packing solvent. The columns were washed on-line with methanol–acetic acid (9:1, v/v) mixture at a flow rate of 1.0 mL/min until no further template bleed could be detected by HPLC.

HPLC analysis

Solution or mixture of the KET and MEF prepared in acetonitrile, was injected for analysis in a total volume of 20 µL and eluted isocratically under 220 nm UV detect. The void volume of the column was determined by toluene injection. The mobile phase was prepared by adjusting the pH of a 0.034 mol/L sodium phosphate solutionand mixing this with acetonitrile to the desired proportion.

The separation factor (α) and retention factor (k) were calculated as follows: α was determined using the relationship $\alpha = k_{KET}/k_{MEF}$, where k_{KET} and k_{MEF} were the retention factors of the KET and MEF, respectively. The retention factors were determined as $k_{MEF} = (t_{MEF} - t_o)/t_o$ and $k_{KET} = (k_{KET} - t_o)/t_o$, where k_{MEF} and k_{KET} were the retention times of the MEF and KET, respectively, and the elution time of the void marker, which was determined by the injection of toluene.

Evaluation of the binding ability

HPLC analysis was used to determine the binding capacity of KET and MEF after the adsorption experiments. The concentration of standard solutions was 10 mmol/L ~1.0 mmol/L. A calibration graph was generated using concentration of subsrate and the absorbance. An accurately weighed 0.1 g portion of the imprinted or non-imprinted polymer particles was transferred into a 10 mL centrifuge tube, 5 mL of 5 mmol/L standard solution was added, and the tube rotary for 12 h. This solution was centrifuged at 2000 rpm for 10 min. The centrifugate was transferred into a 10-mL volumetric flask. The absorbance of the solution was measured by HPLC equipped with UV detector.

Preparation of standard solution

For calibration standards preparation, all reagents were distilled to remove the impurities. KET and MEF stock standard solutions were prepared in 1.0 mg/mL acetonitrile. Working solution of the KET and MEF were prepared in acetonitrile by dilution from the stock solution within the studied range of 0.2~0.8 mg/mL.

Solutions of pharmaceutical dosage forms

A commercial pharmaceutical preparation (KET and MEF) was assayed. Tablets of ketprofen and MEF were purchased from a local pharmacy. The amount of KET and MEF present in the tablet was 50 mg. The KET-containing tablets include the following ingredients: cornstarch, magnesium stearate, stearic acid, and hydroxypropyl cellulose, and MEF-containing tablets are comprised of: starch, stearic acid, magnesium stearate, microcrystalline cellulose, and cellulose. A total of five tablets were accurately weighed and powdered in a mortar and transferred to a 500-mL volumetric flask, 150 mL of 0.05 mol/L sodium phosphate solution was added, and then made up with acetonitrile to 500 mL by volume. After 45 min of mechanical shaking, a portion of the suspension was centrifuged for 3 min at 1000 × g to obtain the clear supernatant solution. It is then filtrated in a 500 mL calibrated flask through Whatman no. 42 filter paper. Sample injection solution was used without treatment.

Results and Discussion

Evaluation of imprinted effect with HPLC

In this work, KET was used as the template molecule, and MAA was chosen as the functional monomer. After being imprinted, the polymer particles were packed in a column. In order to clarify molecular recognition properties of the MIP particles, the polymers were evaluated using HPLC with the actonitrile aqueous solution containing phosphate buffer as the mobile phase. KET and MEF were utilized for the evaluation solutes. 1 g/L of KET and MEF solution or mixture of these two compounds was injected for analysis in a total volume of 20 µL and eluted isocratically. Subsequently, the imprinting effect was evaluated by HPLC with UV detection. The results are shown in Table I.

As shown in the Figure 3, although the polymer particles for packing were irregular in shape, the 15-cm long columns with a linear volumetric flow-rate and retention time for KET and MEF were achieved. Moreover, validation revealed that the retention time of KET and MEF were 20.05 min and 5.04 min when the flow rate was 3 mL/min. Table I shows the retention time, retention factor, and separation factor (α) values obtained after separation by HPLC. In addition, the KET and MEF are readily and rapidly separated when 34 mmol/mL NaH_2PO_4 buffer solution–acetonitrile (3:2, v/v) is used as the mobile phase. From Table I, the retention times for MEF and KET are 4.83~5.04 min and 19.43~20.05 min, respectively. Besides, the difference of retention time (Δt_R) between KET and MEF were 14.55~15.01 min. The chromatographic run was accomplished in less than 25 min and completely separated from the other peaks under a flow rate of 3 mL/min. These results indicate sharp, symmetrical, and well-resolved peaks for KET from MEF. Therefore, we attribute the observed results in these two compounds to their retention factor and selectivity (α) = k_{KET}/k_{MEF} value. The separation factor for KET from MEF under different combinations of concentrations with a total of 1 g/L ranged from 4.36 to 4.39, which is nearly a constant. Furthermore, the retention time for KET and MEF were 1.51 and 1.68 min by employing blank polymer (non-MIP) as the stationary phase. In other words, the difference of retention time (Δt_R) between KET and MEF was 0.17 min. Thus, it can be concluded that the blank polymer revealed little selectivity for MEF and KET, while the molecular imprinted polymer displayed strong retentivity and selectivity for these two molecules. In fact, the good imprinting property was likely due to the carbonyl group on the monomer and the electrostatic interaction or hydrogen bonding between imprinted polymer and template molecules in the polymerization. For the investigation of the comparison results, we have used C18 column to separate KET and MEF under the same conditions, such as mobile phase and flow rate. The values of the separation factor obtained in the experiment was 2.35 are much lower than the ones used MIP column.

Table I. The Chromatographic Data of KET and MEF when MIP and non-MIP* as Used the Stationary Phase†

	Concentration in sample (g/L)		Retention time (min)		Retention factor (k)		Separation factor
Solution	MEF	KET	MEF	KET	MEF	KET	(α)
1	1.0	0.0	4.83	–	6.91	–	–
2	0.2	0.8	4.96	19.53	7.13	31.01	4.39
3	0.3	0.7	4.93	19.48	7.08	30.93	4.36
4	0.5	0.5	5.04	20.05	7.26	31.86	4.38
5	0.7	0.3	4.95	19.62	7.11	31.16	4.38
6	0.8	0.2	5.02	19.96	7.22	31.72	4.39
7	0.0	1.0	–	19.43	–	30.85	–

* The retention time for KET and MEF were 1.51 and 1.68 min when non-MIP was used as the stationary phase and test solution is No. 4. The retention factors for KET and MEF were 1.47 and 1.75. The separation factor of non-MIP was 0.84.

† Composition of solution with a total of 1 g/L.

MEF and KET were well resolved under 220 nm using MIP as the stationary phase when the flow rate was 3.0 mL/min. In addition, the optimum amount of template in this study is about 2 mol % of the total amount in the monomer. Each component had the same retention time as that eluted in a mixed sample. In particular, the retentions of KET were higher on the imprinted polymers. These results indicated that imprinted polymers formed recognition sites for the KET compound as the template molecules. The imprinting effect for the template molecule was observed when MIPs were prepared with KET as the template molecule. The significant difference in the retention was due to the fact that KET molecules were removed by washing the polymer matrix with an acetic acid–methanol solution. As a result, the polymers left cavities of a complementary size and shape providing a great deal of free carbonyl groups in the copolymer matrix. The higher population of free carbonyl group in the polymer particles seems to increase the affinity of the solutes. Therefore, the selective recognition of KET and MEF was achieved by utilizing KET as the template for the imprinted. From this study, HPLC enables the analysis of KET and MEF without derivation or purification with minimum sample preparation and results in high sensitivity and selectivity with MIPs as the stationary phase.

Effect of mobile phase on liquid chromatography

Since the template KET has a simple structure, thereby the mobile phase plays a very important role on the resolution of the template and MEF. To investigate the role of acetonitrile on the separation of KET from MEF, liquid chromatographic runs are carried out using various percentages of acetonitrile in the mobile phase to achieve the best resolution. From Figure 4, six different mobile phases were used. It is apparent that the optimization proportion of buffer solution to the acetonitrile was 3:2 (v/v), when the mobile phase was changed to 25% acetonitrile in buffer, no separation was observed. The mobile phase was changed back to 30% acetonitrile in buffer, separation factor was regained (α = 2.98). It is well known that the molecular recognition of most MIPs is based on the hydrogen-binding between the target and the stationary phase. In this MIP system, the template (KET) could hydrogen bond strongly with carbonyl group of the polymers. Hydrogen bonding has been shown to be very effective in the creation of recognition sites.

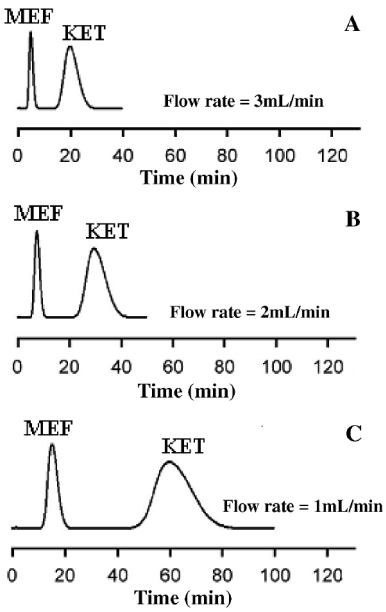

Figure 3. Comparison of retention time for ketoprofen and mefenamic acid. Flow rate: 3.0 mL/min (A), 2.0 mL/min (B), and 1.0 mL/min (C). HPLC conditions-mobile phase: NaH_2PO_4–H_3PO_4–CH_3CN (3:2, v/v); column size: 150 mm × 4.6 mm i.d.; injection volume: 20 µL with UV detect (220 nm).

In this study, the mobile phase consists of buffer solution and acetonitrile. Chromatographic results indicated that the solute molecules were retained in the column due to the possible hydrophilic interaction between the solutes and the stationary phase. Toluene is considered as the non-retained component with a retention time of 0.61 min and results from the stationary phase which did not contribute to the retention of hydrophobic substances. Because the interaction between target molecules and the stationary phase weaken by acetonitrile, solutes on the imprinting cavity of the stationary phase, and displaces the molecule would be released. As a result, the template molecules are finally eluted from the column.

Comparison of the binding abilities of KET and MEF by KET-MIP

Binding abilities to the template molecule KET with MIP and their corresponding non-imprinted polymers were evaluated by a batch adsorption method. Binding recovery (%) calculated according to the equation: $(C_i - C_f)/C_i \times 100\%$, where C_i is the initial concentration of KET or MEF (mmole/L), C_f is the final concentration (mmole/L) after batch adsorption.

Comparison of the data demonstrates the difference in their selectivities. Apparently, KET-MIP shows higher binding capacity toward template, especially the PA1(11~25 µm) particles. High binding recoveries for KET were obtained in the 95.46% and 94.59% for PA1 and PA2 (25~44 µm) polymers. PA1 and PA2 could almost completely absorb the imprint molecule KET. By contrast, low binding recoveries were obtained with the MEF at 51.67% and 47.53% for PA1 and PA2 polymers, respectively. Adsorption with the two non-imprinted polymers was much lower, only about 12%, showing that the binding of blank

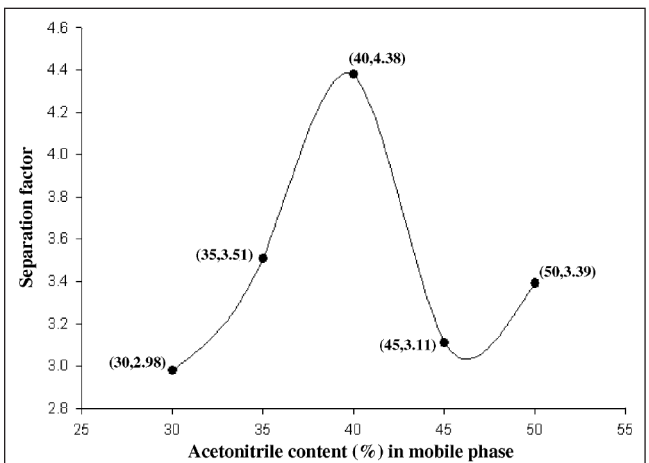

Figure 4. Influence of acetonitrile content in mobile phase on the separation factor. 25% acetonitrile in buffer, no separation was observed.

polymer was due to physical adsorption (non-selective). Overall, it can be seen that all the MIPs prepared in this research showed significant selectivity to the imprint molecule alone.

Limits of detection and quantitation

Linearity of the calibration curve for KET and MEF in acetonitrile was determined. The calibration graph was established for KET or MEF using seven concentrations (0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 g/L) as the standard. The straight line passing through the origin, and the determination coefficients (r^2) were 0.9978 and 0.9982; therefore, the linearity of the calibration graphs would be adequate. Limits of detection (LOD) and quantitation (LOQ) were calculated as a peak area to the concentration on the chromatograms, corresponding to signal-to-noise ratios of 3 and 10. LOD of KET and MEF were found to be 0.029 and 0.038 (g/L), while LOQ were 0.097 and 0.127 (g/L), respectively.

The KET and MEF contents in the tablets obtained from a local pharmacy were quantitated by the HPLC method described earlier, and the results are shown in Table II. Using molecularly imprinted polymer as a stationary phase for HPLC at a flow rate of 3.0 mL/min, the total analysis time was less than 25 min. The RSD values were found to be 1.26~2.42% for the KET tablets and 0.48~2.16% for the MEF tablets. This is an advantage over the current analysis methods, which involved separate quantitation of these compounds.

Conclusion

We developed a novel separation medium for selective separation of KET and MEF. The medium was prepared for molecular imprinting technique with MAA as the functional monomer and KET as the template. The selective recognition of KET is due to the higher binding capacity of KET-MIP toward the template. Therefore, our results indicate that KET and MEF would be determined by HPLC instead of derivation and purification in drug analysis, minimum sample analysis especially. Besides, MIPs, which use a stationary phase procure with high sensitivity and selectivity. Furthermore, our experience shows that this method could be employed directly for NSAID determination in tablets with low cost and high speed.

Table II. Results from Quantitation Analysis of KET and MEF in Tablet Formulation

Compound	Amount found (mg/tablet)	RSD (%)*	Recovery (%)
KET-Tablet (50 mg/tablet)			
KET-Tablet 1	49.02	1.96	98.54 ± 0.24
KET-Tablet 2	48.79	2.42	95.23 ± 0.13
KET-Tablet 3	49.37	1.26	96.48 ± 0.47
MEF-Tablet (50 mg/tablet)			
MEF-Tablet 1	48.92	2.16	95.79 ± 0.19
MEF-Tablet 2	49.12	1.76	96.56 ± 0.35
MEF-Tablet 3	49.76	0.48	96.37 ± 0.67

* RSD (%) = Relative standard deviation. RSD values were estimated from repeatability.

Acknowledgments

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