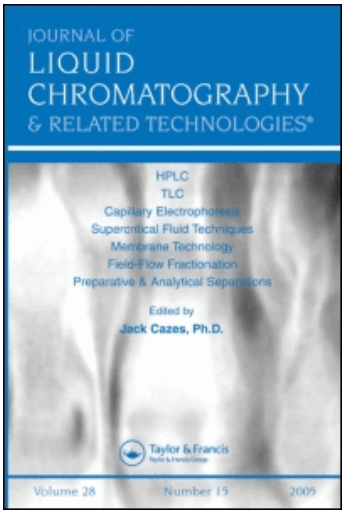

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 19 September 2000

To cite this Article Park, Jeong -Sook , Park, Kyung -Mi and Kim, Chong -Kook(2000) 'DETERMINATION OF TRIFLUSAL IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH AUTOMATED COLUMN SWITCHING SYSTEM', Journal of Liquid Chromatography & Related Technologies, 23: 16, 2513 — 2524
To link to this Article: DOI: 10.1081/JLC-100100505
URL: http://dx.doi.org/10.1081/JLC-100100505

J. LIQ. CHROM. & REL. TECHNOL., 23(16), 2513–2524 (2000)

DETERMINATION OF TRIFLUSAL IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH AUTOMATED COLUMN SWITCHING SYSTEM

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ABSTRACT

An analytical method of triflusal in human plasma was developed using semi-microbore HPLC equipped with automated column switching system. *p*-Toluic acid which is the structural analogue of triflusal, was used as an internal standard and 2 M HCl was employed as a stabilizer. The load phase and mobile phase were prepared using acetonitrile and 20 mM KH_2PO_4 with the volume ratios of 10:90 (pH 2.5) and 43:57 (pH 2.3), respectively. The signals were monitored by a UV detector at 275 nm with flow-rate of load phase, 500 μL/min, and mobile phase, 100 μL/min, respectively. The retention time of triflusal and *p*-toluic acid was about 20.2 min and 16.4 min, respectively. The detection limit of triflusal in human plasma was 0.01 μg/mL and the limit of quantitative analysis was 0.05 μg/mL. The accuracy of the assay was from 97.76% to 116.51% while the intra-day and inter-day coefficient of variation of the same concentration range was less than 15%. This analytical method demonstrated excellent sensitivity, reproducibility, specificity, and speed using the plasma sample. This method could be successfully applied to evaluate the bioavailability of triflusal in human subjects without time-consuming sample clean-up after oral administration of low dose.

INTRODUCTION

Triflusal [2-acetoxy-4-(trifluoromethyl) benzoic acid, $C_{10}H_7F_3O_4$] (Figure 1) is a salicylate that is a structural analogue of acetylsalicylic acid,[1] but has a different pharmacological and pharmacokinetic behavior. Triflusal is an antithrombotic agent that decreases the production of B_2 thromboxane and inhibits platelet aggregation induced by various agents.[2,3] Triflusal is the irreversible inhibitor of the platelet cyclo-oxygenase and c-AMP phosphodiesterase activity.

Recently, various brands of triflusal have been frequently prescribed and dispensed to the patients for inhibiting platelet aggregation. Nevertheless, it is not easy to carry out the bioequivalence test for triflusal after oral administration of normal dose (300 mg), since triflusal is rapidly metabolized and the concentration of intact triflusal in plasma is very low. Due to the limitation of quantitative analysis, the pharmacokinetic studies have been performed using intake of more than 900 mg.[4-6] Therefore, to evaluate the bioavailability or pharmacokinetics of triflusal after intake equivalent to 300 mg, more reliable and sensitive analytical method of triflusal in plasma sample should be developed. Furthermore, the stability of triflusal in plasma should be improved during the assay period, since triflusal is readily decomposed by pH or metabolized by esterase. Although, common reversed-phase HPLC methods with UV detection have been used for the analysis of triflusal in plasma,[4-6] they are inconvenient due to requiring the evaporation of solvents with helium or nitrogen gas and dilution. Furthermore, the detection limits were unsatisfactory. Recently, it was reported that on-line technique improved reliability and sample throughput, and reduced total time for analysis, as well as sample loss or change.[7] Therefore, column switching, which is composed of the on-line technique, is one of

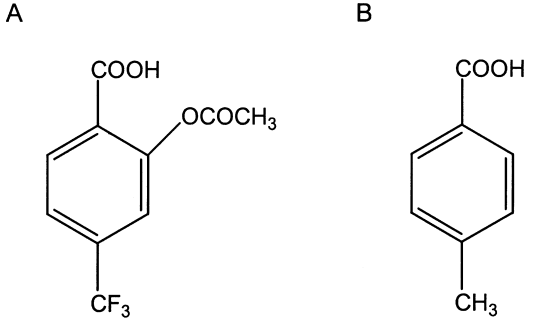

Figure 1. Structure of triflusal (A) and *p*-toluic acid (B).

the candidates for the analysis of triflusal from plasma samples. In the present study, the analysis of triflusal in plasma has been applied by an automated semi-microbore HPLC system equipped with a column switching. This could reduce the analytical procedure, particularly including pre-purification steps.

EXPERIMENTAL

Materials

Triflusal was supplied by Whanin Pharm. Co. (Ahnsung, Korea). *p*-Toluic acid (4-methyl-benzoic acid) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Acetonitrile was of HPLC grade from Fisher Sci. (Fair Lawn, NJ, USA). All other chemicals were the highest purity available, and water was deionized, distilled, and degassed in house.

Preparation of Standard Solutions

Triflusal was dissolved in acetonitrile to make a stock solution at a concentration of 1 mg/mL. Standard solutions of triflusal in human plasma were prepared by spiking the appropriate volume (less than 10 μL per mL) of various diluted stock solutions giving final concentrations of 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg/mL. Internal standard, *p*-toluic acid, structurally similar to triflusal, as shown in Figure 1, was dissolved in acetonitrile to make a stock solution at a final concentration of 50 μg/mL.

Preparation of Sample for HPLC Analysis

Each 10 μL of internal standard solution, and 2 M HCl as a stabilizing agent for triflusal in plasma, was added to 500 μL of standard and sample solutions. The mixture was diluted with an equal volume of load phase and vortexed for 30 s, then, the samples were centrifuged with 2000 *g* at 4°C for 10 min. Aliquots (300 μL) of the supernatant were filtered using nylon microcentrifuge filters (0.45 μm, 500 μL, XPERTEX®, P.J. Cobert Associates, Inc., St. Louis, MO, USA). 240 μL of filtered aliquot was injected into the HPLC system with an autosampler at 4°C.

HPLC System

The determination of triflusal was carried out using semi-microbore HPLC Nanospace SI-1 series (Shiseido, Tokyo, Japan), equipped with two 2001 pumps, a 2002 UV-Vis detector, a 2003 autosampler, a 2004 column oven, a

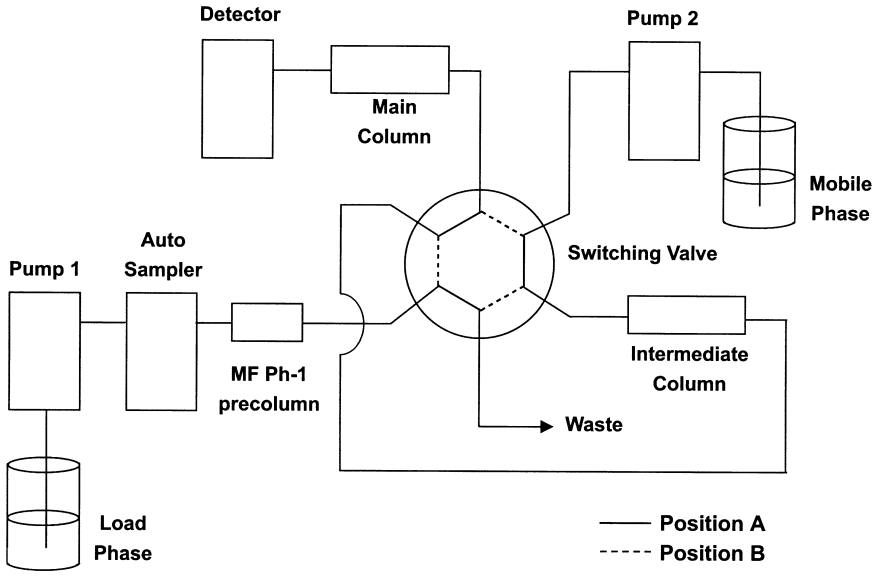

Figure 2. Schematic diagram of a column switching system.

2012 high-pressure switching valve, and a 2009 degassing unit as schematically described in Figure 2.

A Capcell Pak MF Ph-1 column (10 × 4 mm), Capcell Pak C_{18} UG 120 (35 × 2 mm) and Capcell Pak C_{18} UG 120 (250 × 1.5 mm) from Shiseido (Tokyo, Japan) were used as a pre-column for on-line sample preparation, intermediate column, and analytical column, respectively.

Analytical Procedure

Acetonitrile-20 mM KH_2PO_4 with the volume ratio of 10:90 (pH 2.5) and 43:57 (pH 2.3) were used as a load phase for on-line trace enrichment and as a mobile phase for the separation, respectively. The column was maintained at 30°C and the MF Ph-1 pre-column was equilibrated with a load phase. In the meanwhile, the intermediate and analytical column were equilibrated using the mobile phase.

The plasma sample (240 μL) was injected onto a MF Ph-1 pre-column and plasma proteins were removed from the pre-column using a load phase at a flow-rate of 500 μL/min (valve position A). From 4.5 min to 7.8 min after sam-

ple injection (valve position B), triflusal zone and internal standard zone were transferred from pre-column onto the head of an intermediate C_{18} column by the load phase at a flow-rate of 500 μL/min. Continuously, from 7.8 min to 25 min (valve position A), the analytes enriched on an intermediate C_{18} column were separated on an analytical column with 100 μL/min of mobile phase. The signals were monitored using a UV detector at 275 nm and recorded by DS-Chrom® (Donam Intl., Korea).

Stability of Triflusal

Due to the lack of stability of triflusal in a solution,[5] its degradation during the analytical process was evaluated by preparing concentrations of 100 μg/mL of triflusal at different pH conditions (pH 2.0 and 7.4). These solutions were maintained at room temperature for approximately 4 hr. At different time intervals, the concentration of triflusal was determined.

Method Validation

The linearity was examined by analyzing plasma standards containing predetermined amounts of triflusal over the concentration range of 0.05-10 μg/mL. The detection limit of triflusal was determined as the concentration of drug giving a signal-to-noise ratio greater than 3:1. The precision [defined as the coefficient of variation (C.V.) of replicate analysis] and the accuracy (defined as the bias between added and calculated concentration) of method were also evaluated.[8]

Preparation of Biological Samples

After oral administration of triflusal (equivalent to 300 mg) to 16 healthy males aged 21-25 years, blood samples (6 mL) were collected from the individual subject at designated time intervals. The blood samples were allowed to stand in an ice bath for 30 min and were centrifuged at 2000 *g* for 10 min. Each plasma sample was collected, stored at –20°C, and subjected to the HPLC procedure described above. Maximum plasma concentration of triflusal (C_{max}) and time to reach the peak (t_{max}) were compiled from the plasma concentration of triflusal-time curve.

RESULTS AND DISCUSSION

Stability of Triflusal

Since triflusal is hydrophilic and a weak acid, as shown in its structure, it is liable to be hydrolized. Furthermore, due to the instability of triflusal, which

is readily decomposed in plasma by esterase, its degradation needs to be ceased during the analytical period. While, triflusal was relatively stable in pH 2.0 buffer at 25°C for 4 hr, about 35% of triflusal was decomposed in pH 7.4 buffer under the same experimental conditions. Therefore, 2 M HCl was used as a stabilizer in plasma to adjust pH 2.0, and as an inactivator of esterase. Even though, triflusal in plasma was hydrolized by pH or metabolized by esterase, triflusal had not been decomposed at low pH at 4°C during the assay period (at least 8 hr). It could be thought, that the acetoxy group of triflusal is not dissociated at lower pH than pKa of triflusal. Moreover, it could be due to the inactivation or inhibition of esterase.

Chromatography and Column Switching Procedure

Triflusal in human plasma was analyzed using the column switching HPLC. In order to determine the composition of load phase and the appropriate valve switching time, the retention of triflusal on MF Ph-1 pre-column was examined under the various pH conditions with 20 mM KH_2PO_4, such as pH 2.5, 2.8, 2.9, and 3.0 as shown in Figure 3A. At pH 2.5, the retention time of triflusal was 8 min, and it became shorter by gradually increasing the pH as shown in Figure 3A. However, the retention time of *p*-toluic acid was constant at 5 min under the different pH conditions (Figure 3B). From the results, the mixture of acetonitrile and pH 2.5 buffer solution was selected for the composition of load phase. To remove plasma proteins and concentrate triflusal in the required time, 10% acetonitrile in 20 mM KH_2PO_4 (pH 2.5) was chosen as a load phase (Figure 3C).

Figure 4 illustrates typical chromatograms of (A) drug-free human plasma, (B) *p*-toluic acid spiked into human plasma, (C) triflusal spiked into human plasma, and (D) triflusal in plasma collected at 1 hr after oral administration of 300 mg to human subject. The retention times of triflusal and *p*-toluic acid were 20.2 min and 16.4 min, respectively. From the results of blank and raw tests of *p*-toluic acid and triflusal in plasma, the triflusal in human plasma was able to analyze quantitatively. In particular, no interference from endogenous substance was observed in all the biological samples. After 100 injections of plasma samples equivalent to 24 mL plasma, the MF Ph-1 pre-column was exchanged.

Method Validation

The calibration curve was made by the ratio of peak height versus the concentrations of *p*-toluic acid and triflusal in plasma with the range of 0.05-10 μg/mL. The mean (± SD) regression equation from 5 replicate calibration curves on different days was $y = 1.6128\ (\pm 0.208)x - 0.091\ (\pm 0.052)$ (where, y = triflusal concentration, x = ratio of peak height) with the correlation coefficient

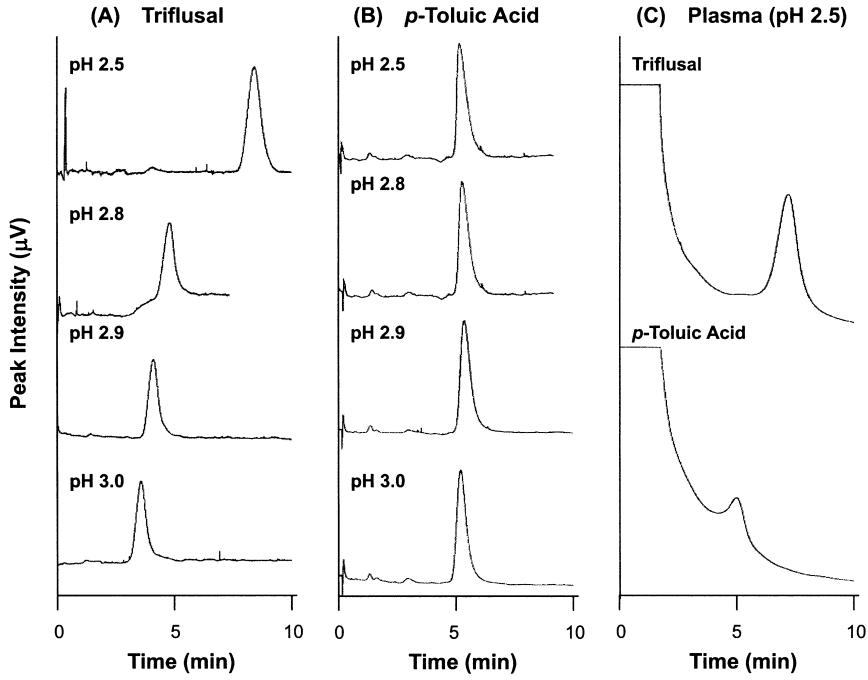

Figure 3. Retention of triflusal, *p*-toluic acid, and triflusal and *p*-toluic acid in plasma on MF Ph-1 column (10×4 mm I.D.). Conditions of mobile phases: potassium dihydrogen phosphate (20 mM); (A) triflusal, (B) *p*-toluic acid and (C) triflusal and *p*-toluic acid in blank plasma, pH 2.5; flow-rate, 500 μL/min; injection volume, 100 μL.

of 0.9993 (± 0.0004). The detection limit of triflusal was 0.01 μg/mL. The precision and accuracy of assay are listed in Table 1. The accuracy deviated from 97.76% to 116.51% of the added amount in the spiked plasma samples. The assay was precise because the C.V. was less than 15%.

Application of the Method to Biological Samples

The semi-microbore HPLC with automated column switching system has been successfully applied to the analysis of more than 600 plasma samples collected from human subjects after oral administration of triflusal capsule. Figure 5 shows a mean plasma concentration versus time plot of triflusal following an oral administration (300 mg) to human subjects. The C_{max} of triflusal was reached at 0.63 ± 0.14 hr as 4.89 ± 1.86 μg/mL. In general, triflusal in plasma

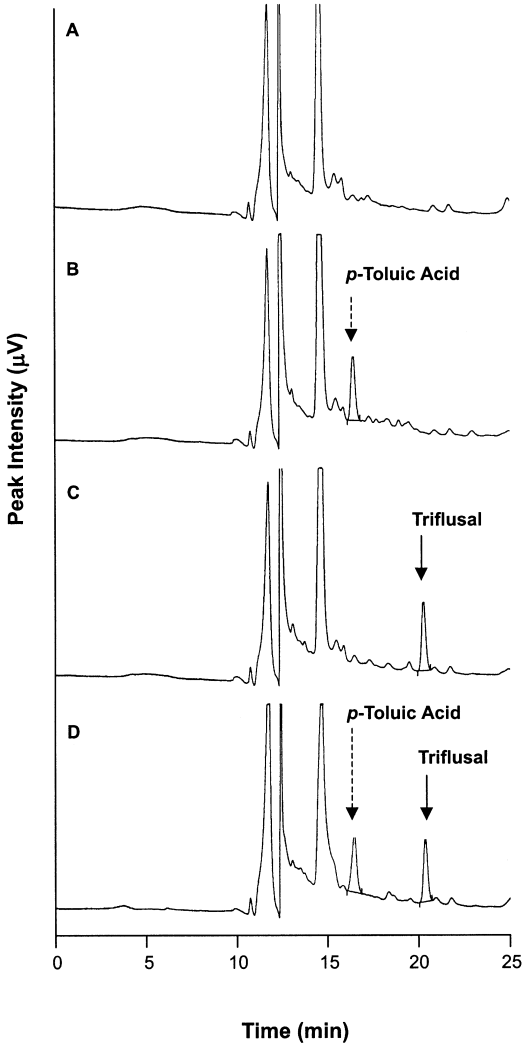

Figure 4. Chromatograms of (A) blank plasma, (B) blank plasma spiked with *p*-toluic acid as internal standard (1 μg/mL), (C) blank plasma spiked with triflusal (2 μg/mL), and (D) plasma sample from a human subject at 1 hour after an oral administration of 300 mg triflusal.

Table 1

Reproducibility of Triflusal Determination in Human Plasma

Added Amount (μg/mL)	Response Factor[a]	Accuracy (%) Intra-Day[b]	Accuracy (%) Inter-Day[c]	Precision (%) Intra-Day[b]	Precision (%) Inter-Day[c]
0.05	0.497 ± 0.181	105.28	116.51	4.919	12.963
0.1	0.521 ± 0.119	110.00	109.21	2.952	6.581
0.2	0.541 ± 0.132	103.50	105.50	5.017	4.022
0.5	0.587 ± 0.100	101.20	99.83	0.613	1.209
1	0.600 ± 0.075	102.10	105.30	1.916	2.161
2	0.527 ± 0.047	99.85	98.83	4.085	2.192
5	0.583 ± 0.076	100.62	97.76	1.160	1.760
10	0.603 ± 0.092	101.20	100.73	0.049	0.304

[a] Peak height (μV) divided by its concentration ratio. [b] n = 3. [c] n = 5.

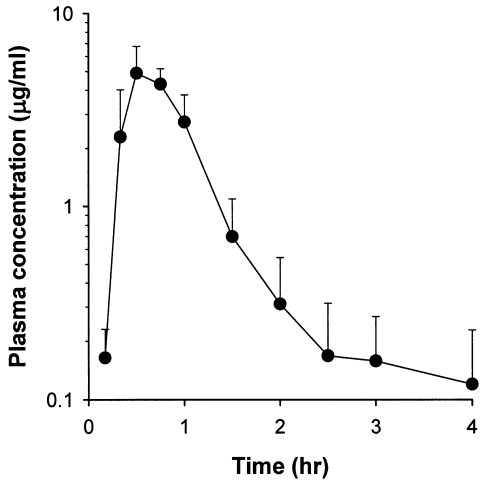

Figure 5. Mean plasma concentration versus time plot of triflusal in human subjects after oral administration of 300 mg triflusal. Each point with vertical bar represents the mean and standard deviation of 16 human subjects.

samples could be readily analyzed between 10 min (the first sampling time, 0.16 ± 0.07 μg/mL) to 240 min (the last sampling time, 0.12 ± 0.08 μg/mL) after the oral administration. This observation indicates that this analytical method could be applied to determine the concentration of triflusal in human plasma with excellent sensitivity and without time-consuming sample clean-up after oral administration of low dose, compared with the previously reported method.[5]

In the column switching technique,[7,9-11] the condition of pre-column packing, load phase, or valve switching time is crucial to obtain complete recovery and remove interference factors. The Capcell Pak MF Ph-1 has been used as the pre-column since it has long polyoxyethylene chains; as well as phenyl groups on the surface of 80 Å silica, and they limit the access of large molecules such as proteins and retain drug molecules longer.[12]

The present method offers practical advantages over the liquid-liquid[13,14] or solid-phase extraction[7] in the viewpoint of the speed, sensitivity, sample throughput, and sample volume. Moreover, compared with previously reported liquid chromatographic determination of triflusal,[5] this method improved the limits of detection and analysis by more than 10 times and maintained the stability of triflusal. The on-line trace enrichment using the column switching technique is appropriate for the sample clean-up of triflusal from plasma samples due to low concentration.

CONCLUSION

Determination of triflusal directly from plasma sample has been developed using semi-microbore HPLC with an automated column switching system. This analytical method demonstrated excellent sensitivity, reproducibility, specificity, and speed using the plasma sample. This method could be successfully applied to evaluate the pharmacokinetics of triflusal in human subjects without time-consuming sample clean-up.

ACKNOWLEDGMENTS

This research is supported by the grant from the Research Institute of Pharmaceutical Sciences in College of Pharmacy, Seoul National University. The authors are grateful to Young Jin Biochrom. Co. Ltd. for their expert technical assistance.

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Received March 10, 2000 Author's Revisions May 15, 2000
Accepted April 15, 2000 Manuscript 5272