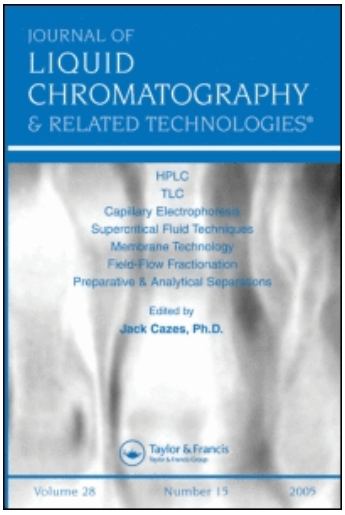

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Semi-Microbore HPLC for the Determination of Baclofen in Human Plasma Using Column Switching

Eunmi Ban[a]; Jeong-Sook Park[a]; Chong-Kook Kim[a]
[a] National Research Lab for Drug and Gene Delivery, College of Pharmacy, Seoul National University, Seoul, Korea

Online publication date: 20 September 2004

To cite this Article Ban, Eunmi , Park, Jeong-Sook and Kim, Chong-Kook(2005) 'Semi-Microbore HPLC for the Determination of Baclofen in Human Plasma Using Column Switching', Journal of Liquid Chromatography & Related Technologies, 27: 19, 3051 — 3064
To link to this Article: DOI: 10.1081/JLC-200032681
URL: http://dx.doi.org/10.1081/JLC-200032681

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 27, No. 19, pp. 3051–3064, 2004

Semi-Microbore HPLC for the Determination of Baclofen in Human Plasma Using Column Switching

Eunmi Ban, Jeong-Sook Park, and Chong-Kook Kim*

National Research Lab for Drug and Gene Delivery, College of Pharmacy, Seoul National University, Seoul, Korea

ABSTRACT

Using a column switching technique, a semi-microbore high performance liquid chromatographic (HPLC) method was developed for the determination of baclofen in human plasma. Methoxamine was used as an internal standard. Following precipitation of the plasma sample with zinc sulfate–acetonitrile, the samples were directly injected onto the system. The analyte was retained in an enrichment column while endogenous plasma components were eluted out to waste. Baclofen was then back-flushed to a semi-micro C_{18} analytical column for separation and quantification with a UV detector at 220 nm. The eluents for pretreatment and separation were 20 mM potassium phosphate (pH 2.0) and 20 mM potassium phosphate (pH 3.6)–methanol (82 : 18, v/v),

*Correspondence: Chong-Kook Kim, National Research Lab for Drug and Gene Delivery, College of Pharmacy, Seoul National University, San 56-1, Shillim-Dong, Kwanak-Gu, Seoul 151-742, Korea; E-mail: ckkim@plaza.snu.ac.kr.

DOI: 10.1081/JLC-200032681 1082-6076 (Print); 1520-572X (Online)

respectively. The retention time of baclofen was about 20.8 min. The mean recovery was 98.5% and the calibration curves were linear in the investigated concentration ranges of 25–800 ng/mL ($r^2 = 0.9993$). The detection limit of baclofen was less than 5 ng/mL. The inter- and intra-day reproducibility (RSD, %) is less than 12%, even at the limit of quantification (LOQ) of the method. This analytical method showed good sensitivity and reproducibility. This method could be successfully applied to evaluate the pharmacokinetics of baclofen in healthy volunteers after the oral dose of 25 mg baclofen.

Key Words: Baclofen; Column switching; High performance liquid chromatography.

INTRODUCTION

Baclofen (4-amino-3-*p*-chlorophenylbutyric acid, Lioresal®), a chemical analogue of γ-aminobutyric acid (GABA), is a selective $GABA_B$-receptor agonist.[1] Clinically, baclofen is used as a skeletal muscle relaxant to alleviate spasticity associated with a range of conditions, including brain and spinal cord injury, multiple sclerosis, and cerebral palsy.[2,3]

Recently, various brands of baclofen have been frequently prescribed and dispensed to patients for treatment of spasticity. To study the pharmacokinetics of baclofen, a selective and sensitive analytical method was required for the determination of baclofen in plasma samples. Several methods have been used in the pharmacokinetic study of baclofen, including gas chromatography,[4] gas chromatography-mass spectrometry,[5] and high performance liquid chromatography (HPLC) with ultraviolet,[6,7] fluorescence,[8,9] or electrochemical detection.[10] Moreover, a sensitive liquid chromatographic method for the determination of baclofen in plasma was developed using tandem mass spectrometry.[11] Zhu and Neirinck recently reported a HPLC-UV detection method for the determination of baclofen R-(−)- and S-(+)-enatiomers in human plasma using a chiral separation technique.[12] However, these methods required sample clean-up procedures, such as liquid–liquid extraction or solid phase extraction and derivatization steps prior to instrumental analysis. Although these pretreatment steps are needed for a sensitive assay, they are complex and time consuming. Therefore, a column switching procedure is an alternative to analyze large numbers of samples and to obtain higher sensitivity.

HPLC with column switching system offers a powerful tool in analytical chemistry due to its convenience without extraction, concentration, or derivatization. Column switching is an on-line trace enrichment technique that can directly analyze biological samples in the hundreds of microliters for

semi-microbore liquid chromatography (LC) as well as conventional LC. The semi-microbore HPLC method[13,14] with a column switching system[15–17] is one of the most useful methods for automated and convenient analysis of drugs in biological fluid. Up to now, semi-microbore HPLC for the determination of baclofen in human plasma has not been reported.

Here, we developed a sensitive and accurate semi-microbore HPLC method, in combination with column switching, for the determination of baclofen in human plasma with relatively simple pretreatment steps. The applicability of the assay method was demonstrated in the pharmacokinetic study of baclofen in healthy volunteers.

EXPERIMENTAL

Materials

Baclofen tablets (Barapa tablets, 25 mg) were obtained from Hana Pharm. Co. (Seoul, Korea). Baclofen and methoxamine (internal standard, IS) (Fig. 1) were obtained from Sigma (St. Louis, MO). Acetonitrile and methanol of HPLC grade were purchased from Burdick & Jackson (Muskegon, MI). HPLC grade water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA). All other chemicals were the highest purity available.

Preparation of Standard Solutions

Baclofen was dissolved in water to make a stock solution at a concentration of 1 mg/mL. Standard solutions of baclofen in human plasma were prepared by spiking the appropriate volume (less than 10 μL/mL) of various diluted stock solutions giving final concentrations of 5, 25, 50, 100, 150, 200, 400, and 800 ng/mL. The IS, methoxamine was dissolved in water to make a stock solution at a final concentration of 100 μg/mL.

Preparation of Sample for HPLC Analysis

Each 5 μL of IS solution was added to 1 mL of plasma. To prevent the precipitation of plasma protein in the HPLC system, the plasma sample was deproteinized by simply adding 100 μL of a zinc sulfate solution (500 mg/mL) and 100 μL of acetontrile.[6] The mixture was vortexed for 15 sec and the samples were centrifuged at 3000*g* at 4°C for 10 min. Aliquots

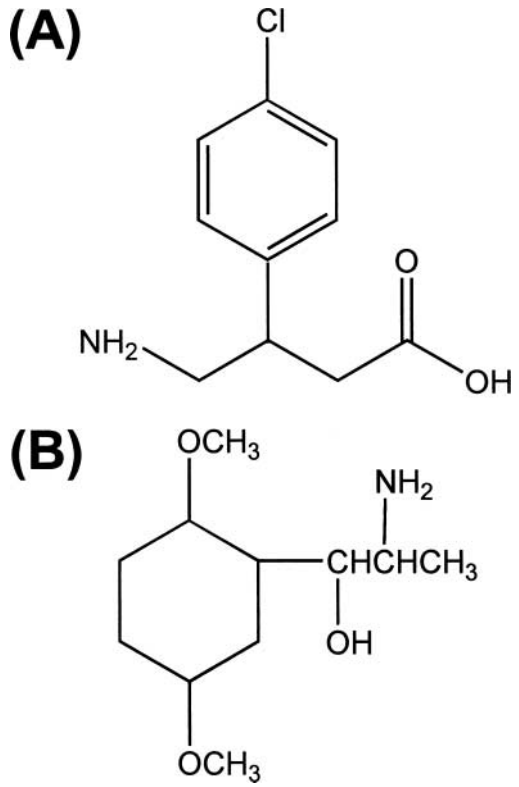

Figure 1. Chemical structures of (A) baclofen and (B) methoxamine.

(50 μL) of the supernatant were injected into a column switching HPLC system.

HPLC System

All experiments were performed using an automated 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 2012 high-pressure switching valve, and a 2009 degassing unit, as schematically described in Fig. 2.

A Capcell Pak MF C_8 SG 80 column (150 mm × 4.6 mm, 5 μm), Capcell Pak C_{18} UG 120 (35 mm × 2.0 mm, 5 μm), and Capcell Pak C_{18} UG 120 (250 mm × 1.5 mm, 5 μm) from Shiseido (Tokyo, Japan) were used as

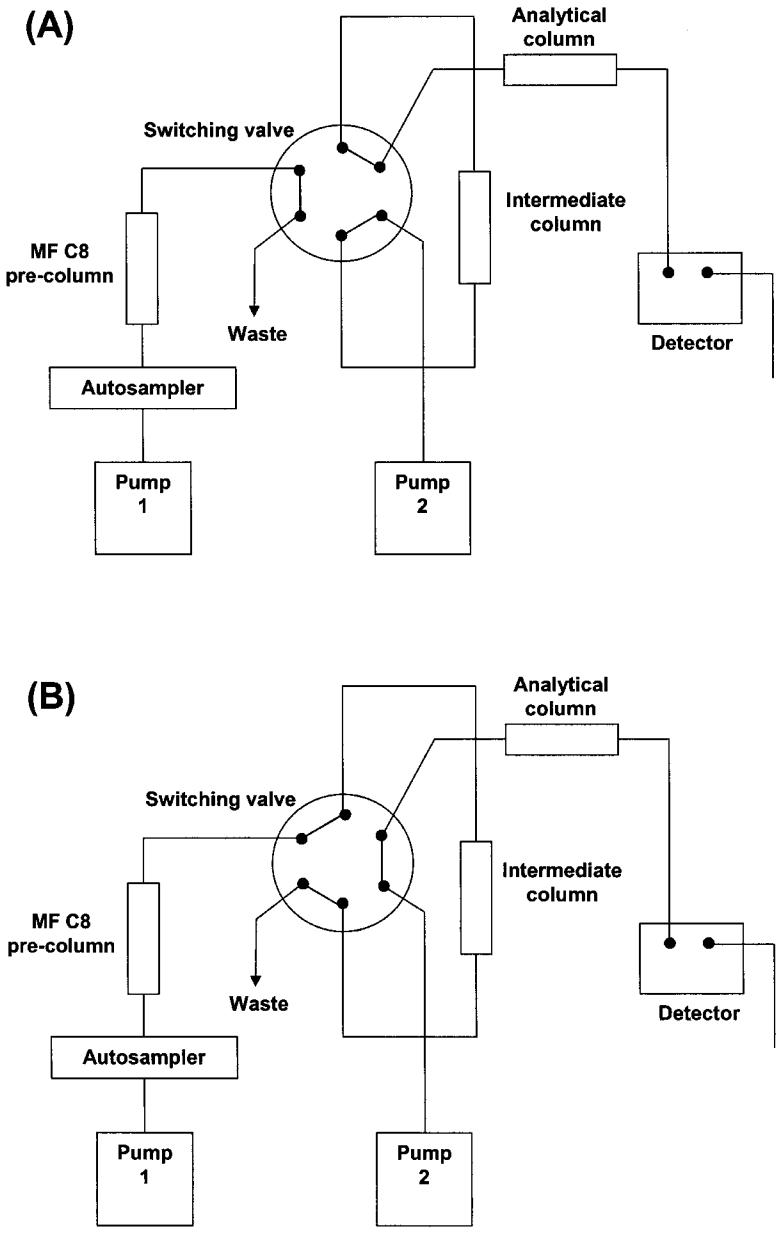

Figure 2. Schematic representation of a column switching system. (A) Sample loading, precolumn wash and re-equilibration position; (B) elution, separation. See Table 1 for switching valve position.

pre-column for on-line sample preparation, intermediate column, and analytical column, respectively.

Analytical Procedure

A phosphate solution (20 mM, pH 2.0) was used as a mobile phase for pretreatment (eluent A). Methanol (18%) in 20 mM phosphate solution (pH 3.6) was used as a mobile phase for analysis (eluent B). The column was maintained at 40°C and a pre-column was equilibrated with eluent A. Meanwhile, the intermediate and analytical columns were equilibrated using eluent B. The plasma sample (50 μL) was injected onto a MF C_8 pre-column and plasma wastes were removed from the pre-column using eluent A at a flow-rate of 0.5 mL/min, according to Fig. 2(A). From 5.8 to 8.0 min after sample injection [see Fig. 2(B)], the baclofen zone and IS zone were transferred from the pre-column onto the head of an intermediate C_{18} column by eluent A, at a flow-rate of 0.5 mL. Continuously, from 8.0 to 27 min [see Fig. 2(A)], the analytes, enriched on an intermediate column, was separated on an analytical column using a separation phase at a flow-rate of 0.1 mL/min. The time program for the column switching HPLC was summarized in Table 1. The signals were monitored with UV detection at 220 nm and data were recorded by DS-Chrom® software (Donam Int., Korea).

Stability

The stability of baclofen was evaluated by comparing the deproteinized plasma samples that were injected immediately (time zero), with the

Table 1. Time program for the column switching HPLC.

Time after injection (min)	Valve position (pathway)	Comments
0.0–5.8	Loading (A)	Sample loading (50 μL) by eluent A at 0.5 mL/min
5.8–8.0	Concentration (B)	Transfer of baclofen and IS from precolumn to intermediate column by eluent A at 0.5 mL/min
8.0–27.0	Separation (A)	Intermediate column backflush onto the analytical column followed by eluent B at 0.1 mL/min

Note: Eluent A: 20 mM phosphate solution (pH 2.0). Eluent B: methanol (18%) in 20 mM phosphate solution (pH 3.6).

samples that were re-injected 72 hr after sitting in the autosampler at room temperature. Evaluation was based on back-calculated concentration.

Assay Validation

The linearity was examined by analyzing plasma standards containing pre-determined amounts of baclofen over the investigated concentration ranges of 25–800 ng/mL. The recovery of baclofen from plasma was determined by the analysis of fixed amounts of baclofen in plasma, followed by a replicate injection of the same amount of a standard in 50 μL of water, directly onto the microbore column providing the 100% value. The detection limit was determined as the concentration of drug giving a signal-to-noise ratio greater than 3 : 1. The precision [the relative standard deviation (RSD) of replicate analysis] and accuracy (defined as the bias between added and calculated concentrations) of the method were assessed at four concentrations of 25, 50, 100, and 800 ng/mL. The limit of quantification (LOQ) was also determined as the concentration of drug giving a signal-to-noise ratio greater than 10, with an accuracy of between 80% and 120%, and with a precision less than 20%.

Pharmacokinetics of Baclofen in Volunteers

After a single oral dose of baclofen (equivalent to 25 mg) to eight healthy males aged 20–30 years, blood samples (10 mL) were withdrawn from the forearm vein at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 hr post dosing. The blood samples were transferred to a Vacutainer® tube (BD, NJ) and centrifuged at 1200*g* for 10 min at 4°C. The plasma samples were separated and stored at −70°C prior to analysis. Maximum plasma concentration of baclofen (C_{max}) and time to reach the peak (T_{max}) of baclofen was determined by visual inspection from the plasma concentration–time plots for baclofen. The other parameters were calculated by K-BE Test 2002 for windows.[18]

RESULTS AND DISCUSSION

Chromatography and Column Switching Procedure

To determine the concentration of baclofen, three kinds of columns were used, including a pre-column, intermediate column, and analytical column. Moreover, to remove the endogenous materials interfering with the analyte

in plasma, the appropriate switching time was determined. In this experiment, to remove plasma wastes and retain baclofen in intermediate column, the low pH without organic solvent, such as 20 mM phosphate buffer (pH 2.0), was used as mobile phase due to the high aqueous solubility of baclofen. Moreover, in order to prevent the precipitation of plasma protein in the HPLC system at low pH, the plasma sample was deprotenized by adding 0.1 mL of acetonitrile and 50 mg of zinc sulfate to 1 mL of plasma, prior to injection into the pre-column. Baclofen and IS in blank plasma were separated in the pre-column, which is unusually long. In most column switching, a short MF column or cartilage was used as a pre-column to clean up plasma samples.[13,14] However, we used a longer pre-column to retain a target drug because it was difficult to separate baclofen and IS from plasma. The analytes, fractionated from the pre-column by a valve switching step, were focused in the top of intermediate column and then separated to the analytical column. The pre-column, intermediate, and main column did not show significant deterioration in efficiency after atleast 100 injections of plasma samples.

Specificity

Using the above determined column switching HPLC system, typical chromatograms were obtained from drug-free plasma (A) and baclofen of 100 ng/mL, and IS spiked into blank plasma (C), as shown in Fig. 3. Under this condition, the sensitivity of baclofen was satisfactory with the LOQ of 25 ng/mL [Fig. 3(B)]. Also, there were no chromatographic interferences derived from endogenous substances in the plasma sample and so good specificity was obtained. The retention time of baclofen and IS was about 21 and 23 min, respectively. Good separation and baseline were observed.

Linearity

The plasma calibration curves were constructed by plotting the ratios of peak area vs. the concentration of baclofen and methoxamine in human plasma with the concentration ranges of 25–800 ng/mL. The linearity of baclofen was evaluated over the investigated concentration ranges of 25–800 ng/mL. The mean ($\pm$SD) regression equation from five replicate calibration curves was $y = 0.0021\ (\pm 0.001)x + 0.0142\ (\pm 0.0122)$ (where, $y =$ baclofen concentration, $x =$ ratio of peak area) with the correlation coefficient of 0.9993 (± 0.0031).

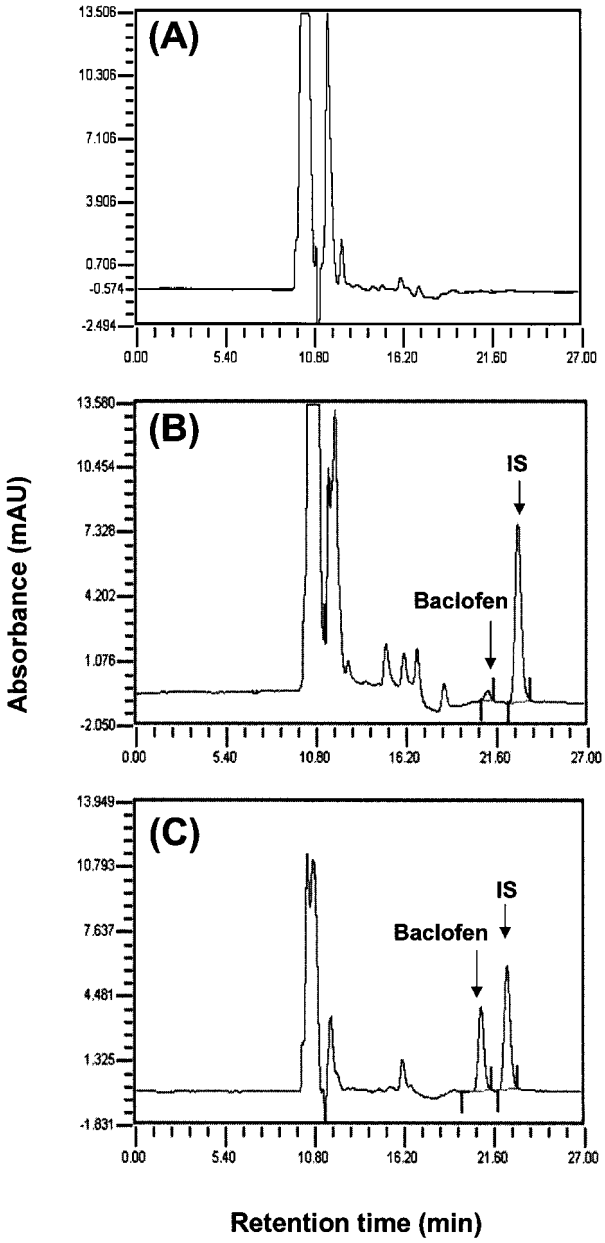

Figure 3. Chromatograms of (A) blank plasma, plasma sample spiked at (B) 25 ng/mL (LOQ) of baclofen, and (C) 200 ng/mL of baclofen.

Assay Validation

The mean recovery of baclofen from human plasma samples was 98.5%. The inter- and intra-day precision and the accuracy were determined by analyzing plasma samples spiked at 25, 50, 400, and 800 ng/mL. Inter-day precision was determined by analyzing five calibration curves on five different days. The intra-day precision was determined by analyzing five replicates in the same day. Precision of baclofen calculated as coefficient of variation (CV) was always below 11.80%. The accuracy of baclofen expressed as percentage (percentage of the measured concentration to the theoretical concentration) ranged from 90.32% to 102.53%. The inter-day and intra-day precision and accuracy for baclofen in human plasma are provided in Table 2. The LOQ was estimated as 25 ng/mL, as shown in Table 2 and Fig. 3(B).

Stability

Deproteinized plasma samples containing baclofen and IS were stable on the autosampler tray at room temperature for 3 days, as determined by reinjection. They can also be stored in a refrigerator for at least 90 hr before injection.

Pharmacokinetics of Baclofen in Humans

The present method was applied to the analysis of baclofen in plasma of volunteers after an oral administration of baclofen tablet. Eight healthy volunteers were administered a single oral of baclofen tablet (25 mg, Barapa tablets). The plasma chromatogram of a volunteer administered baclofen is

Table 2. Precision and accuracy of baclofen in human plasma ($n = 5$).

Concentration (ng/mL)	Precision (RSD, %)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
25	11.21	11.80	101.63 ± 9.90	90.32 ± 8.18
50	7.64	7.49	99.13 ± 7.07	98.93 ± 9.83
400	4.46	6.83	100.01 ± 4.42	102.53 ± 5.74
800	0.66	0.99	96.98 ± 0.65	99.39 ± 1.35

shown in Fig. 4, which shows a typical chromatogram of baclofen in plasma collected at 4 hr after oral administration of 25 mg to human subjects. The plasma concentration of baclofen at 1 hr was 219.89 ng/mL.

Figure 5 shows the mean plasma concentration–time profiles of baclofen following an oral administration (25 mg) to human subjects. The C_{max} of baclofen was reached at 1.75 $\pm$ 0.38 hr as 489 $\pm$ 113 ng/mL after administration. The area under the curve (AUC) was 1870 $\pm$ 322 ng hr/mL. The pharmacokinetic parameters of baclofen are shown in Table 3. These values are comparable with the corresponding parameters obtained by a single oral dose of 20 mg baclofen in the previous reports.[6,10] From these results, it is suggested that the present column switching semi-microbore HPLC analysis can be applied to the routine monitoring of baclofen in biological fluids.

CONCLUSION

We have established a simple and sensitive analytical method for the determination of baclofen from human plasma using the semi-microbore HPLC method with column switching system. This method has the major

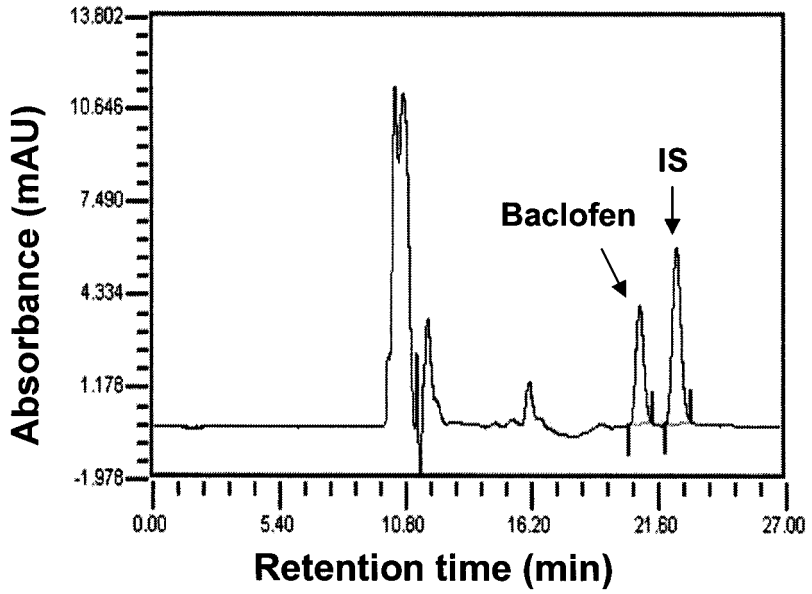

Figure 4. Chromatogram of baclofen in a plasma sample from a volunteer at 1 hr (219.89 ng/mL) after a singleoral dose of 25 mg baclofen.

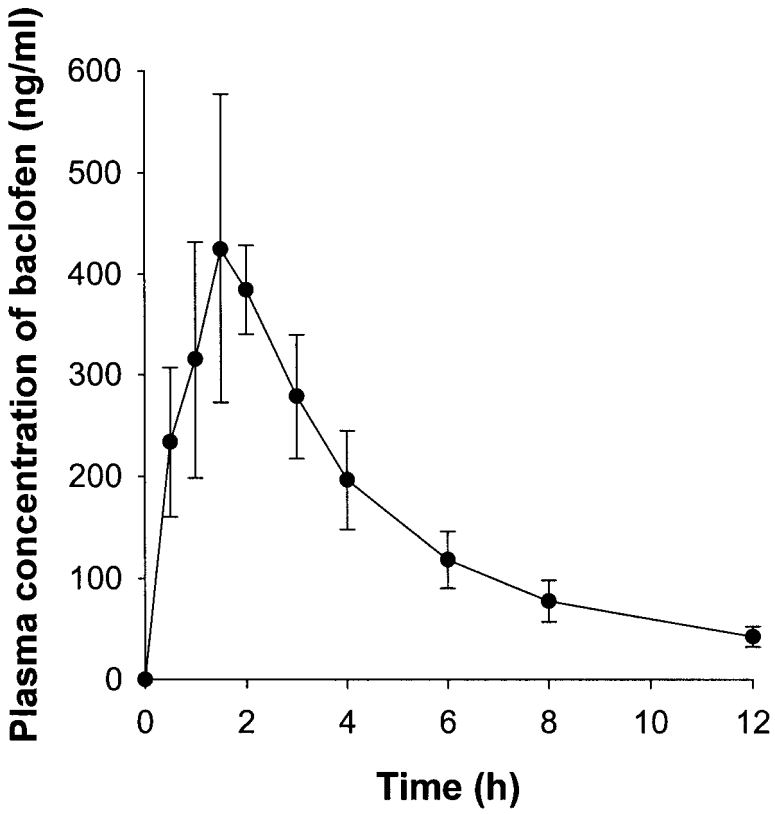

Figure 5. Mean plasma concentration–time profiles of baclofen in eight healthy subjects after oral administration of 25 mg baclofen. Each point represents the mean ± SD of human subjects.

Table 3. Pharmacokinetic parameters of baclofen in plasma of eight healthy subjects after oral administration of 25 mg baclofen.

Parameters	Mean ± SD
AUC_{12} (ng hr/mL)	1870 ± 322
C_{max} (ng/mL)	489 ± 113
T_{max} (hr)	1.75 ± 0.38
$T_{1/2}$ (hr)	4.01 ± 1.12

advantage of eliminating manual extraction and concentration steps for analysis of baclofen in biological samples. Also, this method shows excellent sensitivity, reproducibility, specificity, and speed. The method has been successfully used to provide pharmacokinetic studies of baclofen in human plasma.

ACKNOWLEDGMENT

This work was supported by the Korea Food and Drug Administration Grant (KFDA-02142-EQI-513).

REFERENCES

1. Bowery, N.G. $GABA_B$ receptor pharmacology. Annu. Rev. Pharmacol. Toxicol. **1993**, *33*, 109–147.
2. Bowery, N.G.; Bettler, B.; Froestl, W.; Gallagher, J.P.; Marshall, F.; Raiteri, M.; Bonner, T.I.; Enna, S.J. International Union of Pharmacology. XXXIII. Mammalian gamma-aminobutyric acid(B) receptors: structure and function. Pharmacol. Rev. **2002**, *54*, 247–264.
3. Dario, A.; Di Stefano, M.G.; Grossi, A.; Casagrande, F.; Bono, G. Long-term intrathecal Baclofen infusion in supraspinal spasticity of adulthood. Acta Neurol. Scand. **2002**, *105*, 83–87.
4. Kochak, G.; Honc, F. Improved gas–liquid chromatographic method for the determination of baclofen in plasma and urine. J. Chromatogr. **1984**, *310*, 319–326.
5. Swahn, C.G.; Beving, H.; Sedvall, G. Mass fragmentographic determination of 4-amino-3-*p*-chlorophenylbutyric acid (baclofen) in cerebrospinal fluid and serum. J. Chromatogr. **1979**, *162*, 433–438.
6. Rustum, A.M. Simple and rapid reversed-phase high-performance liquid chromatographic determination of baclofen in human plasma with ultraviolet detection. Application to a pharmacokinetic study. J. Chromatogr. B. **1989**, *487*, 107–115.
7. Harrison, P.M.; Tonkin, A.M.; McLean, A.J. Determination of 4-amino-3-(*p*-chlorophenyl)butyric acid (baclofen) in plasma by high performance liquid chromatography. J. Chromatogr. **1985**, *339*, 424–428.
8. Wuis, E.W.; Dirks, R.J.; Vree, T.B.; van der Kleyn, E. High-performance liquid chromatographic analysis of baclofen in plasma and urine of man after precolumn extraction and derivatization with *o*-phthaldialdehyde. J. Chromatogr. **1985**, *337*, 341–350.

9. Spahn, H.; Krauss, D.; Mutschler, E. Enantiospecific high performance liquid chromatographic (HPLC) determination of baclofen and its fluoro analogue in biological material. Pharm. Res. **1988**, *5*, 107–112.
10. Millerioux, L.; Brault, M.; Gualano, V.; Mignot, A. High performance liquid chromatographic determination of baclofen in human plasma. J. Chromatogr. A. **1996**, *729*, 309–314.
11. Flardh, M.; Jacobson, B.M. Sensitive method for the determination of baclofen in plasma by means of solid-phase extraction and liquid chromatography-tandem mass spectrometry. J. Chromatogr. A. **1999**, *846*, 169–173.
12. Zhu, Z.; Neirinck, L. Chiral separation and determination of R-(−)- and S-(+)-baclofen in human plasma by high performance liquid chromatography. J. Chromatogr. B. **2003**, *785*, 277–283.
13. Tanaka, M.; Kato, K. Sensitive semi-microcolumn high performance liquid chromatographic method for the determination of DU-6681, the active parent drug of a new oral carbapenem antibiotic, DZ-2640, in human plasma and urine using a column switching system as sample clean-up procedure. J. Chromatogr. B. **1999**, *724*, 73–82.
14. Park, J.S.; Park, K.M.; Kim, C.K. Determination of triflusal in human plasma by high performance liquid chromatography with automated column switching system. J. Liq. Chromatogr. Rel. Technol. **2000**, *23*, 2513–2524.
15. De Nardi, C.; Ferrari, L.; Nardin, E.; Ruffo, A.; Braggio, S. Development and validation of a column switching high performance liquid chromatographic method for the determination of sanfetrinem in rat and dog plasma by direct injection. J. Chromatogr. B. **2001**, *752*, 133–139.
16. Cass, Q.B.; Gomes, R.F.; Calafatti, S.A.; Pedrazolli, J., Jr. Determination of amoxycillin in human plasma by direct injection and coupled-column high performance liquid chromatography. J. Chromatogr. A. **2003**, *987*, 235–241.
17. Sachse, J.; Hartter, S.; Weigmann, H.; Hiemke, C. Automated determination of amisulpride by liquid chromatography with column switching and spectrophotometric detection. J. Chromatogr. B. **2003**, *784*, 405–410.
18. Lee, Y.J.; Kim, Y.G.; Lee, M.G.; Chung, S.J.; Lee, M.H.; Shim, C.K. Analysis of bioequivalence study using a log-transformed model. Yakhakhoeji **2000**, *44*, 308–314.

Received May 22, 2004
Accepted June 28, 2004
Manuscript 6408