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Simple and sensitive liquid chromatography–tandem mass spectrometry method for determination of the *S*(+)- and *R*(−)-enantiomers of baclofen in human plasma and cerebrospinal fluid

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Abstract

A simple and sensitive liquid chromatography–tandem mass spectrometry (LC/MS/MS) method to determine the enantiomers of the muscle relaxant baclofen in human plasma and cerebrospinal fluid (CSF) has been developed. A commercially available ultrafiltration membrane is used to prepare the sample. A chiral CROWNPAK CR(+)® stationary phase column is then used to perform complete resolution of the *S*(+)- and *R*(−)-enantiomers of baclofen. This method was used to analyze human plasma and CSF spiked with baclofen, and the calibration curves for both biologic samples were linear over a concentration range of 0.15–150 ng enantiomer/ml. The lower limit of quantification was 0.15 ng enantiomer/ml in both fluids. Finally, the method was tested with an artificial CSF as an alternative to authentic human CSF. The results showed that no matrix effects and no interfering peaks were observed using this artificial CSF.

Keywords: Enantiomer separation; Baclofen

1. Introduction

Baclofen (4-amino-3-*p*-chlorophenylbutyric acid), a skeletal muscle relaxant, has been widely given to patients suffering from multiple sclerosis or spasticity caused by spinal or cerebral injury since its introduction for therapy in 1967 [1]. Recently, continuous intrathecal administration of baclofen by means of an implantable pump has been successfully used to treat spasticity. This technique is gaining wider acceptance because it allows treatment with lower doses of baclofen, which more effectively treats severe spasticity and causes fewer side-effects than oral administration [2–6]. However, these lower doses of baclofen, less 100 μg of baclofen per person, make simultaneous evaluation of baclofen pharmacokinetics in plasma and cerebrospinal fluid (CSF) difficult because of the very low baclofen concentration level in plasma compared with that in CSF. Moreover, baclofen is administered clinically as a racemic mixture although the baclofen enantiomers are known to have different properties; for example, both the efficacy in treating spasticity and the toxicity associated with baclofen have been attributed to the *R*(−)-baclofen enantiomer alone [7], and the pharmacokinetic disposition of the baclofen enantiomers might also be different.

So far few analytical methods have been developed to determine each baclofen enantiomer in biologic material [8–11]. Those methods that do exist require troublesome extraction and derivatization procedures for sample cleanup, enantioselective separation, or sensitive fluorescence detection. The lower limit of quantification of these methods is over 25 nmol/l (equivalent to approximately 5 ng/ml) in plasma. This sensitivity is insufficient to determine low baclofen concentration levels in plasma when baclofen is intrathecally administrated by means of an implantable pump. Consequently, a more sensitive method is needed to evaluate baclofen pharmacokinetics in both plasma and

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CSF simultaneously, and an enantioselective method is also needed to evaluate the different properties of each enantiomer. Furthermore, a less complex sample preparation method than those currently used would use less time, cause less trouble, and provide easier measurement.

With these needs in mind, the objectives of this study were first, to develop a simple yet sensitive liquid chromatography–tandem mass spectrometry (LC/MS/MS) analytical method for the enantioselective determination of baclofen in human plasma and CSF, and second, to determine whether artificial CSF [12] can be used as an alternative to authentic human CSF for calibration tests for this technique, because of the difficulty in obtaining authentic human CSF.

2. Experimental

2.1. Materials

R(+)- and *S*(−)-Baclofen hydrochloride, racemic baclofen, and the internal sntadard L-*p*-chlorophenylalanine were obtained from Sigma (St. Louis, MO, USA). Serial reverse osmosis and a Milli-Q purification system (Nihon Millipore; Tokyo, Japan) was used to prepare ultrapure water. The following reagents were used without further purification: HPLC-grade methanol (Kanto Chemicals; Tokyo, Japan); ammonium acetate, sodium chloride, potassium chloride, magnesium chloride hexahydrate, potassium dihydrogen phosphate, sodium hydrogen carbonate, and sodium DL-lactate (Nacalai Tesque; Kyoto, Japan); analytical-grade calcium chloride dihydrate (Wako Pure Chemical Industries; Osaka, Japan); and anhydrous D-(+)-glucose (Sigma).

Ultrafree-MC centrifugal filter units housing Biomax-5, Biomax-10, PL-5, PL-10, 0.1 μm Durapore, 0.22 μm Durapore, or 0.22 μm PTFE membranes (Millipore; Bedford, MA, USA) were evaluated for sample preparation. Chiral stationary phase columns tested for separation of baclofen enantiomers included a CROWNPAK CR(+) (150 mm × 4.0 mm i.d., 5 μm; Daicel Chemical Industries; Osaka, Japan); a Chiral NEA (R) (250 mm × 4.6 mm i.d., 5 μm; YMC; Kyoto, Japan); OA-2500 and OA-7500 (250 mm × 4.6 mm i.d., 5 μm; Sumika Chemical Analysis Service; Osaka, Japan); Chiralcel OD-RH and Chiralpak AD-RH (150 mm × 4.6 mm i.d., 5 μm; Daicel Chemical Industries); a Chiral CD-Ph (250 mm × 4.6 mm i.d., 5 μm; Shiseido; Tokyo, Japan); and Cyclobond I 2000, Cyclobond I 2000 RSP, and Cyclobond II (250 mm × 4.6 mm i.d., 5 μm; Aztec; Whippany, NJ, USA). The achiral reverse-phase column used to evaluate the preparation procedure for biologic samples was a CAPCELL PAK C_8 (UG120; 150 mm × 4.6 mm i.d., 5 μm; Shiseido, Tokyo, Japan).

Human plasma was prepared from blood obtained from healthy volunteers, and was stored at −20 °C or below until use. Human CSF was kindly donated from Dr. Marge Gillespie, of the US NIH, and artificial CSF was prepared according to the method of Oka et al. [12] as follows. Sodium chloride (7.592 g/l), potassium chloride (0.146 g/l), magnesium chloride hexahydrate (0.24 g/l), calcium chloride dihydrate (0.174 g/l), potassium dihydrogen phosphate (0.154 g/l), sodium DL-lactate (1.69 g/l), anhydrous D-(+)-glucose (0.719 g/l), and sodium hydrogen carbonate (0.924 g/l) were dissolved in ultrapure water. The solution was stored at −20 °C until use.

2.2. Apparatus

A stand-alone HPLC system consisting of a PU610 pump (GL Science; Tokyo, Japan), a UV620 UV detector (GL Science), a CO630 column oven (GL Science), an AS640 autosampler (GL Science), and a VStation system controller (GL Science) was used to separate the enantiomers of baclofen, and to evaluate the preparation procedure for biological sample by mean of Ultrafree-MC centrifugal filter units housing one of several different kinds of filter membranes. The mobile phase was an aqueous 10 mM ammonium acetate solution (pH 6.8)–methanol mixture (9:1, v/v). UV detection at 254 nm was used to detect baclofen.

For LC/MS/MS measurement, the apparatus consisted of a Waters 2690 integrated HPLC system (Waters; Milford, MA, USA) and a 7750 TPMV control valve (Rheodyne; Cotati, CA, USA). The mobile phase was an aqueous 10 mM ammonium acetate solution (pH 6.8)–methanol mixture (9:1, v/v). Mass spectrometry was performed in the positive-ion mode using an API 365 triple-stage quadrupole instrument (Perkin-Elmer Sciex; Concord, Ont., Canada) equipped with a heated-nebulizer ion source. The heated nebulizer gas (air) pressure was 70 psi (approx. 483 kPa). The probe temperature was set at 500 °C. The total eluent flow from the HPLC system was directed to the heated-nebulizer ion source without splitting. Multiple reaction monitoring (LC/MS/MS-MRM) was used to acquire mass spectra. In the MRM analyses, the collision energy was set at −23.5 eV (RO2–RO1), and MRM transitions of baclofen (*m*/*z* 214 → 151) and the internal standard (*m*/*z* 200 → 154) were monitored with dwell times of 500 ms for each channel. The data were processed using MacQuan software (Version 1.6, Perkin-Elmer Sciex) on a Macintosh PowerPC 7300/180 computer.

2.3. Preparation of stock and working solutions

Separate stock solutions were prepared to yield an approximately 2 mg/ml concentration of each compound by separately dissolving each compound in ultrapure water. Working solutions were obtained by diluting the corresponding stock solution in purified water. For calibration tests, a calibration series of racemic baclofen was prepared with concentrations of 1.5, 5, 15, 50, 150, 500, and 1500 ng enantiomer/ml of water. The calibration series of racemic baclofen with concentrations of 1.5, 5, 50, and 500 ng enantiomer/ml of water were also used to prepare the quality control (QC) samples.

All solutions were stored at 4 °C and were stable at least 1 month.

2.4. Sample preparation procedure

A 100 μl aliquot of control drug-free human plasma, human CSF, or artificial CSF was transferred by pipette into sample cups of separate Ultrafree-MC centrifugal filter units. Working solutions of racemic baclofen (10 μl) and 10 μl of the internal standard working solution were added to the biologic each sample in sample cup. The cap of each filter unit was then closed tightly. The filter units were placed into a centrifuge and spun at 6000 × *g* for more than 15 min at approximately 25 °C in order to collect more than 60 μl of filtrate for plasma, and more than 100 μl for human or artificial CSF. The filter cups were removed from each unit, and the filtered samples were transferred to separate sample tubes for measurement. A 50 μl aliquot of the filtrate from a plasma sample or 100 μl of the filtrate from a CSF sample was loaded onto the LC/MS/MS system.

3. Results

3.1. Enantioseparation of baclofen

Columns with one of four different kinds of chiral stationary phase, a Pirkle type, a cellulose or amylose type, a cyclodextrin type, or a crown ether type, were tested with a working solution of racemic baclofen for the ability to separate baclofen enantiomers on a stand-alone HPLC-UV system. The CROWNPAK CR(+) was the only column to perform a complete separation of baclofen enantiomers under the initial conditions of 20 °C column temperature and 0.5 ml/min eluent flow rate (Table 1). Next, the effect of column oven temperature on the retention time and the resolution of baclofen enantiomers was investigated in order to shorten run time on the CROWNPAK CR(+) column. Increasing the column oven temperature from 20 to 50 °C reduced the retention time of each enantiomer; the retention time of *S*(+)-baclofen at a column temperature of 20 °C was 10.8 min, at 30 °C was 9.3 min, at 40 °C was 8.1 min, and at 50 °C was 7.1 min, and the retention time of *R*(−)-baclofen at a column temperature of 20 °C was 18.9 min, at 30 °C was 14.2 min, at 40 °C was 11.0 min, and at 50 °C was 8.8 min (Table 2). This temperature increase also reduced the resolution factor, R_s, of baclofen enantiomers; the R_s at a column temperature of 20 °C was 6.5, at 30 °C was 5.3, at 40 °C was 3.8, and at 50 °C was 2.7. However, each of these R_s values is thought to provide sufficient resolution of the baclofen enantiomers. Next, the effect of flow rate on the signal-to-noise ratio of *S*(+)-baclofen (50 pg) at LC/MS/MS measurement was examined using the same mobile phase at the column temperature of 50 °C. Although increasing the flow rate from 0.5 to 1.0 ml/min reduced the run time, it also reduced the sensitivity by about half;

Table 1
Separation of baclofen enantiomers on several different chiral stationary phase columns

Stationary phase	Chiral stationary column	Retention time (min)		Resolution factor (R_s)
		S(+)-Baclofen	*R*(−)-Baclofen	
Type I (Pirkle type)	Chiral NEA (R)	14.2	14.2	–
	OA-2500S	13.2	14.0	0.8
Type II (cellulose or amylose type)	Chiralpak AD-RH	4.3	4.3	–
	Chiralcel OD-RH	7.7	7.7	–
Type III (cyclodextrin type)	Chiral CD-Ph	18.1	18.1	–
	OA-7500	7.6	7.6	–
	Cyclobond I 2000	8.3	8.3	–
	Cyclobond I 2000 RSP	8.7	8.7	–
	Cyclobond II	7.9	7.9	–
Type IV (crown ether type)	Crownpak CR(+)	10.7	19.0	6.5

LC conditions: flow rate of 0.5 ml/min at column oven temperature of 20 °C.

Table 2
Retention times, resolution factors, and signal-to-noise ratios of baclofen enantiomers on a Crownpak CR(+) column

Column temperature	Retention time (min)[a]		R_s	S/N of *S*(+)-baclofen (50 pg)		
	S(+)-Baclofen	*R*(−)-Baclofen		0.5[b]	0.75[b]	1.0[b]
20	10.8	18.9	6.5	–	–	–
30	9.3	14.2	5.3	–	–	–
40	8.1	11.0	3.8	–	–	–
50	7.1	8.8	2.7	17.4	12.4	8.5

[a] Flow rate was 0.5 ml/min.
[b] Flow rate in ml/min.

the signal-to-noise ratio of *S*(+)-baclofen at a flow rate of 0.5 ml/min was 17.4, at 0.75 ml/min was 12.4 and at 1.0 ml/min was 8.5. These results show that when using a CROWNPAK CR(+) column, a column oven temperature of 50° C and a flow rate of 0.5 ml/min column provide sufficient enantioselective resolution of baclofen.

3.2. Evaluation of biologic sample preparation procedure

Ultrafree-MC centrifugal filter units housing one of several different kinds of filter membranes were evaluated for their ability to prepare human plasma and human CSF samples containing baclofen for LC/MS/MS analysis. The reproducibility of the peak area ratio of baclofen to L-*p*-chlorophenylalanine in samples filtered through membranes by centrifugation was evaluated using a stand-alone HPLC-UV system that employed an achiral reverse-phase CAPCELL PAK C_8 column and a flow rate of 0.5 ml/min for the mobile phase consisting of 10 mM ammonium acetate solution-methanol mixture (9:1, v/v). The column temperature was set at 40 °C in order to avoid an interfering peak observed near the retention time of baclofen. When ultrafiltration membranes (Biomax-5 and Biomax-10, and PL-5 and PL-10) were used to prepare human plasma, the peak area of L-*p*-chlorophenylalanine (186 μg/ml of human plasma) was reduced compared to the peak area of a control working solution (Table 3). Since adsorption of L-*p*-chlorophenylalanine to the ultrafiltration membranes was not observed when the standard solution was analyzed (data not shown), this phenomenon might be caused by L-*p*-chlorophenylalanine binding to protein in human plasma. Nonetheless, stable reproducibility, expressed as the coefficient of variation (%CV) of peak area ratio of baclofen (186 μg/ml of human plasma) to L-*p*-chlorophenylalanine (212 μg/ml of human plasma), was observed for all membranes. Additionally, filtrates obtained using microporous membranes were not transparent and had more endogenous human plasma component peaks on UV chromatograms than filtrates obtained using ultrafiltration membranes observed (Fig. 1). Consequently, ultrafiltration membrane filter units were thought to be the best choice for sample preparation prior to LC/MS/MS analysis. Furthermore, the Biomax-5 filter unit was selected because precision (%CV) of the peak area ratio of baclofen to L-*p*-chlorophenylalanine for samples filtered through this membrane was the smallest (0.9%), and the nominal molecular weight limit (NMWL) of a Biomax-5 membrane, 5000, was less than NMWL of PL-10, 10,000.

3.3. Enantioselective determination of baclofen in human plasma

The determination of the *S*(+)- and *R*(−)-enantiomers of baclofen in human plasma by LC/MS/MS was carried at a column oven temperature of 50 °C and a flow rate of 0.5 ml/min; Ultrafree-MC centrifugal filter units with Biomax-5 membrane were used to pretreat the samples. To evaluate the selectivity of the method, six individual blank human plasma samples were prepared. Representative mass chromatograms of a blank human plasma sample and a human plasma sample containing 0.15 ng enantiomer/ml of each baclofen enantiomer and 10.6 ng/ml of the internal standard, L-*p*-chlorophenylalanine are shown in Fig. 2. No significant interfering peak caused by endogenous components was observed at the retention time of either baclofen enantiomer or at the retention time of the internal standard, L-*p*-chlorophenylalanine in any plasma sample. In order to construct a calibration curve, the peak area ratio (that is, the peak area of baclofen to the peak area of the internal standard; *y*) for each theoretical concentration was plotted against *x*, the corresponding theoretical concentration of the calibration standard (ng/ml). The least-squares method with $1/x^2$ weighting was used to construct a regression line for each baclofen enatiomer to determine the regression equation and the correlation coefficient of the calibration curve. The calibration curve for each baclofen enantiomer was linear over the concentration range of 0.15–150 ng enantiomer/ml of human plasma; the equation for the calibration curve of *S*(+)-baclofen was $y = 0.0278x + 0.000704$, which had a correlation coefficient of 0.999, and the

Table 3
Reproducibility of the peak area ratio of baclofen (186 μg/ml of human plasma) to L-*p*-chlorophenylalanine (212 μg/ml of human plasma) in spiked human plasma filtered through a membrane

Filter membrane	Baclofen (peak area (mean ± S.D., $n = 3$))	L-*p*-Chlorophenylalanine (peak area (mean ± S.D., $n = 3$))	Peak area ratio of baclofen to L-*p*-chlorophenylalanine (mean + S.D., $n = 3$)	Precision (%CV)
Control (working solution)	438623 ± 6037	459249 ± 4523	0.96 ± 0.011	1.1
Biomax-5	434671 ± 8507	342319 ± 6797	1.27 ± 0.011	0.9
Biomax-10	450094 ± 3811	342673 ± 2794	1.31 ± 0.015	1.1
PL-5	455628 ± 17433	361270 ± 7584	1.26 ± 0.023	1.8
PL-10	442985 ± 13563	350083 ± 13062	1.27 ± 0.011	0.9
0.1-μm Durapore	474684 ± 5868	447330 ± 36516	1.07 ± 0.075	7.0
0.22 μm Durapore	470341 ± 6924	461553 ± 25390	1.02 ± 0.045	4.4
0.22 μm PTFE	479879 ± 19782	457696 ± 62245	1.06 ± 0.113	10.7

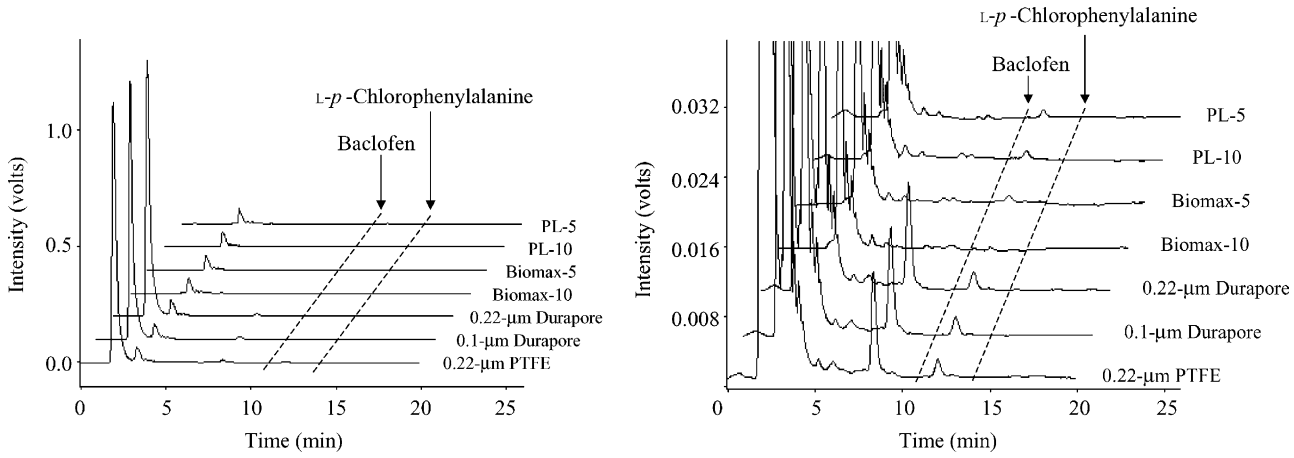

Fig. 1. Representative UV chromatograms of blank human plasma samples filtered through membranes by centrifugation.

equation for a calibration curve of *R*(−)-baclofen was $y = 0.0298x + 0.000181$, which had a correlation coefficient of 0.996. Within-day precision, expressed as the coefficient of variation (%CV), was below 15%, and the within-day accuracy, expressed as the percentage of the observed to the theoretical concentration, was within ±15% of the theoretical concentrations in all QC samples including the LLOQ (Table 4). Additionally, the day-to-day precision also had a variation less than 15%, and the day-to-day accuracy was within ±15% of the theoretical concentrations in LQC and HQC samples (Table 5).

3.4. Enantioselective determination of baclofen in human CSF

Because it is difficult to obtain human CSF samples, an artificial CSF [12] was evaluated as an alternative to human CSF. First, the linearity of the calibration curve over a

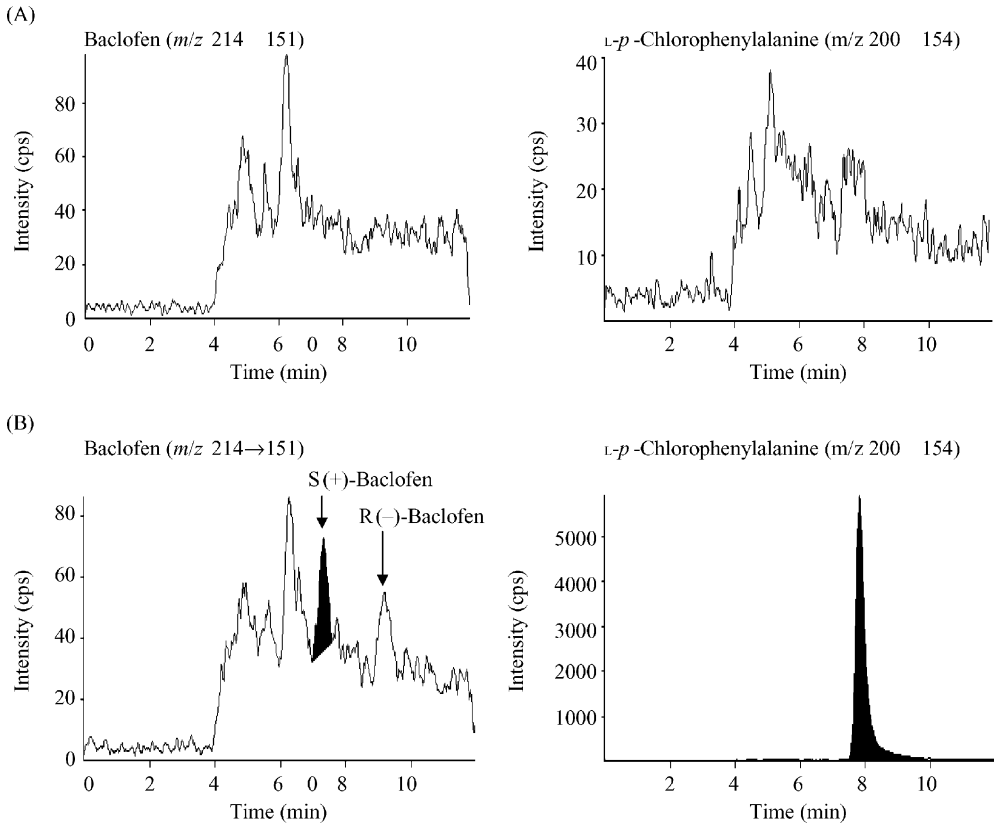

Fig. 2. Representative mass chromatograms of (A) a blank human plasma sample and (B) a human plasma sample containing 0.15 ng enantiomer/ml of each baclofen enantiomer and 10.6 ng/ml of the internal standard, L-*p*-chlorophenylalanine. A six-port control valve was used to direct the total eluent flow from the HPLC system between 4.0 and 11.9 min after injection to the heated-nebulizer ion source.

Table 4
Within-day precision and accuracy of the LC/MS/MS method for the determination of baclofen enantiomers in human plasma

Enantiomer	QC sample	*n*	Theoretical concentration (ng enantiomer/ml)	Mean observed concentration (ng enantiomer/ml)	S.D.	Precision (%CV)	Accuracy (%)
S(+)-Baclofen	LLOQ	5	0.15	0.136	0.012	8.8	90.7
	LQC	3	0.50	0.451	0.027	6.0	90.2
	MQC	3	5.0	4.44	0.19	4.3	88.8
	HQC	3	50	52.9	0.79	1.5	105.8
R(−)-Baclofen	LLOQ	5	0.15	0.157	0.007	4.5	104.7
	LQC	3	0.50	0.490	0.062	12.7	98.0
	MQC	3	5.0	4.32	0.08	1.9	86.4
	HQC	3	50	53.8	0.64	1.2	107.6

Table 5
Day-to-day precision and accuracy of the LC/MS/MS method for the determination of baclofen enantiomers in human plasma

Enantiomer	QC sample	*n*	Theoretical concentration (ng enantiomer/ml)	Mean observed concentration (ng enantiomer/ml)	S.D.	Precision (%CV)	Accuracy (%)
S(+)-Baclofen							
Day 1	LQC	3	0.50	0.451	0.027	6.0	90.2
Day 2		3		0.497	0.043	8.7	99.4
Day 3		3		0.474	0.006	1.3	94.8
Day 1	HQC	3	50	52.9	0.8	1.5	105.8
Day 2		3		53.5	2.1	3.9	107.0
Day 3		3		53.4	2.1	3.9	106.8
R(−)-Baclofen							
Day 1	LQC	3	0.50	0.490	0.062	12.7	98.0
Day 2		3		0.467	0.039	8.4	93.4
Day 3		3		0.498	0.017	3.4	99.6
Day 1	HQC	3	50	53.8	0.6	1.1	107.6
Day 2		3		54.7	0.2	0.4	109.4
Day 3		3		51.6	0.8	1.6	103.2

Table 6
Evaluation of artificial CSF as an alternative matrix to human CSF

Enantiomer	Enantiomer	Theoretical concentration (ng enantiomer/ml)	Mean observed concentration (ng enantiomer/ml)	S.D.	Precision (%CV)	Accuracy (%)
Human CSF ($n = 3$)	*S*(+)-Baclofen	0.50	0.430	0.0012	0.3	86.0
	R(−)-Baclofen	0.50	0.429	0.0006	0.1	85.8
Artificial CSF ($n = 3$)	*S*(+)-Baclofen	0.50	0.443	0.0182	4.1	88.6
	R(−)-Baclofen	0.50	0.436	0.0085	1.9	87.2

concentration range from 0.15 to 150 ng enantiomer/ml was confirmed using the artificial CSF, and within- and day-to-day accuracy and precision of the method was confirmed using the artificial CSF for preparing QC samples. Representative mass chromatograms of a blank human CSF sample, a blank artificial CSF sample, and an artificial CSF sample containing 0.15 ng enantiomer/ml of each baclofen enantiomer and 10.6 ng/ml of the internal standard are shown in Fig. 3. A six-port control valve was used to direct the total eluent flow from the HPLC system between 4.0 and 11.9 min after injection to the heated-nebulizer ion source. No interfering peaks were observed in chromatograms of either drug-free human CSF or the artificial CSF samples. Next, LQC samples (0.50 ng enantiomer/ml) were prepared from both human CSF and artificial CSF to evaluate the method selectivity and to check for matrix effects. The observed concentrations of both human CSF and artificial CSF samples spiked with baclofen and L-*p*-chlorophenylalanine were almost the same with good precision (within 5%). The accuracy of the observed concentrations of both samples was within ±15% of the theoretical concentration (Table 6). These results indicate that the artificial CSF described in this paper can be used as alternative to human CSF for calibration tests.

4. Discussion

Although many methods based on gas chromatography–liquid chromatography (GC–LC) [13,14], HPLC [15–18],

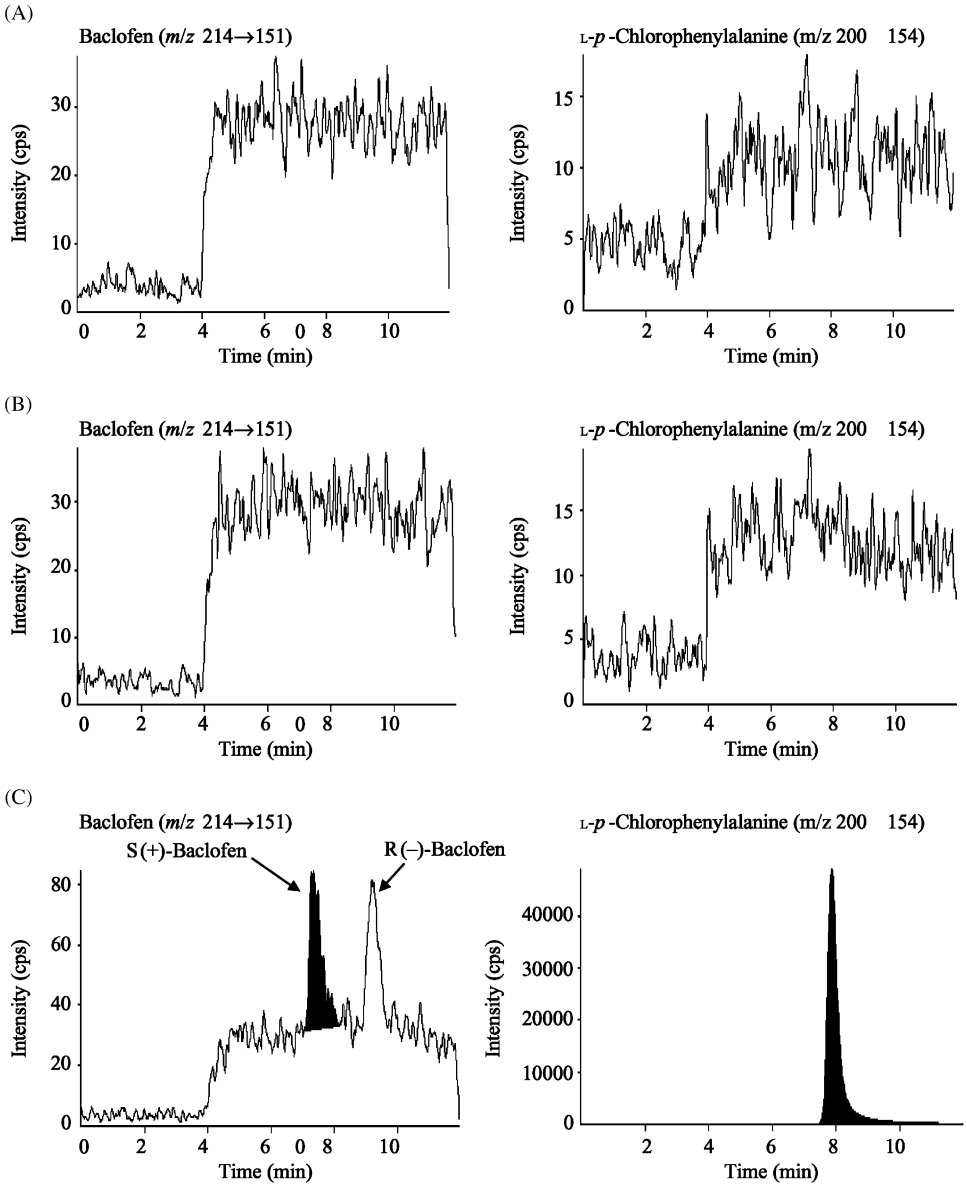

Fig. 3. Representative mass chromatograms of (A) a blank human CSF sample, (B) a blank artificial CSF sample, and (C) an artificial CSF sample containing 0.15 ng enantiomer/ml of each baclofen enantiomer and 10.6 ng/ml of the internal standard, L-*p*-chlorophenylalanine.

or MS [19,20] have been reported for the determination of baclofen in biologic fluids, a few successful enantioselective methods for baclofen analysis in biologic materials exist. Spahn et al. [8] developed an enantiospecific HPLC method to determine *S*(+)-naproxen-derivatized baclofen enantiomers in human plasma, urine, and CSF; Sioufi et al. [9] employed a chiral capillary column to separate *N*-heptafluorobutyryl isobutyl ester derivatives of baclofen enantiomers in human plasma and urine; and Chiang et al. [10,11] described an enantiospecific method based on capillary electrophoretic (CE) separation of naphthalene-2,3-dicarboxaldehyde (NAD)-derivatized baclofen enatiomers. All these methods require troublesome derivatization procedures for sample preparation, enantioselective separation, or sensitive fluorescence detection. In contract, an enantioseparation method for underivatized baclofen was developed using CE with a chiral crown ether by Nishi et al. [21]; however, this technique was not tested for use with biological materials. Recently, an HPLC-UV method for the enantioseparation of underivatized baclofen in human plasma was reported by Zhu and Neirinck [22], but the lower limit of quantification of this method was 20 ng/ml of human plasma, which is similar to those of the previously described derivatization methods.

In contract to these methods, the results of this study show that a CROWNPAK CR(+) column, which contains a chiral crown ether, can be used to separate underivatized baclofen enantiomers. This column is highly efficient in separating compounds possessing primary amino groups near asymmetric centers [23,24]; however, it has certain limitations

in its operating characteristics such as mobile phase composition, and flow rate, among others. Although addition of methanol to the mobile phase effectively shortens the retention time of baclofen, methanol cannot compose more than 15% by volume when using a CROWNPAK CR(+) column, and other organic solvents are not recommended. Although this column is usually operated under acidic conditions, the method developed employs a mobile phase of 10 mM ammonium acetate (pH 6.8)–methanol (9:1, v/v) in order to shorten run time while providing satisfactory separation of because the separation of each baclofen enantiomer.

This method also established a simple preparation procedure for human plasma and CSF samples that uses Ultrafree-MC centrifugal filter units with Biomax-5 membrane. This centrifugation only procedure avoids sample-handling troubles, and a method employing this simple preparation procedure to determine each baclofen enantiomer in human plasma has been successfully validated although there is a concern about variations in recovery through Biomax-5 membrane at the lower plasma concentration of baclofen and L-*p*-chlorophenylalanine (ng/ml of human plasma). The other ultrafiltration filter units tested (Biomax-10, PL-5, and PL-10) are also as potentially useful in this simple preparation procedure as the Biomax-5 unit. In contrast, the microporous membranes (0.1 μm Durapore, 0.22 μm Durapore, and 0.22 μm PTFE) might not be suitable for preparing samples for LC/MS/MS measurements because human plasma samples filtered through microporous membranes had more endogenous human plasma component peaks as measured by UV detection, and filtrates obtained using microporous membrane filtration were not transparent, while those obtained using ultrafiltration membrane filtration were transparent. Additionally, although no data are presented in this report, this simple preparation procedure has also been used for plasma and CSF samples obtained from animals such as mice, rats, dogs, or monkeys.

The high sensitivity of the method results from using LC/MS/MS with APCI ionization. The lower limit of quantification in human plasma and CSF is 0.15 ng enantiomer/ml. This sensitivity is more than 20-fold sensitive than that of the methods described before. Müller et al. [25] measured baclofen concentrations in plasma and in CSF collected from the lumber region of patients receiving continuous intrathecal baclofen and found that the plasma concentration was about 200 times smaller than the lumbar CSF concentration. This sensitivity enables measurement of baclofen levels in human plasma and CSF samples obtained from same subject after continuous intrathecal infusion of baclofen, even though such patients would probably have a very low plasma baclofen level. Additionally, the developed method resolves the enantiomers of baclofen. Therefore, the different properties of each enatiomer at low concentrations can be investigated more detail by evaluating the pharmacokinetics of each enantiomer.

Finally, the results show that an artificial CSF can be used as an alternative to human CSF for calibration tests with this method. Additionally, although the results are not presented in this report, artificial CSF can be used to dilute human CSF. Moreover, artificial CSF can be used as an alternative to animal CSF, which will reduce the need to collect CSF from human and animals for blank sample or calibration tests.

In conclusion, a simple, sensitive, and enantioselective method to resolve baclofen enantiomers in biologic fluid samples was developed. It is hoped this method will be useful in future clinical and pharmacokinetic studies of baclofen.

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