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SIMPLE AND RAPID REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF BACLOFEN IN HUMAN PLASMA WITH ULTRAVIOLET DETECTION

APPLICATION TO A PHARMACOKINETIC STUDY

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SUMMARY

A sensitive, selective, and rapid reversed-phase high-performance liquid chromatographic method for separation and quantitation of intact racemic baclofen in human plasma has been described. Baclofen is very soluble in water and cannot be extracted efficiently by an organic solvent. Therefore, baclofen was isolated from plasma endogenous materials by adding 0.50 ml of acetonitrile and 50 mg of zinc sulfate to 1.0 ml of plasma. A 15 cm × 4.6 mm, 10-μm octyl (C_8) column was slurry packed and used throughout the study. An isocratic mobile phase containing 0.01 *M* monobasic potassium phosphate (pH ≃ 3.5)–acetonitrile (80:20, v/v) was delivered at a flow-rate of 1.0 ml/min through the chromatographic system. Baclofen was detected with an ultraviolet–visible variable-wavelength detector at 220 nm and 0.50–0.005 a.u.f.s. The time needed to complete the analysis of one sample was approximately 15 min. The limit of detection for the assay of racemic baclofen was 35 ng/ml. After an oral dose of 20 mg of baclofen, blood samples were collected at several time points and plasma was analyzed using the method developed in this study. Various pharmacokinetics parameters were determined from the plasma concentration versus time profile of baclofen.

INTRODUCTION

Baclofen, *rac*-4-amino-3-(*p*-chlorophenyl)butyric acid, is a skeletal muscle relaxant and has been extensively used in the treatment of spastic disorders [1]. The mechanism of pharmacological action of baclofen has not been clearly established [2–4]. It has also been proposed that baclofen may be effective in the treatment of schizophrenia, trigeminal neuralgia, and tardive dyskinesia [5–10].

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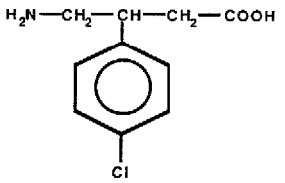

Fig. 1. Chemical structure of baclofen.

The commercially available drug is obtained in a racemic mixture as Lioresal®. Baclofen (Fig. 1) is a *p*-chlorophenyl analogue of *γ*-aminobutyric acid (GABA) which makes the center carbon atom chiral and therefore has a center of asymmetry. It has been established that the (−)-(*R*)-baclofen is the active ingredient for treating spastic disorders [11]. The rate of metabolism of baclofen in humans is not much known and is very slow. At least 85% of the parent drug is excreted through urine. The major metabolite is the deaminated product, which is pharmacologically inactive in animals [12].

Due to the amino acid structure of baclofen, it is extremely difficult to develop an analytical method that is selective in determining baclofen levels in human body fluids. Body fluids also have amino acids; therefore, it becomes a challenging task to separate (and measure) low levels of baclofen from the interfering body fluid. Baclofen, like other amino acids, does not have a strong ultraviolet (UV) chromophore; therefore, monitoring baclofen at nanogram levels using UV detection is also very difficult.

Gas chromatography (GC) with electron-capture detection has been used to measure baclofen concentrations in biological samples. Gas chromatography–mass spectrometry (GC–MS) has also been used to monitor baclofen [13]. High-performance liquid chromatography (HPLC) has been used to determine baclofen in biological fluids [14,15]. All of these methods used derivatization of baclofen with *o*-phthaldialdehyde (OPA) or other derivatization agents. The HPLC methods used fluorescence detection of the derivatized baclofen molecule. Derivatization and poor recovery of the derivatized baclofen makes all of these methods complex and not very suitable for a clinical laboratory set-up. Recently Wuis et al. [14] published a paper to determine baclofen in urine by reversed-phase HPLC and UV detection without derivatization. Harrison et al. [15] also described an HPLC method to determine baclofen in human plasma.

The extraction procedure and the chromatography of all the published methods are poor, complex, time-consuming or all of these. This paper describes a rapid, selective, and sensitive reversed-phase HPLC method to determine baclofen in human plasma using UV detection.

EXPERIMENTAL

Equipment

The HPLC system consisted of a Waters M-6000 solvent delivery pump (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.), equipped with a Rheodyne 7275 sample injector (Rheodyne, Cotati, CA, U.S.A.) with a 100-*μ*l

loop. A reversed-phase, 15 cm×4.6 mm column packed with 10-μm octyl (C_8) particles was used. A slurry packer from Micromeritics (Norcross, GA, U.S.A.) was used to pack the analytical column. A Kratos Spectroflow 783 UV–visible variable-wavelength absorbance detector with a flow cell path length of 8.0 mm (ABI Analytical, Kratos Division, Ramsey, NJ, U.S.A.) was used. UV–visible chromatograms were recorded on a Houston Instrument D5000 strip chart recorder (Houston instruments, Austin, TX, U.S.A.). A Model 2200 Branson sonicator was used to degas the mobile phase (Branson Cleaning Equipment, Shelton, CT, U.S.A.). A Gilson P-1000 digital pipette was used for all quantitative sampling (Gilson International, Middleton, WI, U.S.A.). Deionized water was collected from a Milli-Q® system (Millipore). An analytical balance from Mettler instruments (Highstown, NJ, U.S.A.), Model AE100, was used for weighing reagents. Samples were centrifuged by an IEC centrifuge, Model HN (Damon, IEC Division, Needham Heights, MA, U.S.A.). A vortex mixer from Scientific Industries (Bohemia, NY, U.S.A.), Model Vortexer-2®, was used. A C-130B Upchurch guard column (Upchurch Scientific, Oak Harbor, WA, U.S.A.) packed with 10-μm C_8 particles was used throughout. A 5.0-ml gas-tight syringe was used to filter the samples (Hamilton, Reno, NV, U.S.A.). A 0.45-μm Nylon 66 filter tip for the syringe was purchased from Rainin Instruments (Woburn, MA, U.S.A.).

Materials

The reversed-phase 10-μm octyl (C_8) particles were purchased from Alltech Assoc. (Deerfield, IL, U.S.A.). The aged pooled plasma was obtained from the Blood Center of Southeastern Wisconsin (Milwaukee, WI, U.S.A.). Spectrograde acetonitrile and methanol were purchased from EM Science (Cherry Hill, NJ, U.S.A.). The borosilicate glass culture tubes and the disposable glass pipettes were purchased from Curtin Matheson Scientific (Elk Grove, IL, U.S.A.). Baclofen (brand name Lioresal) in powder form was obtained from Ciba-Geigy, Pharmaceuticals Division (Suffern, NY, U.S.A.). The phosphoric acid and hydrochloric acid were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Spectrograde isopropanol and reagent-grade ammonium acetate were obtained from Aldrich (Milwaukee, WI, U.S.A.). Zinc sulfate was purchased from Sigma (St. Luis, MO, U.S.A.). All the solvents and reagents were used as received without further clean-up or purification, except for the borosiliate culture test tubes and zinc sulfate.

Chromatographic conditions

The mobile phase consisted of solvent A (0.01 *M* monobasic potassium phosphate) and solvent B (100% acetonitrile) (80:20, v/v). The pH of the mobile phase mixture (mixture of solvents A and B) was adjusted to 3.5 by adding 85% phosphoric acid. The rate of mobile phase delivery through the HPLC system was 1.0 ml/min. Baclofen was monitored at 220 nm at a sensitivity of 0.50–0.005 a.u.f.s. The analytical and guard columns and the mobile phase were all maintained at room temperature (25±2°C) during analyses. Quantitation of baclofen in samples (patients' plasma) was done by comparing the peak height of the baclofen in the sample with a standard calibration curve of baclofen in human plasma.

Preparation of stock solution

Baclofen (10.0 mg) was dissolved in 10.0 ml of a 0.001 *M* aqueous hydrochloric acid solution. A 1-ml volume of this stock solution was diluted to 20.0 ml in deionized Milli-Q filter water. Both the original stock dilution and the diluted solution were used to prepare plasma and water standards to construct the calibration curves. A 20-μl aliquot of a 5-ppm baclofen solution was injected into the HPLC system to determine the retention time of baclofen under the experimental chromatographic conditions.

Cleaning of zinc sulfate and borosilicate culture tubes

Cleaning of zinc sulfate and borosilicate culture tubes has been described elsewhere [16].

Packing of the analytical column by slurry method

The 15 cm$\times$4.6 mm analytical column was packed using a slurry packing method described elsewhere [17].

Pre-chromatographic isolation of baclofen from plasma

Isolation of baclofen from plasma constituents prior to chromatography was conducted by adding 0.25 ml of acetonitrile and approximately 25 mg of zinc sulfate crystals to 0.50 ml of plasma. The aged plasma was stored at $-25\,^{\circ}$C. This plasma was thawed at room temperature and 0.50 ml of thawed plasma was pipetted into a clean disposable borosilicate test tube. An aliquot of the standard stock solution of baclofen was added to the 0.50 ml of plasma and mixed on a vortex mixer for at least 30 s. A 250-μl aliquot of acetonitrile and approximately 25 mg of zinc sulfate were added to the plasma baclofen solution, mixed on a vortex mixer for at least 1 min, and centrifuged at 2000 *g* for 5 min. The supernatant was decanted into another fresh borosilicate culture test tube. This solution was filtered through a 0.45-μm Nylon 66 filter tip using a 5.0-ml gas-tight syringe. An aliquot of this filtered solution was injected directly onto the chromatographic system.

Construction of the calibration curves in three matrices

The stock solutions of baclofen described in *Preparation of stock solution* was stored at $-25\,^{\circ}$C and was found to be stable for at least six months. At least six solutions of baclofen were prepared by adding enough stock solution to give concentrations of about 0.05, 0.10, 0.50, 1.0, 5.0, and 10.0 μg/ml. These solutions were prepared in each of three matrices: pure water, acetonitrile–water (1:2), and acetonitrile–plasma (1:2) treated with 50 mg of zinc sulfate per ml of the solution. The plasma solutions were treated as described in *Pre-chromatographic isolation of baclofen from plasma.* If plasma samples of the patient had baclofen concentrations higher than 10.0 μg/ml, then additional higher-concentration solutions were used to construct the calibration curve (the calibration curve was linear to at least 50 μg/ml). A linear calibration curve of peak height versus concentration of baclofen was constructed and was used for quantification of baclofen in patients' plasma samples.

RESULTS AND DISCUSSION

Chromatograms of blank plasma and a patient's plasma analyzed for baclofen were obtained using the chromatographic technique described and are depicted in Fig. 2. The total time consumed for sample preparation and chromatography was less than 15 min for one sample.

Inspection of the blank and patient's plasma chromatograms reveal that the baclofen peak is well resolved from the plasma constituent peaks. A straight calibration curve of baclofen peak height versus concentration was used for all quantitative calculations of baclofen (of unknown concentration) in plasma samples of patients. An internal standard method was not used because statistical calculations showed that the precision and accuracy of the method were quite respectable when a straight calibration curve was used. The straight calibration curve method also eliminated the possibility of peak interference with the internal standard. Three calibration curves were constructed: one in pure water, one in 0.50 ml of acetonitrile in 1.0 ml of water treated with 50 mg of zinc sulfate, and one in 0.50 ml of acetonitrile in 1.0 ml of human plasma treated with 50 mg of zinc sulfide. Table I shows the parameters of the calibration curves. At the 98% confidence interval, the slopes of the calibration curves in the first two matrices were identical. The slope of the calibration curve of baclofen in plasma was at least 15% less than the slopes of the other two calibration curves. This information reveals that some baclofen may have coprecipitated with plasma endogenous substances during sample preparation. therefore, a calibration curve of baclofen in human plasma must be used to quantitate baclofen in human plasma samples.

Cleaning the borosilicate culture test tubes is critical in order to obtain good chromatographic results with no interferences. The addition of 0.50 ml acetoni-

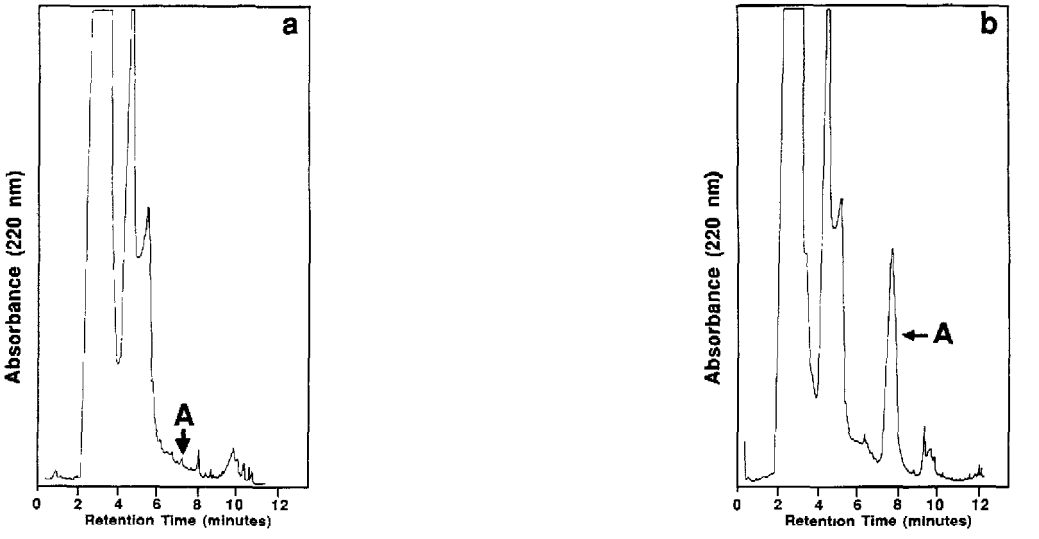

Fig. 2. (a) Chromatogram of an extract of plasma containing no baclofen (arrow A). Detector setting was 220 nm and 0.005 a.u.f.s. (b) Chromatogram of a patient's plasma who was treated with baclofen (peak A). Concentration found was 195 ng/ml. Detector conditions were same as in (a).

TABLE I

CALIBRATION CURVES OF BACLOFEN IN THREE MATRICES

Matrix	Slope	Correlation coefficient	y-Intercept
Pure water	11.30	0.995	−2.76
0.5 ml acetonitrile–1.0 ml water, treated with 50 mg of zinc sulfate	11.40	0.999	0.945
0.5 ml acetonitrile–1.0 ml plasma, treated with 50 mg of zinc sulfate	9.69	0.999	−0.747

TABLE II

WITHIN-DAY ASSAY REPRODUCIBILITY OF BACLOFEN

Actual concentration (μg/ml)	Concentration determined (mean ± S.D., $n=6$) (μg/ml)	R.S.D. (%)
0.25	0.27 ± 0.015	5.5
0.70	0.66 ± 0.042	6.3
1.0	0.93 ± 0.041	4.4
3.0	4.15 ± 0.080	2.5
10.0	9.75 ± 0.220	2.2

trile to 1.0 ml of plasma precipitated a significant amount of plasma proteins. The zinc sulfate caused an additional precipitation of endogenous substances that were not precipitated by acetonitrile. If the cleaning step of plasma samples by zinc sulfate is not conducted, then the sample solution looks turbid and large interfering peaks elute with retention times similar to baclofen.

Using a guard column on top of the analytical column and filtering the plasma samples (before injection) with a 0.45-μm Nylon 66 membrane filter was necessary to avoid the high back-pressure of the chromatographic system (due to sample injections) and also to give the analytical column a longer lifetime. The guard column was dry packed with the same stationary phase as in the analytical column. The selectivity and sensitivity of the analytical column did not show any significant deterioration after at least 400 injections. But the packing of the guard column was replaced with fresh packing every 25–30 injections.

The precision and accuracy of the method were determined by multiple analyses of plasma samples spiked with a known amount of baclofen at different concentrations (Tables II and III). Table II shows the assay reproducibility for the within-day and Table III shows the assay reproducibility for between-day analysis. The relative standard deviation (R.S.D.) ranged from 2.2 to 6.3% for the within-day analyses and from 1.8 to 5.9% for the between-day analyses. It appears that the R.S.D. for within-day analyses is better (lower) at higher concentrations of baclofen in plasma. An R.S.D. of 9% or less was obtained when the concentration of baclofen in plasma was 0.01 μg/ml.

TABLE III

BETWEEN-DAY ASSAY REPRODUCIBILITY OF BACLOFEN

Samples were analyzed every day for five days.

Actual concentration (μg/ml)	Concentration determined (mean $\pm$ S.D., $n=6$) (μg/ml)	R.S.D. (%)
0.20	0.22 $\pm$ 0.013	5.9
0.50	0.46 $\pm$ 0.018	3.9
1.0	1.2 $\pm$ 0.042	3.5
5.0	5.3 $\pm$ 0.170	3.2
10.0	10.4 $\pm$ 0.190	1.8

TABLE IV

RECOVERY EXPERIMENT OF BACLOFEN IN PATIENT'S PLASMA SAMPLES

Mean concentration ($n=3$) (μg/ml)			Recovery (%)
Determined in plasma	Expected after spiking	Determined after spiking	
0.28	0.56	0.60	107
0.16	0.32	0.29	91
0.09	0.18	0.20	111
0.07	0.14	0.13	93

The pK_{a1} and pK_{a2} of baclofen is 3.87 and 9.62 [12]. The best peak shape and resolution was obtained when the mobile phase pH was between 3 to 3.5. A mobile phase pH of greater than 3.5 gave an irreproducible peak height and poor peak shape of baclofen. The capacity factor (k') of baclofen under the chromatographic conditions of this experiment was sensitive toward the ionic strength of the mobile phase. The retention time decreased with an increase of mobile phase ionic strength. Using phosphate buffer in the mobile phase minimized this effect and decreased the variation between retention time of baclofen and injection volume.

The recovery of baclofen from patient's plasma was determined by adding a known amount of baclofen to the plasma samples to result in twice the concentration of baclofen that had been determined before. After adding the known amount of baclofen, the plasma was treated identically as before for sample preparation. The concentration was determined by injecting an aliquot of the sample onto the HPLC system. For quantitative calculations, the peak height was compared with a standard calibration curve of baclofen in plasma. The recovery of baclofen in plasma ranged from 91 to 111%. The results are shown in Table IV.

Determination of baclofen in patient's plasma samples was conducted using the method described here. The plasma concentration of baclofen in four samples

ranged from 0.07 to 0.28 μg/ml. The concentrations and R.S.D. values are given in Table V.

Using this analytical method, the in vivo pharmacokinetic study of baclofen was conducted in a subject who ingested a single 20-mg oral dose of baclofen. Fig. 3 shows the baclofen concentration in the subject's plasma with respect to time. The pharmacokinetic parameters were determined from the data of the elimination portion of the curve in Fig. 3. Because few time points were taken for this portion of the curve, the pharmacokinetic parameters determined may not be very accurate. The elimination rate constant (k_{el}) was calculated from an open two-compartment model with first-order absorption and elimination from the central compartment and was found to be -0.247 ng h^{-1} ml^{-1}. The elimination

TABLE V

BACLOFEN DETERMINED IN PATIENT'S PLASMA SAMPLES

Concentration determined (mean ± S.D., $n=3$) (μg/ml)	R S.D. (%)
0.16 ± 0.0067	4.2
0.28 ± 0.0080	2.8
0.07 ± 0.0039	5.6
0.09 ± 0.0048	5.3

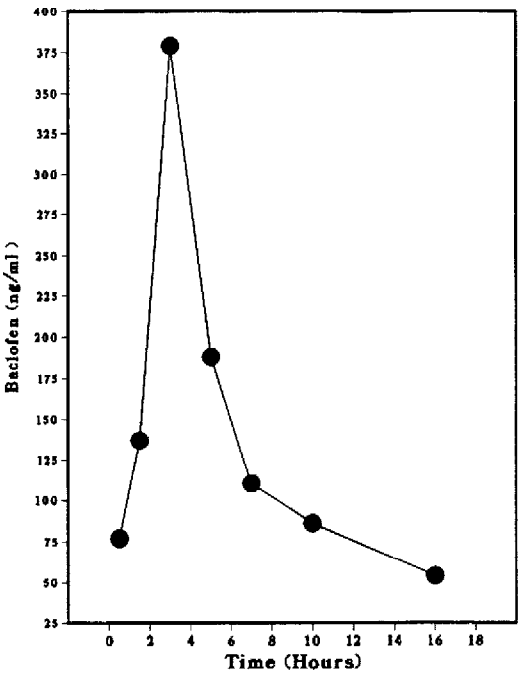

Fig. 3. Plasma concentration versus time curve following an oral administration of 20 mg of baclofen to a healthy subject.

half-life ($t_{1/2}$) was calculated from the equation $t_{1/2} = -0.693/k_{el}$ and was found to be 2.80 h. The maximum concentration (C_{max}) was calculated to be approximately 0.38 μg/ml. The time required to reach the C_{max} (t_{max}) of baclofen in plasma was approximately 3 h. The area under the curve (AUC) was calculated using the trapezoidal method. The $AUC_{0\rightarrow x}$ was approximately 1.58 μg h ml^{-1}.

The interferences of some common drugs such as acetaminophen, aspirin, caffeine, theophylline, erythromycin, tetracycline, and creatinine were tested and were negative.

CONCLUSION

The HPLC method presented here is faster and simpler than any HPLC method described in the literature. The pre-chromatography isolation of baclofen from plasma was very simple and selective. This makes the entire method more efficient with respect to time and chromatography. The evaporation step has been eliminated from the sample preparation procedure. This minimized the manipulative error and resulted in good reproducibility. The limit of detection using UV–visible detection at 220 nm was 35 ng/ml. This sensitivity was adequate for conducting in vivo pharmacokinetics studies in humans. This method will be very suitable for routine analysis of baclofen in plasma samples and also for research studies involving pharmacokinetics and bioavailability.

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