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Chiral separation and determination of *R*-(−)- and *S*-(+)-baclofen in human plasma by high-performance liquid chromatography

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Abstract

The method presented here is a high-performance liquid chromatography (HPLC)–UV detection method for the determination of baclofen *R*-(−)- and *S*-(+)-enantiomers in human plasma using a chiral separation technique. Baclofen enantiomers were extracted from human plasma with a reversed-phase solid-phase extraction (SPE) cartridge. The extract was then injected onto a HPLC system with a UV detection system set at 220 nm. The separation was achieved by using a 150×4.6 mm, 5 μm Phenomenex chirex 3216 chiral column with a mobile phase consisting of 0.4 m*M* $CuSO_4$ in acetonitrile–20 m*M* sodium acetate (17:83). The calibration curves were linear for both *R*-(−)- and *S*-(+)-enantiomers of baclofen in the concentration range of 20–5000 ng/ml. The average regressions were 0.9980 and 0.9991 for *R*-(−)- and *S*-(+)-baclofen, respectively. Inter-day precision was 3.3–5.2% for *R*-(−)-baclofen and 3.5–3.9% for *S*-(+)-baclofen at a concentration range of 60–4000 ng/ml. Intra-day precisions were 0.6–4.4 and 0.5–3.5% for *R*-(−)-baclofen and *S*-(+)-baclofen, respectively. The average extraction recovery was 81.6% for *R*-(−)-baclofen, 83.0% for *S*-(+)-baclofen and 94.0% for the internal standard (*p*-aminobenzoic acid). The limit of quantitation for both *R*-(−)- and *S*-(+)-baclofen in human plasma was 20 ng/ml. The method is simple and easy to operate with accuracy and reproducibility and it is suitable for pharmacokinetic studies.

Keywords: Enantiomer separation; Baclofen

1. Introduction

Baclofen [4-amino-3-(*p*-chlorophenyl) butyric acid] (Fig. 1) is a skeletal muscle relaxant used in the treatment of spasticity of spinal and cerebral origin [1]. Although it is marketed as a racemic mixture, only the baclofen *R*-enantiomer is stereo-specifically active at so called $GAGA_B$-receptors [2].

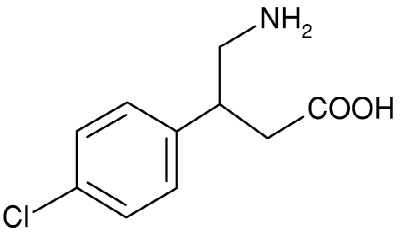

Fig. 1. Chemical structure of baclofen.

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Researchers believe that the enantiomers of baclofen have different properties. It was claimed that the *R*-(−)-enantiomer is about 100 times more active than the *S*-(+)-enantiomer [3,4].

Baclofen has been measured in human biological fluids in many different ways. For example, gas chromatography (GC) following derivatization or with electron-capture detection [5,6], gas chromatography–mass spectrometry (GC–MS) [7], high-performance liquid chromatography (HPLC)–UV detection [8,9], HPLC–florescence detection after derivatization [10,11] and HPLC–electrochemical detection with derivatization [12]. All these published methods were used for the measurement of the racemate only and not for the chiral assay.

Vaccher et al. [13] ha described a method for the separation of baclofen *R*-(−)- and *S*-(+)-enantiomers by using HPLC with a chiral crown-ether stationary phase. However, baclofen was prepared in a solution, and not in a biological matrix. Therefore, it cannot be applied to the analysis of baclofen in plasma samples. One method for the determination of *R*-(−)- and *S*-(+)-enantiomers of baclofen in plasma was done by gas chromatography using a chiral fused-silica capillary column and electron-capture detection [14]. However, this method involved a complex sample processing procedure and derivatization process.

Up to now, no HPLC method for the chiral separation of baclofen *R*-(−)- and *S*-(+)-enantiomers in human plasma has been published. Therefore, a HPLC method for the analysis of *R*-(−)- and *S*-(+)-baclofen in human plasma was developed.

The method presented here is a HPLC chiral separation for the determination of baclofen *R*-(−)- and *S*-(+)-enantiomers in human plasma using UV detection. The column we have used is a chirex 3126 D-penicillamine ligand-exchange chiral column that was purchased from Phenomenex. According to the supplier, this column requires the presence of Cu(II) ion (0.2–3 m*M*) and a limited organic concentration (<15%) in the mobile phase to have a proper function. This method involves a reversed-phase solid-phase extraction (SPE) procedure. It is simple and easy to operate with a run time of 12 min, which allows analysis of over 100 samples per day. This method is suitable for the routine analysis of both of baclofen *R*- and *S*-enantiomers in human plasma samples.

2. Experimental

2.1. Reagents

R-(−)-Baclofen was obtained from Pharmascience (Montreal, Canada), *S*-(+)-baclofen was from RBI (Oakville, Canada) and *p*-aminobenzoic acid was purchased from Sigma (Oakville, Canada). Sodium phosphate monobasic, sodium acetate, copper sulfate and dodecyl sodium sulfate, as well as HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Nepean, Canada). HPLC-grade water was obtained from a laboratory Nano-pure water purification system.

The drug-free human plasma was obtained from Biological Specialty (Colmar, PA, USA). All plasma samples were stored at -20 ± 5 °C.

2.2. Stock solutions and standards

Stock solutions of *R*-(−)-baclofen (1.00 mg/ml), *S*-(+)-baclofen (1.00 mg/ml) and internal standard (1.00 mg/ml *p*-aminobenzoic acid) were prepared in 0.01 *M* HCl water solutions. A seven-point non-zero calibration standard curve, ranging from 20.0 to 5000.0 ng/ml, was prepared by spiking the drug-free plasma with appropriate amounts of *R*-(−)- and *S*-(+)-baclofen. The quality control (QC) samples (at three concentration levels, i.e., 60, 1500 and 4000 ng/ml) were prepared in a similar manner from the stock solution. Before the spiking, the drug-free plasma was tested to make sure that there was no endogenous interference at retention times of *R*-(−)-baclofen, *S*-(+)-baclofen and internal standard. The quality control samples were extracted with the calibration standards to verify the integrity of the method.

2.3. Solid-phase extraction

Plasma samples (1 ml) were placed in glass culture tubes and 0.1 *M* sodium phosphate monobasic buffer was added. The solid-phase extraction cartridge (C_{18}) was activated with 1 ml methanol followed by 0.5 ml H_2O. Sodium dodecyl sulfate (SDS) solution (10 m*M*, 0.5 ml) was passed through the C_{18} cartridge and 0.5 ml sodium phosphate

monobasic buffer was then added to rinse away access SDS. The plasma and buffer mixture was then passed through the conditioned and modified cartridge by gravity. The cartridge was then washed with 2 ml of rinse solution A (0.8 m*M* $CuSO_4$ in acetonitrile–20 m*M* sodium acetate buffer, 10:90), followed by a 0.7 ml of rinse solution B (4.5 μg/ml *p*-aminobenzoic acid in water). The cartridge was then transferred to a glass tube and eluted with 1 ml of elution solution (20 m*M* NaOH in methanol–water, 30:70). The eluent was evaporated to dryness under a nitrogen evaporator. The residue was reconstituted with 250 μl of mobile phase and 40 μl was injected into the HPLC system for analysis.

2.4. Instrumentation

An Agilent 1100 series HPLC system with a UV–Vis detector was used for this study. A 150×4.6 mm Chirex, 5 μm chiral column was purchased from Phenomenex (Torrance, CA, USA). The detector was set at 220 nm. The mobile phase consisted of 0.4 m*M* $CuSO_4$ in acetonitrile–20 m*M* sodium acetate buffer (pH 5.5) (17:83) delivered at a flow-rate of 1.1 ml/min. A peak height ratio method (baclofen/internal standard) was used for quantitation. The baclofen concentration in human plasma samples was determined by using a standard curve that was analyzed with a weighted least-squares linear regression (weighting factor $1/x^2$).

3. Results

3.1. Limit of quantitation, linearity and precision

The results of *R*-(−)-baclofen and *S*-(+)-baclofen calibration samples are presented in Tables 1 and 2, respectively. The calibration curves are linear in the concentration range of 20 to 5000 ng/ml. The average regression ($n=5$) was 0.9979 for *R*-(−)-baclofen and 0.9991 for *S*-(+)-baclofen. The limit of quantitation (LOQ) in human plasma for both baclofen enantiomers was 20.0 ng/ml.

Inter-assay precision was determined by analyzing five calibration curves with quality control samples on 5 different days. The intra-assay precision was determined by analyzing six replicates of quality control samples extracted in the same batch. The results of inter-day and intra-day precision for *R*-(−)-baclofen and *S*-(+)-baclofen in human plasma are tabulated in Table 3. In the concentration range of 60–4000 ng/ml, the inter-assay and intra-assay relative standard deviations (RSDs) of *R*-(−)-baclofen ranged from 3.3 to 5.2 and 0.6 to 4.4%, respectively. The inter-assay and intra-assay RSDs of *S*-(+)-baclofen in human plasma were 3.3 to 3.9 and 0.6 to 3.5%.

Table 1
Summary of *R*-(−)-baclofen calibration standards

Concentration added (ng/ml)	Concentration found (ng/ml)	R.E.* (%)	RSD (%)	*n*
20	19.32±0.18	−3.4	0.9	5
40	42.46±0.90	6.2	2.1	5
500	206.64±6.79	3.3	3.3	5
1000	968.46±5.29	−3.2	0.5	5
2000	2014.80±50.62	0.7	2.5	5
3500	3428.40±30.20	−2.0	0.9	5
5000	4916.58±37.79	−1.7	0.8	5

Correlation coefficient=0.9979±0.0003 ($n=5$).
* R.E.=Relative error.

3.2. Recovery

The absolute recovery was determined by comparing the peak height of extracted human plasma samples with the peak height of solutions prepared at the same concentrations. The analysis was done with six replicates at concentrations of 60, 1500 and 4000 ng/ml. The average recovery from spiked human plasma sample that contained 60 ng/ml of *R*-(−)-baclofen was 74.3%. The average recovery of *S*-(+)-baclofen from spiked human plasma sample of 60 ng/ml was 79.9%. The average recovery ($n=18$) of the internal standard was found to be 94.0%. The detailed results of recovery for *R*-(−)- and *S*-(+)-baclofen at different concentration levels are presented in Table 4.

3.3. Specificity

Six different sources of drug-free human plasma were screened and no endogenous interference was observed at the retention time of *R*-(+)-baclofen, *S*-(−)-baclofen and internal standard. A chromatogram of extracted blank human plasma sample as well as a representative chromatogram of an ex-

Table 2
Summary of *S*-(+)-baclofen calibration standards

Concentration added (ng/ml)	Concentration found (ng/ml)	R.E.* (%)	RSD (%)	*n*
20	19.78±0.25	−1.1	1.3	5
40	40.76±1.18	1.9	2.9	5
500	202.64±6.33	1.3	3.1	5
1000	971.22±7.93	−2.9	0.8	5
2000	2029.90±40.93	1.5	2.0	5
3500	3471.36±38.71	−0.8	1.1	5
5000	5001.12±33.63	0.0	0.7	5

Correlation coefficient=0.9991±0.0005 (n=5).
* R.E.=Relative error.

tracted plasma sample containing 3500 ng/ml *R*-(+)- and *S*-(−)-baclofen are provided in Fig. 2.

Commonly used over-the-counter drugs (OTCs) were also tested for possible interference. No interference was observed at the retention time of *R*-(−)-baclofen, *S*-(+)-baclofen and internal standard. The OTCs tested were as follows: caffeine, acetylsalicylic acid, ibuprofen, acetaminophen and dextromethorphan.

3.4. Stability

Extracted plasma samples containing baclofen enantiomers and internal standard were stable at room temperature for 89 h. The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0) with the samples that were re-injected 89 h after sitting in the auto-sampler at room temperature. Evaluation was based on back-calculated concentrations. The human plasma samples containing *R*-(−)- and *S*-(+)-baclofen were also evaluated for freeze–thaw stability. The freeze–thaw stability evaluation was conducted by comparing the stability samples that have been frozen and thawed three times with the plasma samples thawed once only. *R*-(−)- and *S*-(+)-baclofen in human plasma are stable for at least three freeze–thaw cycles. The stability of plasma samples containing *R*- and *S*-baclofen and stored at −20 °C was tested as well. The results indicated that both *R*-baclofen and *S*-baclofen are stable for at least 15 weeks at −20 °C.

Table 3
Assay variability of *R*-(−)- and *S*-(+)-baclofen in human plasma

Concentration added (ng/ml)	*R*-(−)-Baclofen			*S*-(−)-Baclofen			*n*
	Concentration found (ng/ml)	R.E.* (%)	RSD (%)	Concentration found (ng/ml)	R.E.* (%)	RSD (%)	
Inter-day							
60	59.52±3.10	−0.8	5.2	59.49±2.13	−0.8	3.6	18
1500	1458.29±48.62	−2.8	3.3	1467.76±51.29	−2.1	3.5	18
4000	3884.84±141.42	−2.9	3.6	3953.61±152.79	−1.2	3.9	18
Intra-day							
20	20.32±1.49	1.6	7.4	19.68±1.37	−1.6	7.0	6
60	59.82±2.65	−0.3	4.4	60.53±2.12	0.9	3.5	6
1500	1488.78±10.74	−0.7	0.7	1508.82±7.89	0.6	0.5	6
4000	3930.85±23.56	−1.7	0.6	4019.47±22.46	0.5	0.6	6

* R.E.=Relative error.

Table 4
Extraction recovery *R*-(−)- and *S*-(+)-baclofen in human plasma

	Added (ng/ml)	Found) (ng/ml)	Added (ng/ml)	Found (ng/ml)	Added (ng/ml)	Found (ng/ml)
R-(−)-Baclofen	60.0	43.4	1500.0	1290.2	4000.0	3385.2
	60.0	47.7	1500.0	1267.8	4000.0	3365.2
	60.0	41.1	1500.0	1284.6	4000.0	3430.8
	60.0	45.3	1500.0	1271.7	4000.0	3410.1
	60.0	43.3	1500.0	1282.7	4000.0	3418.7
	60.0	46.5	1500.0	1287.0	4000.0	3417.3
Mean	60.0	44.6	1500.0	1280.7	4000.0	3404.6
RSD* (%)	–	4.9	–	0.6	–	0.7
Recovery (%)	–	74.3	–	85.4	–	85.1
S-(+)-Baclofen	60.0	48.7	1500.0	1278.7	4000.0	3366.3
	60.0	49.8	1500.0	1262.4	4000.0	3345.2
	60.0	39.5	1500.0	1266.3	4000.0	3404.2
	60.0	49.3	1500.0	1261.2	4000.0	3392.2
	60.0	52.3	1500.0	1272.4	4000.0	3398.7
	60.0	48.1	1500.0	1272.4	4000.0	3388.2
Mean	60.0	47.9	1500.0	1268.9	4000.0	3382.5
RSD* (%)	–	8.4	–	0.5	–	0.6
Recovery (%)	–	79.9	–	84.6	–	84.6

* RSD: Relative standard deviation.

4. Discussion

A sodium dodecyl sulfate (SDS) modified C_{18} solid-phase extraction cartridge was used in this method to improve the clean-up efficiency of human plasma matrix. It was observed that during the investigation of the extraction of baclofen, a group of impurities from plasma had similar elution characteristics as baclofen when using either a C_{18} or an strong cation-exchange (SCX) cartridge. The chirex analytical column, which is a D-penicillamine ligand type of column, had a strong retention for the plasma

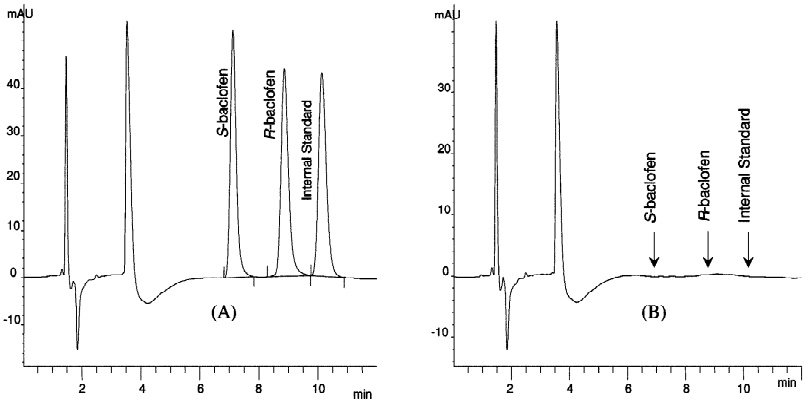

Fig. 2. Chromatograms of extracts of human plasma samples. (A) Plasma spiked with 3500 ng/ml *R*-(−)- and *S*-(+)-baclofen; (B) plasma containing no baclofen.

impurities and required very long run times to elute these impurities. The fact that these impurities had a strong retention on the D-penicillamine ligand column, and that the retention increased with an increase in Cu(II) concentration in the mobile phase suggested the formation of a complex between the impurities and Cu(II). After modifying the C_{18} cartridge with DSS, the behavior of impurities was very different from baclofen. Furthermore, introduction of Cu(II) in the rinse solution resulted in a better cleaning of interfering substances in the sample preparation.

The chiral separation of amino acids on the D-penicillamine ligand column is based on the stereo specificity of the tertiary amino acid–Cu(II)–penicillamine complex. An adequate concentration of Cu(II) is required in the mobile phase to maintain equilibrium of Cu(II) on the separation column. According to the supplier, copper ions in the mobile phase prevent a loss of copper from the stationary phase and influence retention. Higher Cu(II) concentrations will shorten retention times slightly. Copper also functions to produce a coordination complex with the penicillamine and to effect the separation. In addition, copper acts as a fungicide during storage. Due to the fact that we were working at a low concentration of 20 ng/ml and that there is a relatively strong absorption of Cu(II) in the wavelength region used for analysis (220 nm), it was necessary to use a low concentration of Cu(II) to minimize baseline noise. It was determined that a concentration of around 0.4 m*M* Cu(II) allowed for a stable chromatographic baseline and at the same time provided sufficient Cu(II) to satisfy the needs of the column. In order to minimize the distortion of Cu(II) equilibrium caused by sample injection, the alkaline extract solution was neutralized with acetic acid and the mobile phase was used for the reconstitution of the extract.

It is worthwhile mentioning that a new D-penicillamine ligand column needs to be rinsed with a large volume of mobile phase (approximately 1 l) before a stable baseline can be reached. We believe that this is due to a trace amount of free D-penicillamine in the new column which gradually elutes and causes changes in the baseline. However, after the first rinse, a stable baseline is readily established, even after changes in composition or storage of the mobile phase.

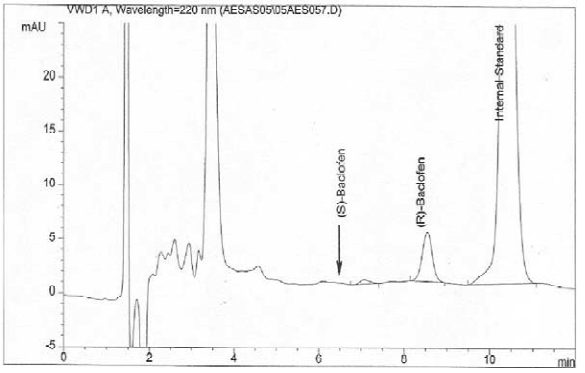

Fig. 3. Chromatogram of plasma sample from a patient dosed with *R*-baclofen.

5. Conclusion

A high-performance liquid chromatography method for the determination of *R*- and *S*-baclofen in human plasma was developed. The *R*- and *S*-baclofen enantiomers were separated with a D-penicillamine ligand exchange chiral column. The total run time for this method is 12 min which allows processing of over 100 samples per day. This method has been successfully applied to the analysis of human plasma samples from a clinical study. A representative chromatogram from a patient dosed with *R*-baclofen is presented in Fig. 3. This method has provided good sensitivity and excellent precision and reproducibility.

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