High-performance liquid chromatographic determination of baclofen in human plasma

Laurette Millerioux, Murielle Brault, Virginie Gualano, Alain Mignot*

Cephac Research Centre, B.P. 28, 86281 Saint-Benoît Cedex, France

Abstract

A reversed-phase isocratic HPLC method is described for the determination of baclofen in human plasma. Solid-phase extraction using a SCX Bond Elut column is used followed by derivatization with o-phthalaldehyde–tert.-butanethiol and electrochemical detection. Both the within- and between-day R.S.D. and inaccuracy are less than 10% and 7%, respectively, even at the limit of quantification of the method, i.e., 10 ng/ml. The method was shown to give optimum performance in terms of sensitivity, precision and accuracy for the pharmacokinetic study of baclofen after a single oral administration to volunteers.

Keywords: Baclofen

1. Introduction

Baclofen (4-amino-3-*p*-chlorophenylbutyric acid) is a chemical analogue of γ-aminobutyric acid, used for the symptomatic relief of muscular spasm. In humans, baclofen is rapidly absorbed after oral administration and is almost completely recovered unchanged in the urine [1]. The initial therapeutic dose is 5 mg three times daily, and might be increased to 20 mg three times daily to achieve the desired effect. Following a single 20-mg oral administration of baclofen to healthy volunteers, plasma concentrations are maximum (about 340 ng/ml) after about 2 h [2]. The variability of the C_{max} (R.S.D.) was reported to be 22%, suggesting that with some individuals the maximum concentrations might be two standard deviations less, i.e. about 200 ng/ml. It is

usually recommended that analytical methods used in pharmacokinetic studies be sufficiently sensitive to measure accurately plasma concentrations after four half-lives have elapsed. It therefore turns that for baclofen about 6% of the C_{max} (about 10 ng/ml) should be measured accurately. This obviously implies that any analytical method for the determination of baclofen in pharmacokinetic samples should reach this target sensitivity.

Several methods have been described in the literature, including gas chromatography [3,4], gas chromatography–mass spectrometry [5] and high-performance liquid chromatography (HPLC) with either ultraviolet [6–9], fluorescence [10–13] or electrochemical detection [14]. Owing to the amino acid structure of baclofen, several HPLC methods involve derivatization for fluorophore formation. Since the early GC method which involved two derivatization steps [3],

* Corresponding author.

analysts have tried to develop an assay which would be rapid, simple and sensitive. With respect to these goals, it appears that all the HPLC methods which were developed hardly combine simplicity and the target sensitivity for pharmacokinetic analysis of baclofen. Indeed, the published methods are either simple with insufficient sensitivity, or more complex and still with too high a limit of quantification (LOQ). Additionally, in almost all reports there is a discrepancy between the lowest concentration for which the precision and accuracy are reported and claimed limits of detection.

One of the most recently published assays consisted of a very simple extraction procedure, i.e., precipitation with zinc sulfate–acetonitrile, but unfortunately, the lowest concentration for which the precision and accuracy were reported was 200 ng/ml. A sensitive method involving solid-phase extraction (SPE) of baclofen, followed by a derivatization step with o-phthalaldehyde and tert.-butanethiol at room temperature and HPLC analysis with amperometric detection, has been published for the assay of baclofen in liver tissue [14]. The signal-to-noise ratios (S/N) reported, with a detection limit of 3.8 ng/ml for $S/N = 3.0$, prompted us to adapt this procedure for the assay of baclofen in human plasma. This paper describes a reversed-phase HPLC method with electrochemical detection, the sensitivity of which (10 ng/ml) was validated with acceptable intra- and inter-day precision and accuracy for its use in pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Baclofen as a racemate and the reagents for derivatization, o-phthalaldehyde (OPA) and tert.-butanethiol (2-methyl-2-propanethiol), were purchased from Sigma (St. Louis, MO, USA). Borate hydroxide buffer (pH 9.3) was prepared by diluting a 0.2 M boric acid solution in 0.1 M sodium hydroxide (solution A, 87 ml) to 100 ml with 0.1 M sodium hydroxide solution (solution B, 13 ml). Borate hydroxide buffer (pH 10.4) was prepared by diluting solution A (54 ml) to 100 ml with the solution B (46 ml). Citrate buffer (pH 2.6) was prepared by diluting 89 ml of 0.1 M citric acid solution (solution C) to 100 ml with 0.2 M disodium hydrogenphosphate solution (solution D, 11 ml). Phosphate buffer (pH 7) was prepared from 0.06 M disodium hydrogenphosphate by adjusting the pH to 7 with 0.06 M potassium dihydrogenphosphate. The derivatization reagent, OPA–tert.-butanethiol, was prepared daily by mixing 75 mg of OPA, 5 ml of methanol, 50 μl of tert.-butanethiol and 5 ml of borate hydroxide buffer (pH 9.3). The mobile phase consisted of phosphate buffer–methanol (36:74, v/v). A stock standard solution of baclofen (100 μg/ml) was prepared in water and all serial dilutions were carried out with water.

Other chemicals were purchased from various sources: methanol, HPLC grade (BDH, Merck, Nogent sur Marne, France); hexane, HPLC grade (SDS, Peypin, France); sodium chloride, citric acid and potassium dihydrogen phosphate, analytical-reagent grade (Merck, Darmstadt, Germany); water was filtered using a Milli-Q system (Millipore, Milford, MA, USA).

2.2. Equipment

The HPLC system consisted of an LC-9A solvent-delivery system (Shimadzu, Tokyo, Japan), equipped with an automatic injector (SIL-10A) with a cooling block (Shimadzu) and an A/D converter (Model 18652A; Hewlett-Packard, Les Ulis, France). The data were collected and analysed via the Laboratory Automation System (LAS) software (Hewlett-Packard). A C_{18} 4-μm Novapak column (150 mm $\times$ 3.9 mm I.D.) (Millipore) was used. The flow-rate of the mobile phase was 0.8 ml/min. The electrochemical detector was a Coulochem II (ESA, Bedford, MA, USA) equipped with a guard cell (Model 5020) and a glassy carbon working cell (Model 5011). The oxidation potential of the guard cell was +1.2 V. The signal was monitored at potentials of +0.2 V (screen electrode) and +0.7 V (quantifying electrode) with a sensitivity of 50 nA.

2.3. Calibration

Standard calibration samples were prepared by spiking drug-free human plasma (1 ml) with the appropriate baclofen standard solutions (100 μl) to achieve final concentrations of 500, 200, 100, 50, 20 and 10 ng/ml. The blank plasma sample was obtained by adding 100 μl of water to 1 ml of control plasma. Calibration lines were obtained by least-squares linear regression of the peak area of baclofen versus the nominal concentration. Concentrations were calculated using the slope and the intercept of the calibration lines. All concentrations are reported as ng/ml of baclofen free base.

2.4. Method

Baclofen was extracted from plasma using 1-ml SCX (strong cation exchanger) Bond Elut columns (Varian, Harbor City, CA, USA). Plasma (1 ml) was added to 100 μl of water and to 1 ml of citrate buffer (pH 2.6) and applied to a column previously conditioned successively with 2 ml of hexane, 2 ml of methanol, 2 ml of water and 3 ml of saturated sodium chloride solution. The column was then washed with 4 ml of water and 1 ml of saturated sodium chloride solution. The cartridge was dried and baclofen was eluted with exactly 1.5 ml of borate hydroxide buffer (pH 10.4). An aliquot of the eluate (200 μl) was transferred into the autosampler vials for derivatization. The derivatization reagent (50 μl) was added automatically in each vial via the autosampler immediately prior to injection (20 μl) into the HPLC system.

3. Results

The chromatographic conditions achieved an acceptable separation of baclofen from plasma endogenous compounds, as shown in Figs. 1 and 2. The average retention time of baclofen was 26 min. Under these conditions, the intrinsic sensitivity after injection of pure baclofen standard was satisfactory with a limit of detection of 2.5 ng/ml. The extraction efficiency of baclofen

spiked in plasma samples at 10, 100 and 500 ng/ml was 92%, 88%, and 85%, respectively. The method was shown to be linear from 10 to 500 ng/ml. The within-day and between-day precision and accuracy were determined by analysing several batches of plasma samples spiked at 10, 50, 200 and 500 ng/ml (Tables 1 and 2). Determinations were performed either with replicates on the same day or with twelve replicates on six separate days. The within- and between -day precision expressed as R.S.D. was always below 10%. The within- and between day inaccuracy expressed as the percentage error (percentage difference between the measured and the theoretical concentration) was always below 7%, indicating that the LOQ was validated at 10 ng/ml. Additionally, the stability of the extracts was verified at two concentration levels (50 and 500 ng/ml) over a 48-h period. As the run time was 35 min, this stability allowed the analysis of large batches of samples. The stability of baclofen during prolonged storage at −20°C in plasma was also verified at two concentration levels (50 and 500 ng/ml) after 6 months.

4. Discussion

When applying the method developed by Wall and Baker [14] for the assay of baclofen in liver to human plasma, we confirmed their observation that the background noise was too high for a correct measurement of low concentrations of baclofen. Alternative extraction procedures were therefore tested. Among them, the most efficient with regard to the signal-to-noise ratio was that published by Wuis et al. [10]. Indeed, under these conditions no interfering peak related to the derivatization reagent was recorded near the retention time of baclofen (see Fig. 1). The HPLC method described here is therefore a combination of the extraction procedure published by Wuis et al. [10] and the electrochemical detection developed by Wall and Baker [14]. Because the internal standard used by Wall and Baker (bromobaclofen) was not commercially available, it was initially planned to develop an
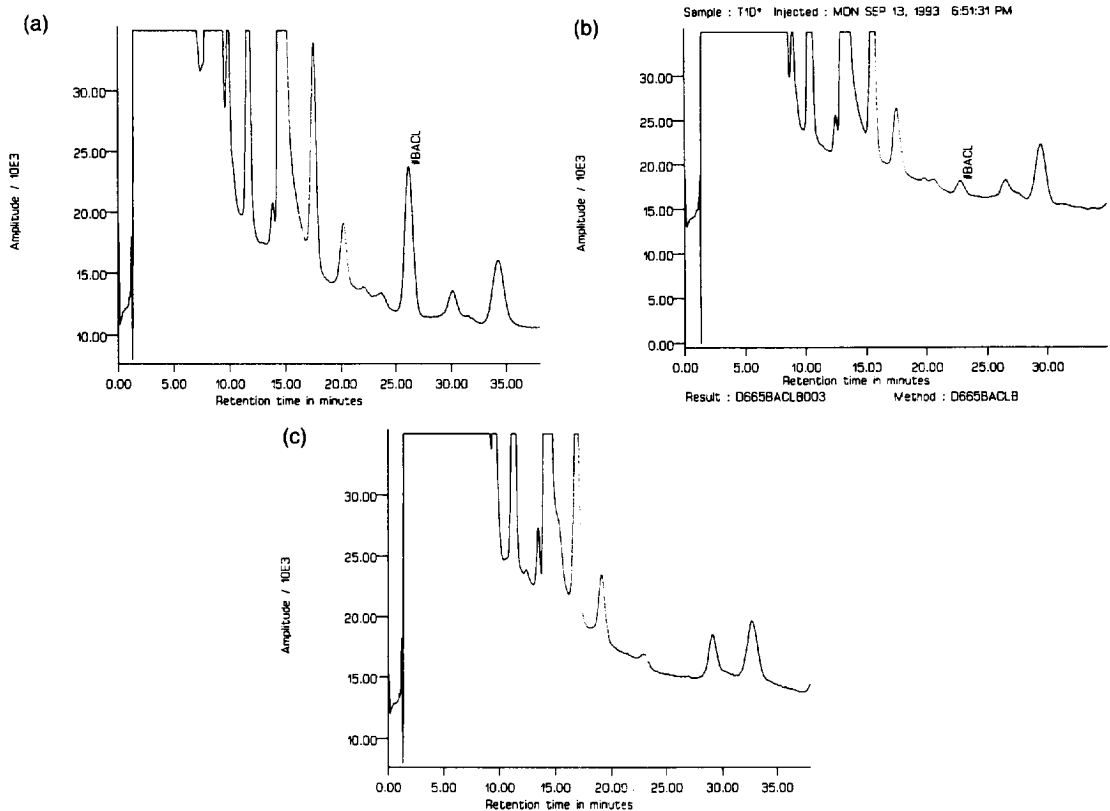
Fig. 1. Chromatograms of plasma sample spiked at (a) 100 ng/ml of baclofen, (b) 10 ng/ml (LOQ) of baclofen and (c) blank plasma.

extraction and derivatization procedure with almost quantitative efficiency. As the extraction efficiency was shown to be higher than 85%

Fig. 2. Chromatogram of baclofen in a plasma sample from a volunteer 8 h after a single 20-mg oral dose of baclofen.

whatever the concentration, it was concluded that the lack of an internal standard did not impair the performance of the method.

This method was shown to be appropriate for pharmacokinetic studies. Indeed, the linearity is sufficient to cover the range of concentrations expected to be found in plasma (10–500 ng/ml) after oral administration of 20 mg of baclofen. A high extraction efficiency is achieved, therefore obviating the use of an internal standard. The precision and accuracy of the method are good and the performance of the method at the LOQ (10 ng/ml) is comparable to that at higher concentrations.

The method was applied to the determination of plasma concentration profiles of baclofen in healthy volunteers after a single 20-mg oral dose. A typical chromatogram obtained after the analysis of a subject sample is presented in Fig. 2,
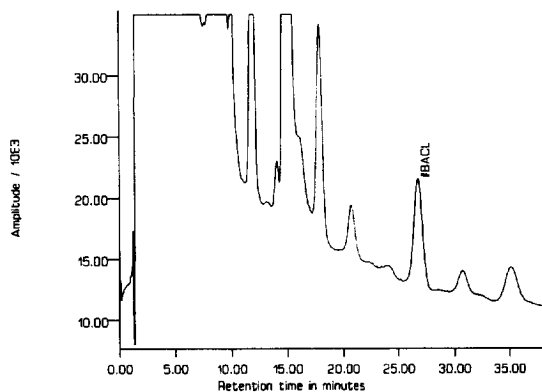
Table 1
Within-day precision and accuracy of the HPLC method for determination of baclofen in human plasma

Parameter	Concentration present (ng/ml)			
	10	50	200	500
Found concentrations (ng/ml)	9.4	52.1	192.9	469.6
	10.8	43.4	191.0	475.8
	10.7	50.3	189.6	473.9
	10.1	52.8	182.8	428.1
	10.8	52.9	190.3	482.5
	10.0	43.6	188.6	475.4
Mean (ng/ml)	10.3	49.2	189.2	467.6
S.D. (ng/ml)	0.6	4.5	3.5	19.8
R.S.D. (%)	5.8	9.2	1.9	4.2
Inaccuracy (%)	3.0	−1.6	−5.4	−6.5

which confirms that the specificity of the method is correct. During this study sixteen calibration lines were generated with correlation coefficients ranging from 0.9986 to 0.9999, thus demonstrating that linearity was reproducible. Forty-six quality control (QC) samples prepared at 15, 150, and 480 ng/ml were analysed along with the clinical samples. Analyses of the QC data dem-

onstrated that the precision and accuracy of the method were good and within the limits previously established during the validation (Table 3). Examination of the individual plasma concentration profiles of baclofen indicates that the maximum concentrations range between 213 and 534 ng/ml for nineteen subjects out of twenty. For one subject only the level reached 120 ng/ml

Table 2
Between-day precision and accuracy of the HPLC method for determination of baclofen in human plasma

Parameter	Concentration present (ng/ml)			
	10	50	200	500
Found concentrations (ng/ml)	9.8	45.0	187.3	468.2
	10.6	48.4	205.0	462.7
	11.3	50.4	182.8	455.8
	11.5	57.2	188.4	439.4
	10.2	47.7	205.6	500.6
	10.1	45.7	211.2	453.3
	9.7	54.5	176.3	462.1
	10.2	53.6	174.0	460.4
	11.9	54.5	194.6	439.1
	11.8	43.9	186.3	479.6
	10.0	53.0	197.4	477.7
	−[a]	51.9	202.8	516.2
Mean (ng/ml)	10.6	50.5	192.6	467.9
S.D. (ng/ml)	0.8	4.3	12.0	22.9
R.S.D. (%)	7.6	8.5	6.2	4.9
Inaccuracy (%)	6.0	1.0	−3.7	−6.4

[a] Aberrant value.

Table 3
Precision and accuracy of the determination of baclofen in quality control samples analysed during a 5-week period

Parameter	Concentrations (ng/ml)		
	15	150	480
R.S.D. (%)	11.3	8.1	6.8
Inaccuracy (%)	−4.6	−1.6	−6.1

at the peak. This therefore confirms that an LOQ of 10 ng/ml is necessary for the correct evaluation of the pharmacokinetics of baclofen. Fig. 3 shows the mean plasma concentration–time profile of baclofen in twenty healthy volunteers after a single 20-mg oral administration.
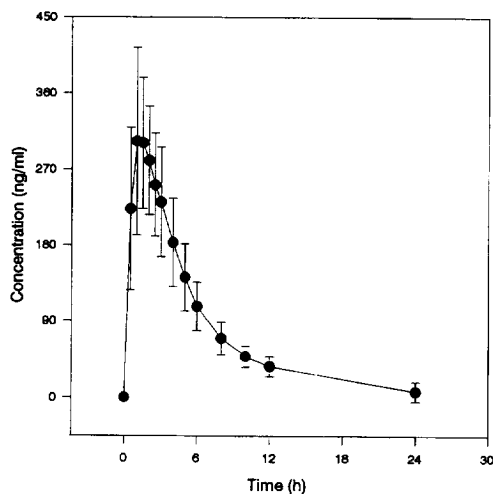

Fig. 3. Mean (±S.D.) baclofen plasma concentrations after a single 20-mg oral administration of baclofen to twenty healthy volunteers.

From this study, it can be concluded that the HPLC method that we have developed, which combines solid-phase extraction and electrochemical detection, reaches the optimum performance in terms of sensitivity, precision and accuracy for pharmacokinetic studies of baclofen.

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