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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF BACLOFEN IN PLASMA AND URINE OF MAN AFTER PRECOLUMN EXTRACTION AND DERIVATIZATION WITH *o*-PHTHALDIALDEHYDE

EVELINE W. WUIS*, RITA J.M. DIRKS, TOM B. VREE and EPPO VAN DER KLEYN

Department of Clinical Pharmacy, Sint Radboud Hospital, University of Nijmegen, Geert Grooteplein Zuid 8, 6525 GA Nijmegen (The Netherlands)

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SUMMARY

A reversed-phase high-performance liquid chromatographic method for the determination of the skeletal muscle relaxant baclofen in human plasma and urine is described. Cation-exchange extraction, precolumn derivatization with *o*-phthaldialdehyde, and on-column concentration precede fluorimetric detection (excitation at 340 nm, emission at 460 nm). The precision of the assay was always better than 6%. Recoveries of standards added to plasma and urine were 92% and 93%, respectively. With a sample size of 0.5 ml, a detection limit of a few nanograms, and the possibility of analysing up to four samples per hour, this method is suitable for pharmacokinetic studies. An example is presented.

INTRODUCTION

Baclofen, 4-amino-3-*p*-chlorophenylbutyric acid, is a skeletal muscle relaxant, which has been used in spastic disorders since its introduction for therapy in 1967 [1]. Several symposia have been dedicated to its pharmacological actions and clinical applications, but no conclusive evidence has yet been acquired for its mode of action [2—4]. Besides motor disorders other indications have also been proposed, such as schizophrenia [5—7], tardive dyskinesia [8, 9], and trigeminal neuralgia, but only the latter shows promising results [10, 11].

Baclofen is a *p*-chlorophenyl analogue of γ-aminobutyric acid (GABA), with the substituent rendering it a centre of asymmetry (Fig. 1). The discussion about its mode of action has been complicated by the different effects of the two enantiomers. The efficacy in spasticity has been attributed to (−)-baclofen, a substance with $GABA_B$-mimetic properties [12, 13]. The commercially available drug (Lioresal®) is the racemic mixture.

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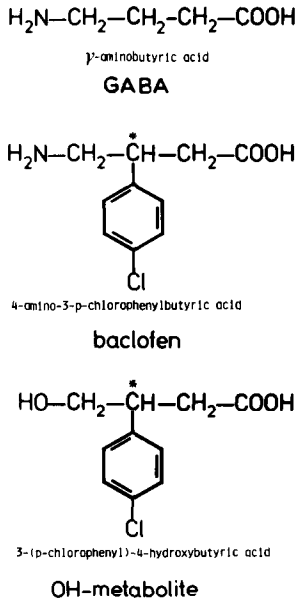

Fig. 1. Structural formulae of γ-aminobutyric acid (GABA), baclofen and its hydroxy metabolite.

Baclofen is metabolized to only a minor extent. Deamination yields 3-(*p*-chlorophenyl)-4-hydroxybutyric acid (Fig. 1), a metabolite which has been identified in the urine of rat, dog, and man. In man about 85% of a ^{14}C-labelled oral dose was found to be excreted unchanged, primarily in the urine. Most of the remaining radioactivity was accounted for by the deaminated metabolite, which was inactive in animals [14, 15].

The development of a procedure to determine baclofen in body fluids encountered several analytical problems due to its amino acid structure. For the measurement of concentrations in the nanogram range, as needed for pharmacokinetic studies, it has to be separated from the endogenous amino acids. Degen and Riess [16] developed a gas—liquid chromatographic method with electron-capture detection, requiring lengthy derivatization reactions and resulting in only a 50% recovery. Gas chromatography in combination with mass spectrometry (GC—MS) has been described by Swahn et al. [17].

No high-performance liquid chromatographic (HPLC) method for the determination of baclofen has yet been published. One problem is that baclofen itself, like other amino acids, cannot be detected in low concentrations in biological material with ultraviolet spectrometry or fluorimetry. However, a considerable number of methods to assay endogenous amino acids, using reversed-phase HPLC in combination with fluorophore formation, have recently been published. Commonly used derivatization agents for pre- or postcolumn fluorescence detection of amino acids are Dns chloride, fluorescamine, and *o*-phthaldialdehyde (OPA) [18]. Derivatization with OPA is relatively simple and rapid. In the presence of alkylmercapto compounds highly fluorescent isoindoles are formed [19—21]. We developed an analytical assay for baclofen based upon precolumn derivatization with OPA. Separation

from the endogenous amino acids was achieved by cation-exchange extraction prior to the derivatization. High sensitivty was obtained with an on-column concentration and cleaning procedure which allows for injection of large volumes [22].

MATERIALS AND METHODS

Reagents and chemicals

Solutions were made in distilled water. All glassware was rinsed with distilled water prior to use. Chemicals were of analytical grade and were used without further purification. A stock solution of baclofen (a gift from Ciba-Geigy, Arnhem, The Netherlands) containing 100 mg/l was diluted with water, urine, or plasma to produce concentrations in the range 0.02—2 mg/l. For the extraction procedure the following solvents and solutions were used: hexane, methanol, saturated sodium chloride solution, citrate buffer pH 2.6 (0.1 *M* citric acid—0.2 *M* dibasic sodium phosphate, 89.1:10.9, v/v), and borate buffer pH 10.4 (0.1 *M* borax adjusted to pH 10.4 with sodium hydroxide). The derivatization reagent consisted of 250 mg of *o*-phthaldialdehyde dissolved in 1.5 ml of methanol, 23 ml of borate buffer pH 10.4 (0.4 *M* boric acid adjusted to pH 10.4 with potassium hydroxide), and 0.5 ml of thioglycolic acid. The pH was adjusted to 10.4 after mixing [23]. Two mobile phases were used: eluent A, 0.9% (w/v) sodium chloride solution; eluent B, methanol—tetrahydrofuran—phosphate buffer pH 8.5 (0.067 *M* dibasic sodium phosphate adjusted to pH 8.5 with monobasic potassium phosphate) (40:2:58, v/v/v).

Apparatus

Extraction was performed with the Baker-10 extraction system (Baker Chemicals, Cat. No. 70180, Deventer, The Netherlands), fitted with 3-ml disposable extraction columns packed with aromatic sulphonic acid bonded to silica gel (Cat. No. 70903).

The chromatographic system consisted of a double-head solvent pump (Orlita, DHP-1515, Bakker, Zwijndrecht, The Netherlands), two sampling valves (Valco, Houston, TX, U.S.A.), and a sampling loop of 1.0 ml. Complete pulse quenching was achieved with a pulsation dampener (Orlita, PDM 3.350 M, Bakker) between pump and injection valve. The analytical column (25 cm × 4.6 mm I.D.) was packed with reversed-phase material Cp-Spher C_8, particle size 8 μm (Chrompack, Cat. No. 28502, Middelburg, The Netherlands). The concentration column (5 cm × 3.0 mm I.D.) was filled with LiChrosorb RP-8, 10 μm (Chrompack). A fluorescence detector (Perkin-Elmer, Model 3000, Delft, The Netherlands), equipped with a red sensitive photomultiplier and a doubly mirrored flow cuvette, was used. The detector was connected to a 10-mV recorder (Kipp & Zonen, BD 40, Delft, The Netherlands).

Extraction and derivatization

The extraction column was conditioned with two column volumes of hexane, two column volumes of methanol, two column volumes of water, and three column volumes of saturated sodium chloride solution. To 0.5 ml of plasma (low concentrations 1.0 ml) or 0.5 ml of urine (high concentrations

were diluted prior to use) an equal volume of citrate buffer pH 2.6 was added. This mixture was loaded onto the column. After five subsequent column washings, four with water and the last with saturated sodium chloride solution, the sample was eluted with four 0.5-ml aliquots of borate buffer pH 10.4. To the collected eluent 0.4 ml of the derivatization reagent was added. After mixing on a vortex mixer and subsequent centrifugation at 2000 *g*, a 1.0-ml sample was taken to be used for HPLC. The time elapsed between the addition of the reagent until injection into the HPLC system was standardized at 150 sec.

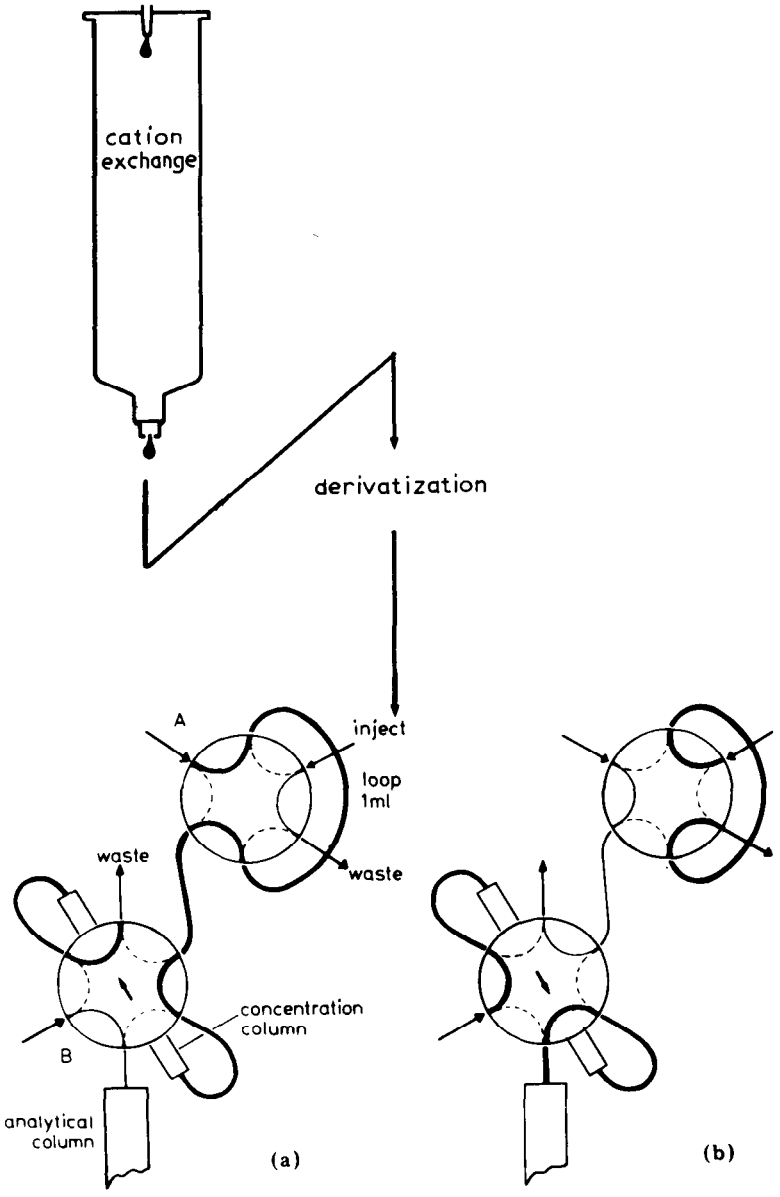

Fig. 2. Schematic diagram of the assay of baclofen. For further explanation, see text.

HPLC and detection

The sample loop was filled as shown in Fig. 2b. By turning the upper valve, solvent A (flow-rate 1.5 ml/min, pressure approx. 5 MPa) transported the sample onto the concentration column (Fig. 2a). After 5 ml of solvent A had been used, the lower valve was turned and with 1.5 ml of solvent B (flow-rate 1.0 ml/min, pressure approx. 10 MPa) the concentrated sample could be flushed onto the analytical column (Fig. 2b). With an excitation wavelength of 340 nm and emission at 460 nm the fluorophore was detected quantitatively by measuring the peak height. The experiments were carried out at room temperature. Between samples the concentration column was flushed with several 1-ml methanol washings.

Recovery

The recoveries of the standards that had been added to water, plasma, and urine were measured in triplicate for three different concentrations in the range 0.10—2.1 mg/l and compared to a direct (i.e. no extraction) assay in water.

Experiment in a healthy volunteer

A 35-year-old Caucasian woman (67 kg) was given a single oral dose of 20 mg baclofen (Lioresal®, two tablets of 10 mg each) 2 h after breakfast. Blood samples of 1—2 ml were drawn at predetermined intervals by fingertip puncture for a total period of 14 h. Samples from spontaneously voided urine were collected for 50 h. All blood specimens were collected in heparinized tubes. Plasma and urine were stored at −20° C until analysis.

RESULTS AND DISCUSSION

Chromatograms of baclofen in plasma and urine are shown in Figs. 3 and 4. Blanks (Fig. 3A and 4A) did not show interfering substances. In Fig. 3B a plasma sample obtained from a patient treated with a daily oral dose of 45 mg is shown. The detection limit in plasma at a signal-to-noise ratio of 3 was approx. 1.5 ng (Fig. 3C). An example of baclofen measured in the urine of a volunteer is given in Fig. 4B. The detection limit in urine was approx. 5 ng (Fig. 4C). The capacity ratio (k') was 3. Calibration curves showed good linearity between peak heights and concentrations (r^2 always > 0.99). The precision of the determination in water, plasma, and urine was measured for three different concentrations in the range 0.10—2.1 mg/l ($n = 4$). Coefficients of variation were always less than 6%. Recoveries in a similar concentration range were 97% for extraction from water, 92% and 93% for extraction from plasma and urine, respectively.

With the cation-exchange extraction procedure interfering endogenous amino acids could be effectively removed, owing to their low $pK_{a,1}$ values [24] as compared to baclofen ($pK_{a,1} = 3.87$, $pK_{a,2} = 9.62$) [15]. Thus, based upon the pK_a values of baclofen and the pH needed for the subsequent derivatization reaction, the different buffers were chosen. When the conditioning with sodium chloride was omitted, baclofen was not reproducibly held on the column. Prior to elution, another wash with sodium chloride was necessary for complete recovery. Derivatization of baclofen with OPA was as simple and rapid as for

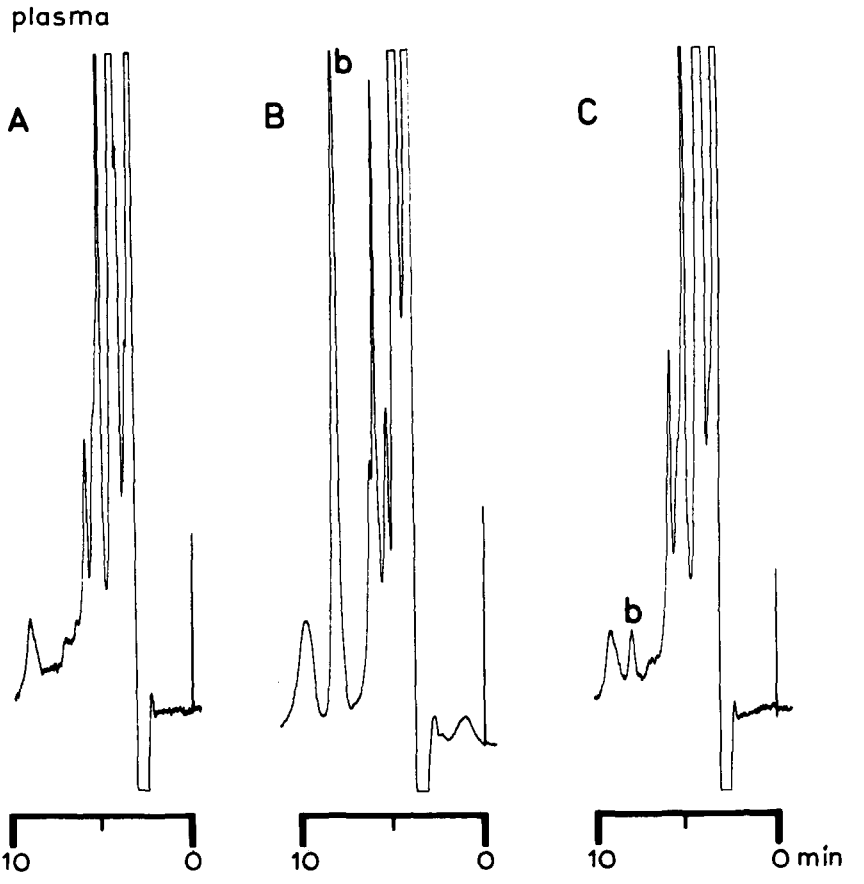

Fig. 3. Chromatograms of plasma samples: (A) plasma blank, (B) plasma of a patient on chronic oral therapy with 0.603 mg/l baclofen, (C) plasma spiked with 0.021 mg/l baclofen. b = baclofen.

endogenous amino acids. The structure of the proposed reaction product is given in Fig. 5. As the stability of the fluorescent derivatives of OPA with amino acids can vary with time [19—21], the baclofen fluorophore was always measured at a fixed time, in this case 150 sec after starting the reaction.

Although baclofen has been in clinical use for over fifteen years, hardly any pharmacokinetic data are available. Only a few studies have been published mentioning pharmacokinetic parameters, in volunteers [14, 15] and in patients [25, 26]. This seems to be mainly due to the lack of analytical procedures suitable for routine measurements.

To test the applicability of the presented HPLC method in pharmacokinetic studies a pilot experiment was done in a healthy volunteer. Fig. 6 shows the plasma concentration—time and renal excretion rate—time profiles of baclofen after a single oral dose of 20 mg. Some pharmacokinetic parameters are listed in Table I, calculated according to standard methods [27, 29]. The values of t_{max} and of C_{max} are similar to those reported by Swahn et al. [17] with the GC—MS method. After 50 h, 85% of the dose administered was recovered as unchanged drug in the urine. This is in agreement with data from experiments

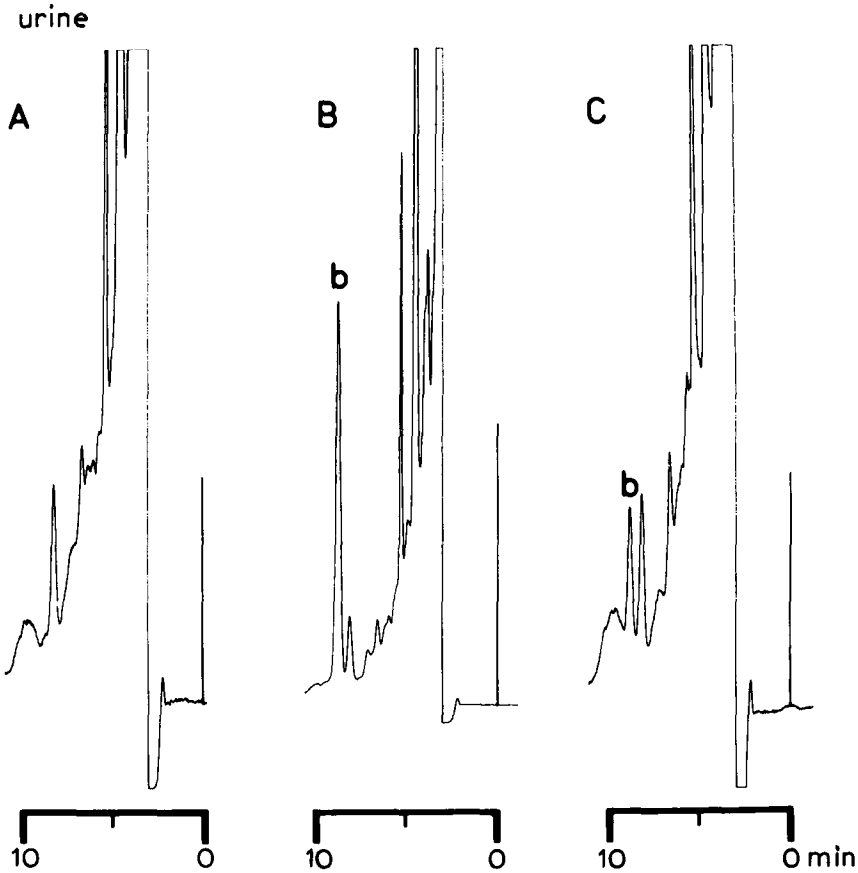

Fig. 4. Chromatograms of urine samples: (A) urine blank, (B) urine sample of a volunteer after a single oral dose of 2.51 mg/l baclofen, (C) urine spiked with 0.205 mg/l baclofen. b = baclofen.

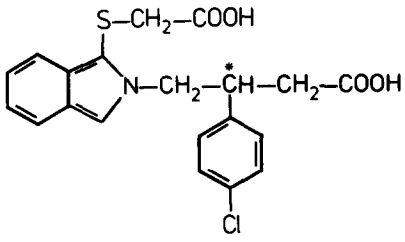

Fig. 5. Proposed reaction product of *o*-phthaldialdehyde and thioglycolic acid with baclofen.

with radioactively labelled baclofen [14, 15]. Clearance values have not been reported earlier. A total plasma clearance (Cl) of 0.16 l h^{-1} kg^{-1} was calculated. In this volunteer the renal clearance of baclofen (Cl_R) was equal to the creatinine clearance. Half-lives from plasma data have been reported to range from 2.5 to 6 h [14, 15]. After massive overdoses, however, half-lives of more than 30 h have been observed [30, 31]. We found a half-life of 5.4 h from terminal plasma data. When the combined plasma and urine data were fitted to a two-compartment open model using NONLIN [27], a higher terminal

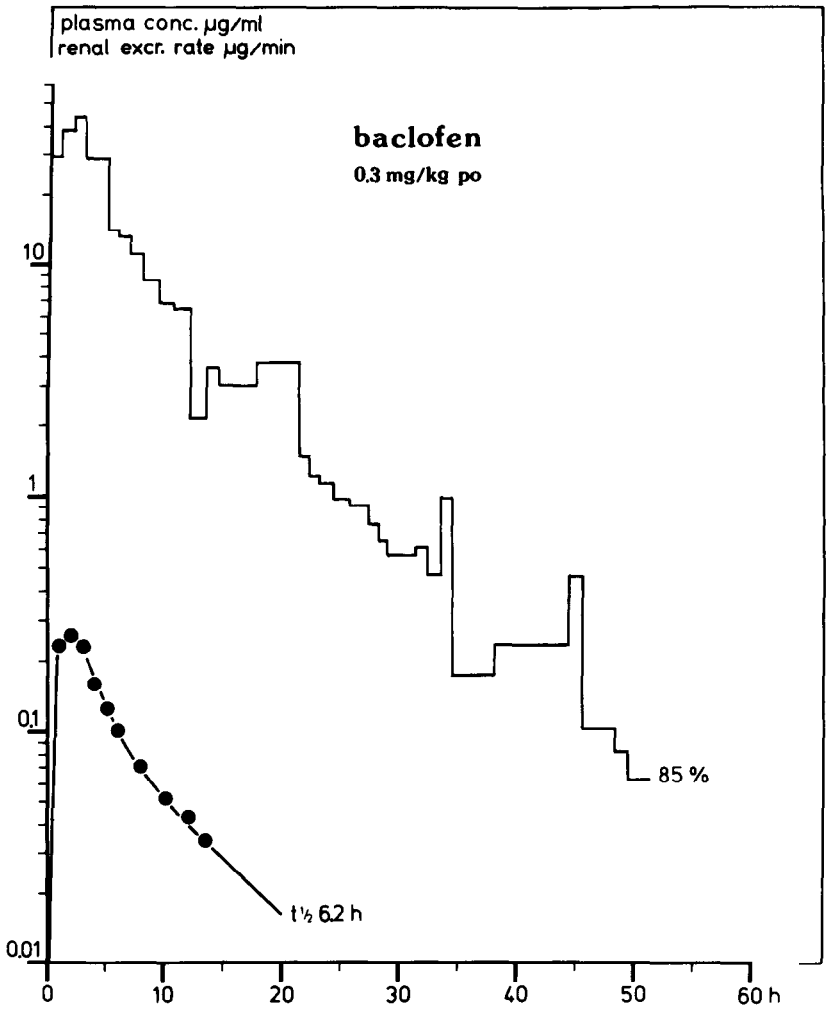

Fig. 6. Plasma concentration—time and renal excretion rate—time profiles of baclofen in a human volunteer following a single oral dose of 20 mg. % = percentage of the dose excreted unchanged in the urine.

TABLE I

SOME PHARMACOKINETIC PARAMETERS OF BACLOFEN IN MAN

Single oral dose of 20 mg (n = 1). Abbreviations according to ref. 28, calculations according to refs. 27 and 29.

C_{max}	0.24 mg/l
t_{max}	2.0 h
$t_{½z}$	6.2 h
Cl^{*}	0.16 l h^{-1} kg^{-1}
Cl_R**	0.12 l h^{-1} kg^{-1}
$A_e(\infty)$***	86% (of dose in urine)

*Assumption F = 1.
**Calculated from total plasma concentration data.
***Extrapolated to infinity.

half-life of 6.2 h was found. In a preliminary study in eighteen patients on chronic oral therapy with daily baclofen doses ranging from 0.26 to 1.2 mg/kg, we measured plasma concentrations varying from 0.078 to 0.60 mg/l, with a mean total plasma clearance of 0.21 ± 0.11 l h^{-1} kg^{-1}. Samples were drawn 3 h after the morning dose.

With the described reversed-phase HPLC method, only the parent drug is measured. The hydroxymetabolite lacks the NH_2 group, which reacts with OPA. Also no separation of the enantiomers is obtained. These disadvantages also apply to the gas–liquid chromatographic methods. From the results presented, the possibility of measuring up to four samples (plasma or urine) per hour, with a sample size of only 0.5 ml, and a detection limit of a few nanograms, it appears that baclofen can be measured with adequate sensitivity and selectivity for pharmacokinetic purposes.

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