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IMPROVED GAS—LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF BACLOFEN IN PLASMA AND URINE

GREGORY KOCHAK* and FRANK HONC

Research Department, Pharmaceuticals Division, Ciba-Geigy Corporation, Ardsley, NY 10502 (U.S.A.)

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SUMMARY

A simple, rapid and sensitive assay for baclofen analysis has been developed. Baclofen and the internal standard are analyzed by gas—liquid chromatography with electron-capture detection after esterification of the carboxyl group to the butyl ester and acylation of the amino group to the pentafluoropropionylamide. Recovery from biological matrixes is accomplished by ion-pair extraction. The limit of quantitation of the entire assay as stated is about 10 ng/ml baclofen in plasma.

INTRODUCTION

Baclofen (Lioresal®) is a centrally acting muscle relaxant which is indicated for the alleviation of signs and symptoms of spasticity resulting from multiple sclerosis, particularly for the relief of flexor spasms and concomitant pain, clonus and muscular rigidity.

A sensitive and specific analytical procedure is necessary for use in bioavailability and pharmacokinetic studies. Following administration of single oral 10-mg commercial tablets resulting plasma concentrations are 30—50 ng/ml at 10 h.

Reported methods [1, 2] for the analysis of baclofen, γ-amino-β-(*p*-chlorophenyl)butyric acid, utilized adsorption of baclofen onto charcoal, XAD-2, or Dowex 50W-X4 resin for recovery from biological matrixes. Recovered baclofen was analyzed by gas—liquid chromatography (GLC) with electron-capture detection following derivatization to the butyl ester and heptafluorobutyrylamide or mass fragmentography.

An improved method for the determination of baclofen in plasma and urine has been developed. Recovery of baclofen and the internal standard, γ-amino-β-

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(2,4-dichlorophenyl)butyric acid from biological matrixes is accomplished by ion-pair extraction with heptanesulfonic acid directly into an organic phase. Analysis is by GLC with electron-capture detection following derivatization to the butyl ester and pentafluoropropionylamide. The use of an ion-pair extraction and formation of the pentafluoropropionylamide derivative results in significant improvement in selectivity, throughput time, reproducibility, and sensitivity compared to the previous GLC method [1] and comparable sensitivity to the other method [2].

EXPERIMENTAL

Preparation of reagents and sources

Heptanesulfonic acid, 90 m*M*, in 1 *M* phosphate buffer pH 3 was prepared by dissolving 68.995 g of sodium phosphate monobasic (J.T. Baker, Phillipsburg, NJ, U.S.A.), 2.5 ml of 85% orthophosphoric acid (Fisher Scientific, Pittsburgh, PA, U.S.A.) and 9.9 g heptanesulfonic acid, sodium salt (Eastman, Rochester, NY, U.S.A.) in 500 ml of distilled water.

Butanolic hydrochloric acid was prepared by mixing 5 ml of 1-butanol with 0.25 ml acetylchloride (both from MCB, Gibbstown, NJ, U.S.A.).

Borate buffer pH 10 was prepared by dissolving 24.6 g boric acid, 29.8 g potassium chloride and 14.1 g sodium hydroxide (all obtained from J.T. Baker) in 1 l of distilled water.

Pentafluoropropionic anhydride (Pierce, Rockford, IL, U.S.A.) was mixed with ethyl acetate (MCB) to give a concentration of 28.6% (v/v).

The following solvents were mixed (v/v) to the indicated concentrations: 40% 1-butanol in dichloromethane (MCB); 20% tertiary amyl alcohol (MCB) in ethyl acetate; 20% dichloromethane in diethyl ether; 2% acetone (both from J.T. Baker) in hexane (MCB); and methanol absolute (J.T. Baker) was used as supplied.

Preparation of standard solutions and calibration standards

The internal standard was prepared by dissolving γ-amino-β-(2,4-dichlorophenyl)butyric acid (Ciba-Geigy, Basle, Switzerland) in 0.1 *M* hydrochloric acid (J.T. Baker) to a concentration of 1 μg/ml.

The baclofen standard solution was prepared by dissolving γ-amino-β-(*p*-chlorophenyl)butyric acid (Ciba-Geigy, Summit, NJ, U.S.A.) in 0.1 *M* hydrochloric acid to a concentration of 1 μg/ml. Standard solutions were refrigerated and remained stable for at least six months.

Calibration standards were prepared by spiking pooled human plasma or urine with the prepared standard solutions.

Sample preparation and recovery

Ion-pair extraction. A 100-μl aliquot of internal standard solution is added to 1 ml plasma or 20 μl urine diluted in 1 ml distilled water, followed by 1 ml heptanesulfonic acid solution, 4 drops of 1 *M* phosphoric acid, 2.5 ml distilled water, and 0.5 ml of 40% butanol in dichloromethane. The resultant pH of this mixture should be 2.5—3.0. The mixture is shaken for 10 min on a horizontal mechanical shaker and centrifuged 10 min. The protein precipitate will

compress at the bottom in the tube and the clear supernatant is transferred into a clean 13-ml centrifuge tube.

The supernatant is extracted twice with 4 ml of 20% tertiary amyl alcohol in ethyl acetate by shaking on a horizontal shaker for 15 min. Both organic phases are pooled and evaporated to dryness under a stream of nitrogen at 45°C.

Derivatization. The dry residue is dissolved in 0.5 ml of butanolic hydrochloric acid, heated at 100°C for 15 min and evaporated to dryness under nitrogen at 45°C. The dry residue is dissolved in 5 ml of 20% dichloromethane in diethyl ether and the solution is extracted with 2 ml of 0.05 *M* sulfuric acid by shaking for 10 min. After a short centrifugation the organic phase is discarded. To the aqueous phase 2 ml of borate buffer pH 10 are added and extracted into 5 ml of 2% acetone in hexane by shaking 10 min. After a short centrifugation 4 ml of the organic phase are evaporated to dryness under nitrogen.

A 0.2-ml aliquot of 28.6% pentafluoropropionic anhydride in ethyl acetate is added to the residue and reacted for 1 h at room temperature. Unreacted reagent is evaporated to dryness and the tube washed with 0.2 ml methanol in order to destroy left-over reagent. The methanol is evaporated and the dry residue dissolved in 1 ml toluene (or heptane) of which 3 μl are injected on column.

Chromatographic conditions and instrumentation

The chromatographic system consisted of a Varian Model 3700 gas chromatograph with a ^{63}Ni (8 μCi) electron-capture detector and Perkin-Elmer Model 023 strip-chart recorder. The column used was a 15 m × 2 mm I.D. glass column packed with 3% OV 225 on Chromosorb W HP 80—100 mesh (Supelco, Bellefonte, PA, U.S.A.). The following temperatures were used: column 218°C, injector 240°C, detector 350°C. Nitrogen was used as the carrier gas at a flow-rate of 32 ml/min. The sensitivity of the electrometer was set at $1 \cdot 10^{-12}$ A.f.s. and attenuation 64.

RESULTS AND DISCUSSION

Chromatography

The retention times of the derivatives of baclofen and the internal standard were 3.1 and 4.2 min, respectively. Typical chromatograms obtained from control and spiked plasma are shown in Fig. 1. Chromatograms obtained from control and spiked urine are shown in Fig. 2.

Derivative formation

Baclofen (I, Fig. 3) and the internal standard are converted easily into their butyl esters (II) by heating with butanolic hydrochloric acid. Subsequent acylation of the primary amine was achieved by reaction of the ester with pentafluoropropionic anhydride. The structure of the resulting baclofen derivative (III) has been verified by mass spectrometry.

Derivatization of baclofen to the methyl ester and then transesterification to the butyl ester as previously reported [1] was found not to be necessary.

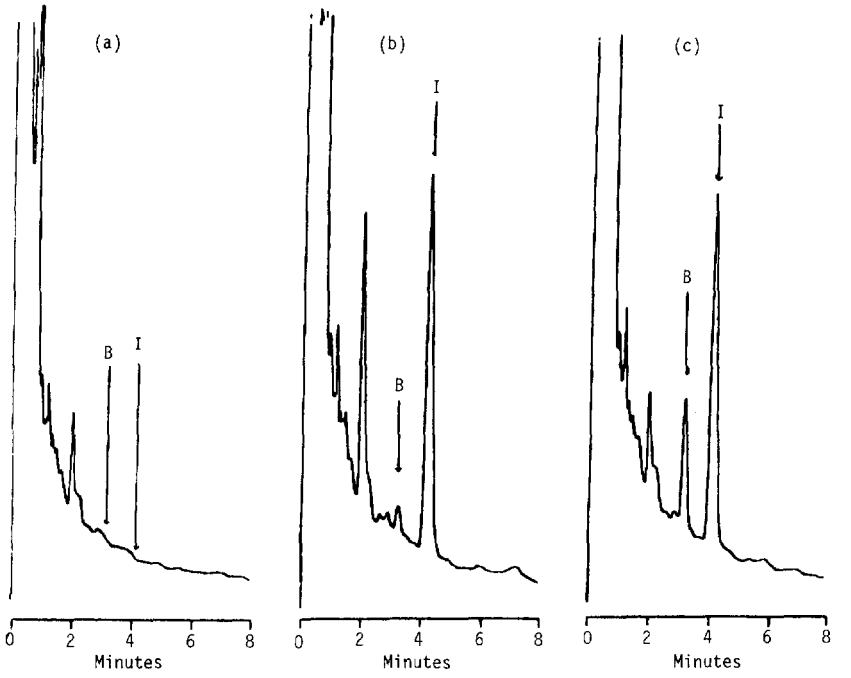


Fig. 1. Typical chromatograms from plasma extract. (a) Blank pooled plasma extract from 1 ml plasma; (b) plasma extract and reaction products from 1 ml plasma spiked with 20 ng/ml baclofen and 100 ng/ml internal standard; (c) plasma extract and reaction products from 1 ml plasma spiked with 100 ng/ml baclofen and 100 ng/ml internal standard. Peaks: B = baclofen reaction product; I = internal standard reaction product.

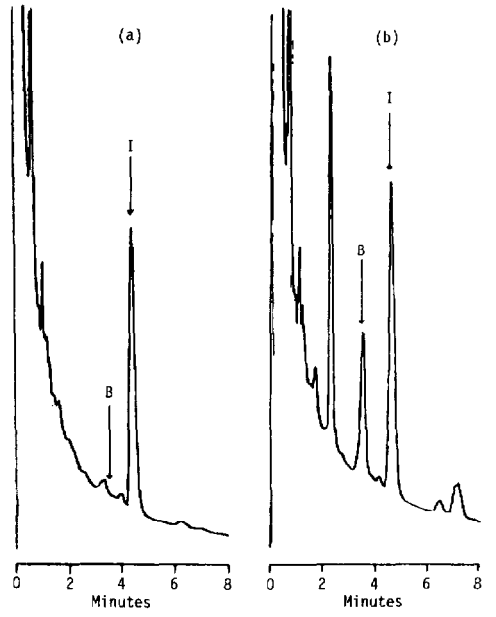
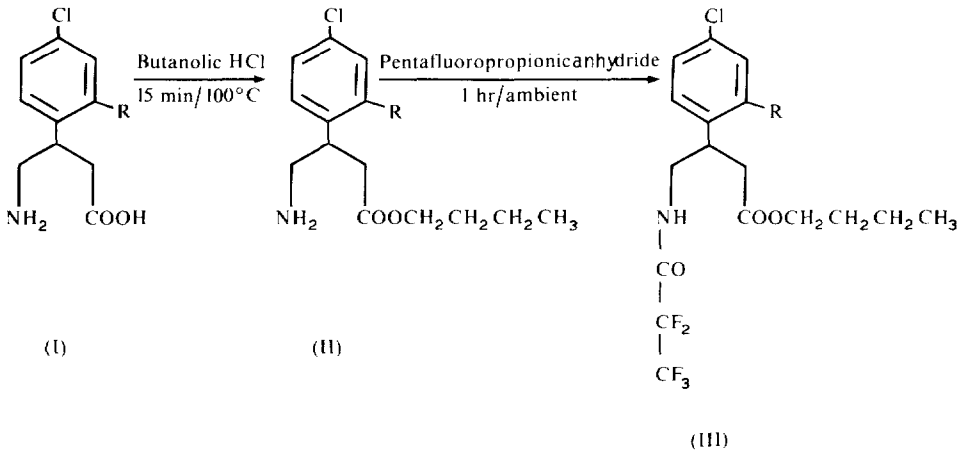


Fig. 2. Typical chromatograms from urine extract. (a) Pooled urine extract from 20 μl urine containing 5 μg/ml internal standard; (b) urine extract and reaction products from 20 μl urine spiked with 5 μg/ml baclofen and 5 μg/ml internal standard. Peaks: B = baclofen reaction product; I = internal standard reaction product.



(I) (II) (III)

Fig. 3. Scheme showing formation of *n*-butyl esters and the N-pentafluoropropionyl derivatives of baclofen (R = H) and the internal standard (R = Cl).

Direct formation of the butyl ester was facile and rapid. The throughput time was decreased by 50% for the acylation step used in this method compared to that previously reported [1]. The N-pentafluoropropionyl derivatives exhibited superior chromatographic performance and throughput time compared to the previously reported N-heptafluorobutyryl derivatives [1]. The N-pentafluoropropionyl derivatives resulted in increased sensitivity both in terms of peak area and cleaner baseline chromatography.

Ion-pair extraction

The ion-pair extraction produced a cleaner extract from plasma due to the increase in selectivity for baclofen and the internal standard. This resulted in an overall increase in sensitivity because of the improved signal-to-noise ratio. Throughput time was decreased by at least 30% compared to charcoal and resin adsorption recovery methods [1, 2]. The extraction efficiency was 35.3 ± 6.3% (n = 14) over the range 20—800 ng/ml. The extraction efficiency decreased if the plasma or urine was not adjusted to a pH below 3. This was due to ionization of the carboxylic groups on baclofen and the internal standard. Ion-pair extraction of urine samples was found to be necessary. Urine sample volumes of 20 μl resulted in an interference under the baclofen peak of approximately 8% peak height relative to a 100-ng baclofen sample. No interference was detected relative to the internal standard.

Linearity of calibration curves

The standard curve for plasma was linear in the range 10—800 ng/ml employing a sample volume of 1 ml and a single standard curve. The standard curve regression was determined as follows: peak height ratio = 0.00313 (concentration baclofen) + 0.00221, r = 0.9999. However, for samples in the range 5—20 ng/ml a separate calibration curve is suggested in order to prevent bias in the regression slope and intercept. Similarly the urine calibration curve was linear in the range 1—40 μg/ml using 20-μl sample volumes. The standard curve regression was determined as follows: peak height ratio = 0.00445 (concentration baclofen) — 0.05876, r = 0.9989.

Selectivity

Control plasma (drug-free) showed no detectable interferences (Fig. 1). Control urine samples (20 μl) showed only a single very low level interference under the baclofen peak (Fig. 2). Due to the high concentration of baclofen in urine (microgram range) after the administration of a single 20-mg oral dose this level of interference is considered negligible. Larger urine sample volumes, however, should only be used cautiously due to the increasing percentage of interference relative to baclofen in the sample.

Since only 3—6% of a dose of baclofen is excreted renally as metabolites in man [3] no detectable metabolic interferences are anticipated. Plasma (1 ml) and urine (20 μl) samples from subjects receiving baclofen indeed showed no detectable interferences with baclofen or the internal standard.

Accuracy and precision

The results of within-day accuracy and precision for the determination of baclofen in independent plasma samples are presented in Table I. The coefficients of variation (C.V.) ranged from 25.0% (n = 3) at 5 ng/ml to 1.4% (n = 4) at 50 ng/ml. Mean absolute error ranged from 20% at 5 ng/ml to 1% at 100 ng/ml. Similarly within-day accuracy and precision for urine determination utilizing 20-μl urine samples are presented in Table II. The C.V. values ranged from 5.0% (n = 3) at 800 ng per 20 μl to 2.8% (n = 3) at 100 μg per 20

TABLE I

WITHIN-DAY ACCURACY AND PRECISION FOR DETERMINATION OF BACLOFEN IN PLASMA BY GLC

Baclofen added (ng/ml)	Baclofen found (ng/ml)				No. of samples	Mean ± S.D.	C.V. (%)	Mean absolute error ± S.D. (%)
5	3	5	4		3	4 ± 1.0	25.0	20 ± 20.0
10	11	11	9	12	4	11 ± 1.3	11.8	13 ± 5.0
20	27	25	22	22	4	24 ± 2.4	10.0	20 ± 12.2
50	44	43	44	43	4	44 ± 0.6	1.4	11 ± 3.5
100	99	101	103	100	4	101 ± 1.7	1.7	1 ± 1.3
200	209	216	204	214	4	211 ± 5.4	2.6	5 ± 2.7
400	441	437	400	441	4	430 ± 19.9	4.6	7 ± 5.0
800	795	748	817	767	4	782 ± 30.4	3.9	3 ± 2.5

TABLE II

WITHIN-DAY ACCURACY AND PRECISION FOR DETERMINATION OF BACLOFEN IN URINE BY GLC

Baclofen added (ng per 20 ml)	Baclofen found (ng per 20 ml)			No. of samples	Mean ± S.D.	C.V. (%)	Mean absolute error ± S.D. (%)
20	22	23	21	3	22 ± 1.0	4.5	10 ± 5.0
100	94	95	90	3	93 ± 2.6	2.8	7 ± 2.6
800	821	768	848	3	812 ± 40.7	5.0	4 ± 1.7

TABLE III

DAY-TO-DAY PRECISION FOR DETERMINATION OF BACLOFEN IN PLASMA AND URINE BY GLC

Day	Regression slope	Regression intercept	No. of standards per curve	Correlation coefficient (r)
*Plasma**				
1	0.00313	0.00221	8	0.9999
2	0.00310	0.00588	7	0.9998
3	0.00336	0.00409	8	0.9995
4	0.00319	0.03487	27	0.9992
5	0.00343	0.03777	7	0.9980
Average	0.00324	0.01696		0.9993
S.D.	0.00015	0.01775		0.0008
C.V. (%)	4.6	104.7		0.1
Urine				
1	0.00445	−0.05876	6	0.9989
2	0.00421	−0.01768	12	1.0000

*Over a period greater than two weeks.

μl. Mean absolute error ranged from 10% at 20 ng per 20 μl to 4% at 800 μg per 20 μl.

Day-to-day variability is demonstrated for a period in excess of two weeks by the change in slope of standard curves (Table III). For plasma determinations of baclofen the C.V. for the regression slope is 4.6% (n = 5) indicating a high degree of reproducibility. The regression slopes for the urine standard curves (Table III) were reproducible for two curves obtained on separate days, two days apart.

Sensitivity

Based on the use of a single calibration curve and the reported instrumental conditions the overall limit of quantitation is 10 ng/ml for the plasma assay and 1 μg/ml for the urine assay. For plasma analysis improved accuracy and precision would be expected utilizing an additional calibration curve in the 5—20 ng/ml range.

Stability of plasma samples

Spiked 50 and 400 ng/ml plasma samples were frozen and stored at −15°C for up to 148 days. The average (± S.D.) recovery at 47 days was 102.6% (n = 2) and at 148 days was 93.5 ± 7.8% (n = 4). These values indicate the stability of baclofen plasma samples for at least 148 days.

CONCLUSION

An improved method for the determination of baclofen in biological matrixes has been developed which provides a sensitive and selective analytical procedure for use in bioavailability and pharmacokinetic studies. This method

provides at least a two-fold increase in sensitivity as well as significant increases in selectivity and throughput time of analysis over previously described methods.

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