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THE DETERMINATION OF γ-AMINO-β-(*p*-CHLOROPHENYL)BUTYRIC ACID (BACLOFEN) IN BIOLOGICAL MATERIAL BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Baclofen and the internal standard are isolated from biological samples by adsorption on charcoal. After elution, the compounds are converted into butyl esters and purified by base-specific solvent extraction. The butyl esters are then N-acylated with heptafluorobutyrylimidazole, and the neutral derivatives are isolated by solvent extraction and subjected to gas–liquid chromatography, with use of an electron-capture detector. Baclofen can be determined in concentrations down to about 30 ng per sample.

INTRODUCTION

The compound γ-amino-β-(*p*-chlorophenyl)butyric acid is the active principle in Lioresal® (generic name baclofen), a drug marketed by Ciba-Geigy Ltd. (Basle, Switzerland). The drug exhibits muscle-relaxing properties and is used in the treatment of spasticity. A sensitive method for the determination of this compound was needed in order to carry out pharmacokinetic studies. Because this amino acid has amphoteric properties, direct extraction from biological material is not possible; however, the charcoal procedure used by Meola and Vanko[1] for isolating drugs was also applicable to baclofen.

To achieve the properties required for gas–liquid chromatography (GLC), the carboxyl group of baclofen is esterified as described by Gehrke and Stalling[2], and the amino group is acylated using heptafluorobutyrylimidazole; the final derivative is stable and exhibits very good GLC properties and high sensitivity in the electron-capture detector.

The internal standard used is γ-amino-β-(2,4-dichlorophenyl)butyric acid (C 36141), which, owing to the close similarity in chemical structure, shows the same extractability and reactivity as baclofen. For the determination of baclofen in plasma, a special purification procedure had to be developed to eliminate GLC signals from the biological background. This was achieved by base-specific extraction of baclofen and the standard after formation of the butyl esters. For the determination in urine,

however, this additional purification is not necessary, and the amino group can be acylated immediately after formation of the butyl ester.

DEVELOPMENT OF THE METHOD

Derivative formation

Baclofen (I in Fig. 1) and the internal standard are easily converted into their methyl esters (II) by heating with methanolic hydrochloric acid. However, the methyl esters are not stable, owing to transformation into lactam. The lactam derivative of baclofen was also tested for its GLC properties, but was inferior to the N-acyl derivative of the butyl ester.

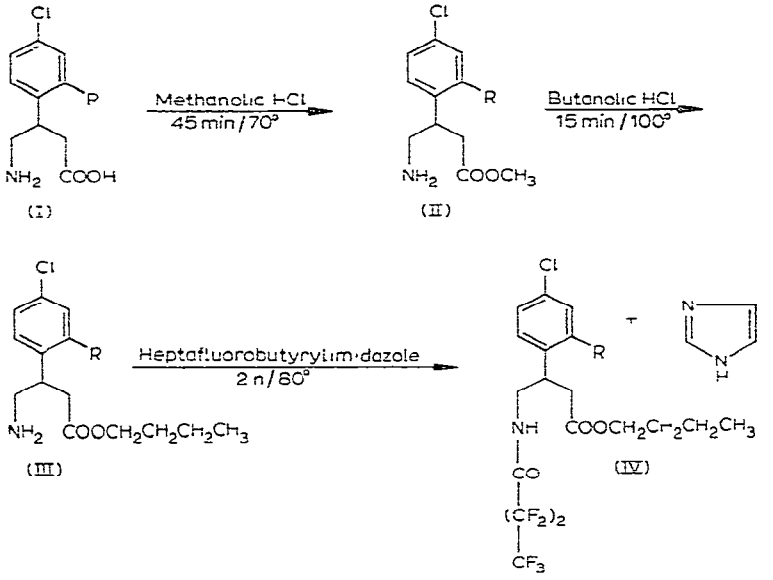

Fig. 1. Scheme showing formation of the *n*-butyl esters and the N-heptafluorobutyryl derivatives of baclofen (R=H) and the internal standard (R=Cl).

The methyl esters can be trans-esterified into butyl esters (III) immediately after methyl ester formation, and acylation of the primary amine is best achieved by treatment with heptafluorobutyrylimidazole. The structure of the resulting baclofen derivative (IV) has been verified by mass spectrometry.

Extraction

The isolation of baclofen from plasma and urine was optimized by using [^{14}C]baclofen; recovery (radioactivity in the eluate) from both types of sample was 80–90%. Good yields were also obtained using XAD-2 resin, but there were disadvantages in comparison with charcoal. First, the XAD-2 resin had to be used in small columns in order to obtain good yields, whereas, with charcoal, the batch meth-

od could be used; secondly, charcoal was easier to free from interfering impurities. The over-all recovery for the entire procedure, including the additional purification, applied to 1 ml of plasma (as tested with 800 ng of [^{14}C]baclofen) was about 48%. For determinations of baclofen in plasma, up to 3 ml of sample could be used.

For determinations in urine, only 0.1 ml (or less) of sample is required, because much of the dose is excreted as unchanged drug. In fact, if the concentrations in urine are high, *i.e.*, above 50 μg/ml, only 10–20 μg of biological material need be used and the isolation procedure via adsorption on charcoal can be omitted. In such instances, the sample is combined with an adequate portion of an alcoholic solution of the internal standard, and the mixture is evaporated to dryness; the dry residue is directly subjected to the derivative-forming reactions. The over-all recovery from 0.1 ml of urine, including the charcoal isolation procedure, as tested with 800 ng of [^{14}C]-baclofen, was about 50%.

MATERIALS AND METHODS

Reagents

The following reagents were used. Ethanol (p.a.; E. Merck, Darmstadt, G.F.R.). Methanol–water (9:1). Carbonate buffer: 21 g of sodium carbonate and 0.42 g of sodium bicarbonate are dissolved in about 700 ml of water, the pH is adjusted to 11 with 2 *N* sodium hydroxide and/or 2 *N* hydrochloric acid, and the mixture is diluted to 1 litre with water. Charcoal slurry: 2 g of active charcoal (Merck) are washed with 200 ml of water and 200 ml of methanol, then dried; 500 mg of the washed charcoal and 200 ml of the carbonate buffer solution are stirred for 30 min at room temperature, the mixture is centrifuged, and the precipitate is suspended in 5 ml of the carbonate buffer solution. Diethyl ether–dichloromethane (4:1). Buffer solution (pH 10): Merck Titrisol (24.6 g of boric acid, 29.8 g of potassium chloride and 14.1 g of sodium hydroxide per litre). Sulphuric acid, 0.1 *N*. Methanolic hydrochloric acid (5 ml of methanol and 0.25 ml of acetyl chloride). Butanolic hydrochloric acid (5 ml of butanol and 0.25 ml of acetyl chloride). Heptafluorobutyrylimidazole (Pierce, Rockford, Ill., U.S.A.). Internal standard: γ-amino-β-(2,4-dichlorophenyl)butyric acid; used as an ethanolic solution (500 ng/ml for urine: 342 ng/ml for plasma).

Isolation

A 1-ml portion of freshly prepared standard solution (500 ng per sample for urine and 342 ng for plasma) (C 36141) in ethanol is evaporated to dryness under a stream of nitrogen, then 1–3 ml of plasma (or 0.1 ml of urine) is added, and the mixture is allowed to equilibrate at room temperature for 1 h. Then 1 ml of carbonate buffer solution of pH 11 and 0.1 ml of charcoal slurry are added, and the mixture is shaken for 20 min on a mechanical rotary shaker (Infors) at 200 strokes per min. The suspension is centrifuged, and the supernatant solution is aspirated off and discarded. The remaining charcoal is washed twice with 2 ml of water, each time with shaking for 5 min, and centrifuged; the water is aspirated off and discarded.

Baclofen and the standard are eluted by shaking the charcoal twice for 15 min with 2 ml of methanol–water (9:1), and the supernatant solvent collected after centrifugation is filtered through a cotton-wool plug and evaporated to dryness in a stream of nitrogen at 45°.

Esterification

The dry residue is dissolved in 0.5 ml of methanolic hydrochloric acid, heated at 70° for 45 min, again evaporated to dryness under nitrogen at 45°, and immediately re-dissolved in 0.5 ml of butanolic hydrochloric acid, heated at 100° for 15 min and evaporated to dryness under nitrogen at 60°.

Purification procedure for plasma samples

The dry residue is dissolved in 5 ml of diethyl ether–dichloromethane (4:1), and the solution is extracted with 2 ml of 0.1 *N* sulphuric acid by shaking for 10 min on the mechanical shaker at 200 strokes per min. After a short centrifugation, the organic phase is discarded. To the aqeuous phase are added 2 ml of pH 10 buffer solution, and the liquid is shaken with 5 ml of the diethyl ether–dichloromethane for 10 min, followed by a short centrifugation. The organic phase is evaporated to dryness in a stream of nitrogen.

Acylation

Heptafluorobutyrylimidazole (25 μl) is added to the dry residue, the mixture is heated at 80° for 2 h, then cooled, and 5 ml of heptane and 2 ml of water are added, and the mixture is shaken briefly and vigorously by hand and immediately placed into a bath of solid carbon dioxide for 10 min. After a short centrifugation, the organic phase is removed and aliquots (1–5 μl) are injected directly into the gas chromatograph.

Gas–liquid chromatography

The instrument used was a Pye Unicam Model 74, Series 104 instrument equipped with a pulsed (150 μsec) electron-capture detector (^{63}Ni; 10 mCi), and a W + W Model 1100 recorder. The borosilicate-glass columns (5 ft. × 4 mm I.D.) were packed with 3% of OV-225 on Chromosorb W-HP (80–100 mesh), the nitrogen carrier-gas flow-rate was 40 ml/min, and injections were made using the on-column technique. The temperatures were as follows: column oven, 210°; detector, 350°; injector, 250°.

The retention times of the derivatives of baclofen and the internal standard were 7.3 and 9.8 min, respectively. Typical chromatograms obtained from urine and from plasma are shown in Fig. 2.

Calibration graphs

Urine. The concentrations of unchanged drug in urine after a single oral dose of 20 mg of Lioresal are high (up to approx. 80 μg/ml); it is therefore possible to work with samples of only 10 to 20 μl of urine. This amount of sample does not require purification after esterification and it is generally possible to omit the isolation step by charcoal.

A calibration graph was prepared using 10 μl of urine. The samples, after addition of internal standard, were evaporated to dryness and the residues were submitted directly to derivative formation. The peak-height ratios (*Hx*) were plotted *versus* initial concentration as shown in Fig. 3.

Plasma. For determinations in plasma after an oral dose of 20 mg of Lioresal, concentrations of up to 300 ng/ml can be expected; it is therefore necessary to employ sample volumes of 1–3 ml.

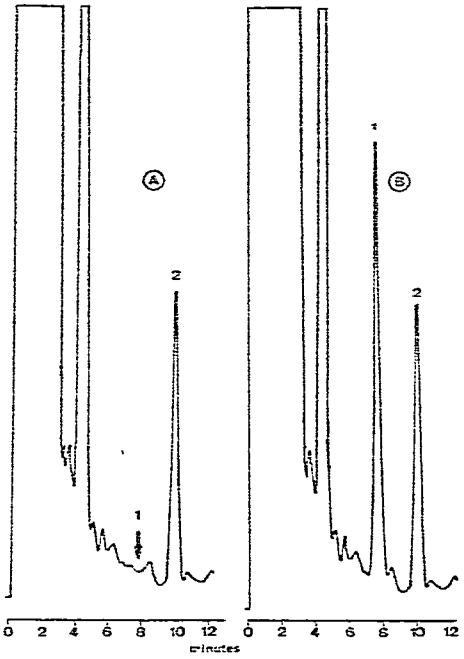

Fig. 2. Typical chromatograms: A, extract and reaction product from 1 ml of plasma containing internal standard (2) only (172 ng of C 36141), dissolved in 5 ml of heptane and 5 μl injected; B, extract and derivatives from 1 ml of plasma containing baclofen (1; 240 ng) and internal standard (2; 172 ng), dissolved in 5 ml of heptane and 5 μl injected.

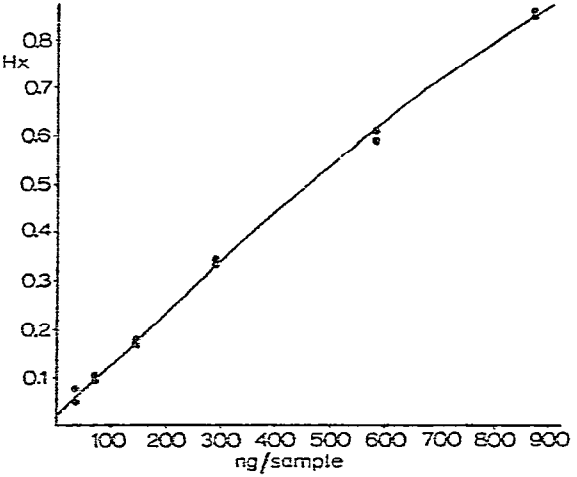

Fig. 3. Calibration graph for urine with samples containing 0 to 874 ng of baclofen per 10 μl with 500 ng of C 36141 (internal standard). *Hx* = ratio of peak-height for the baclofen derivative to that for the C 36141 derivative.

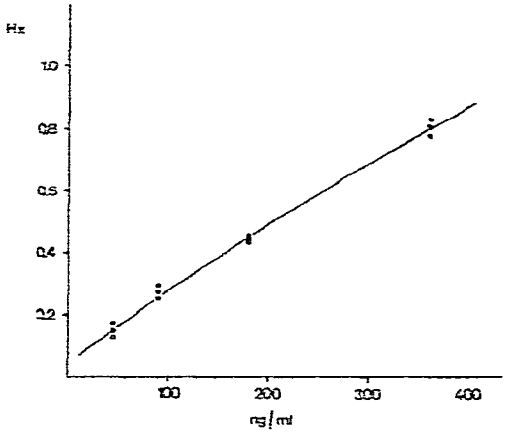

Fig. 4. Calibration graph for plasma with samples containing 0 to 360 ng of baclofen per ml and 342 ng of C 36141. *Hx* = as in Fig. 2.

A calibration graph was prepared using 1-ml samples of fresh human plasma containing 45 to 360 ng of baclofen per ml; 342 ng of C 36141 (internal standard) were added to each sample, and the mixture was processed as described above. The *Hx* values were plotted *versus* initial concentrations as shown in Fig. 4.

Sensitivity and precision

The method was tested by analysing samples of plasma to which baclofen had been added in concentrations unknown to the analyst; three independent determinations were performed on each sample. As shown in Table I and II, the sensitivity and precision were sufficiently satisfactory to permit use of the technique for determining baclofen concentrations following the ingestion of 20 mg of Lioresal.

TABLE I

RECOVERY OF BACLOFEN ADDED TO URINE

Each result is the mean of three determinations.

Baclofen concentration (ng/10 µl)		*Standard deviation (ng/10 µl)*	*Coefficient of variation (%)*	*Deviation of $\bar{x}$ from theory (%)*
Present	*Found ($\bar{x}$)*			
0.0	0.0	0.0	0.0	0.0
48	53.7	2.1	3.9	+11.9
53.5	53.0	5.0	9.4	− 0.94
107	110.0	17.5	15.9	+ 2.8
214	226.0	10.6	4.7	+ 5.6
235	245.6	14.1	4.2	+ 7.7
289	309.0	9.0	2.9	+ 6.9
428	423.0	40.0	9.2	+ 0.9
600	615.0	90.4	14.7	+ 2.7
642	618.0	36.8	6.0	− 3.7

TABLE II

RECOVERY OF BACLOFEN ADDED TO PLASMA

Each result is the mean of three determinations

Baclofen concentration (ng/ml)		*Standard deviation (ng/ml)*	*Coefficient of variation (%)*	*Deviation of $\bar{x}$ from theory (%)*
Present	*Found ($\bar{x}$)*			
0.0	0.0	0.0	0.0	0.0
55	61.6	4.2	6.8	+10.7
93	97.6	7.5	7.7	+ 4.7
184	183.3	15.3	8.3	− 0.4
245	259.3	11.0	4.3	+ 5.5
385	425.3	6.1	1.4	+10.0

ACKNOWLEDGEMENT

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1 J. M. Meola and M. Vanko, *Clin. Chem.*, 20 (1974) 184.
2 C. W. Gehrke and D. L. Stalling, *Separ. Sci.*, 2 (1967) 101.