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DETERMINATION OF BACLOFEN AND α-BACLOFEN IN RAT LIVER HOMOGENATE AND HUMAN URINE USING SOLID-PHASE EXTRACTION, *o*-PHTHALALDEHYDE–*tert.*-BUTYL THIOL DERIVATIZATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

Methods were developed for the determination of the zwitterionic compounds baclofen and α-baclofen in complex biological samples (rat liver homogenates and human urine) in concentration ranges that would be suitable for pharmacokinetic studies of these compounds. In the procedure, the biological samples along with an internal standard were selectively concentrated using C_{18} solid-phase extraction cartridges, evaporated, derivatized with *o*-phthalaldehyde and *tert.*-butyl thiol at room temperature for 2 min, then subjected to high-performance liquid chromatographic analysis. The reversed-phase (C_{18}) chromatographic analysis with amperometric detection (glassy carbon electrode in oxidative mode, +0.6 V vs. Ag/AgCl) was found to be useful for the measurement of both baclofen and α-baclofen from 10 ng/ml to 10 μg/ml in these complex biological samples. The primary advantage of the method was that the derivatives formed using *tert.*-butyl thiol were markedly more stable than the previously reported derivatives prepared using mercaptoethanol.

INTRODUCTION

Baclofen [4-amino-3-(*p*-chlorophenyl)butyric acid, Lioresal®] is a skeletal muscle relaxant used in the treatment of spasticity of spinal origin [1]. Baclofen has been assayed in pharmaceutical preparations by: colorimetry with salicylaldehyde [2] or ninhydrin derivatization [2,3]; fluorimetry with 4-chloro-7-nitrobenzofuran derivatization [4]; gas chromatography (GC) with bu-

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tanol–trifluoroacetic anhydride derivatization [2]; high-performance liquid chromatography (HPLC) with ultraviolet detection [2]; enantioselective HPLC–UV of esterified 3,5-dinitrobenzoyl derivative on a chiral column [5]; enantioselective HPLC–fluorimetry after *o*-phthalaldehyde (OPA)–N-acetylcysteine derivatization [6]; thin-layer chromatography [2,3]; and titrimetry [2].

Baclofen has also been assayed in human biological fluids (reported detection limits shown in parentheses); in plasma and cerebrospinal fluid by GC following derivatization (12 ng/ml) [7]; in plasma or urine by GC–electron-capture detection and butanol–heptafluoroimidazole (300 ng/ml) [8] or pentafluoropropionaldehyde (10 ng/ml) [9] derivatization; in breast milk [10] and in cerebrospinal fluid (5 ng/ml) [11] by GC–mass spectrometry; in plasma by solid-phase extraction followed by HPLC–UV (50 ng/ml) [12]; in cerebrospinal fluid, plasma and urine by enantioselective HPLC–UV of diastereomeric derivatives (50, 50, and 500 ng/ml, respectively) [13]; and in plasma or urine by HPLC–fluorimetry with OPA–thiol derivatization (5 ng/ml) [14].

The primary objective of this study was to develop a sensitive and specific assay method for baclofen and a synthetic analogue, α-baclofen, in liver homogenate supernatant and urine samples for use in drug metabolism studies. A previously described extraction method for plasma samples [12], using solid-phase columns prior to HPLC analysis, was modified for this study. An established method for dietary amino acid analysis using OPA–mercaptoethanol derivatization and fluorescence detection [15] was modified for the present study by utilizing OPA–*tert.*-butyl thiol derivatization (to potentially give better stability for the derivatives) and amperometric (AMP) detection.

EXPERIMENTAL

Chemicals and equipment

Baclofen was supplied by Ciba-Geigy (Summit, NJ, U.S.A.). Bromobaclofen [4-amino-3-(*p*-bromophenyl)butyric acid], a gift from Drs. W. Riess and G. Dorhofer of Ciba-Geigy (Basle, Switzerland), was used as an internal standard. The analogue, α-baclofen [4-amino-2-(*p*-chlorophenyl)butyric acid] was prepared by synthesis in these laboratories and will be the subject of a subsequent paper. Acetonitrile and methanol, HPLC grade, were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). OPA and *tert.*-butyl thiol (2-methyl-2-propanethiol) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Tris buffer [tris(hydroxymethyl)aminomethane] was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were reagent grade. The disposable, 1-ml capacity, 100-mg octadecylsilane-packed Bond-Elut® brand, solid-phase columns and Vac-Elut® vacuum chamber were obtained from Analytichem International (Harbor City, CA, U.S.A.).

The HPLC system consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model

6000A pump, Waters Assoc. Model U6K injector, and a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 3390A integrating recorder. The derivatives of baclofen, α-baclofen, and internal standard were separated using a 25 cm $\times$ 4 mm I.D. octadecyl reversed-phase, 10 μm particle size column (Whatman, Clifton, NJ, U.S.A., Partisil 10 ODS-3), a 6.8 cm $\times$ 2 mm I.D. C_{18}-packed guard column, a mobile phase of 58 to 62% (v/v) methanol in phosphate buffer (0.0095 *M* dipotassium hydrogenphosphate and 0.0154 *M* potassium dihydrogenphosphate in distilled water, observed pH 7) and a flow-rate of 1.5 ml/min. The AMP detector (Bioanalytical Systems, West Lafayette, IN, U.S.A., Model LC-3A amperometric detector), using a glassy carbon working electrode and a silver chloride reference electrode, was set on a range of 10 nA (for f.s.d.) for drug concentrations greater than 50 ng/ml and at 5 nA for drug concentrations less than 50 ng/ml; a potential setting of +0.6 V was used for all applications. A Waters Assoc. fluorescence detector, Model 420-AC, was evaluated but, ultimately, was not used for the standard assay procedure.

Biological samples

For development of the analytical procedure for urine, known quantities of baclofen, α-baclofen, and internal standard were added to freshly voided human urine. For development of the assay of the analytes in a liver homogenate sample, the following liver homogenate was prepared. A fresh rat liver was washed and homogenized with ice-cold 0.05 *M* Tris buffer pH 7.4 containing 0.25 *M* potassium chloride (1 g liver per 3 ml buffer) in a Potter-Elvehjem tube, transferred to polycarbonate tubes and centrifuged at 700 *g* for 10 min. The resultant supernatant was diluted with ice-cold cofactor solution containing 16 mg nicotinamide-adenine dinucleotide phosphate·4.5H_2O, 57.5 mg glucose-6-phosphate, and 20.3 mg anhydrous magnesium sulfate in 25 ml Tris–KCl buffer (1 ml homogenate per 3 ml cofactor). Known quantities of the analytes were added to this mixture.

Standard assay procedure

Baclofen, α-baclofen, and internal standard were extracted from liver homogenate supernatant or urine using solid-phase extraction columns in a modified procedure of Harrison et al. [12]. Solid-phase extraction columns packed with 100 mg of octadecylsilane were employed. Aqueous solutions were passed through these columns into 2-ml centrifuge tubes by suction from the vacuum chamber (ten-column capacity). The columns were placed in the holes in the cap of the chamber and samples were eluted through the stainless-steel needles of the cap into the tubes below when subjected to a vacuum between 3 and 6 kPa applied via a water aspirator. Though successive solutions were eluted completely from the columns, care was taken to not aspirate the columns to dryness for extended periods of time.

Typically, the columns were activated just prior to use by washing with 2 $\times$ 1

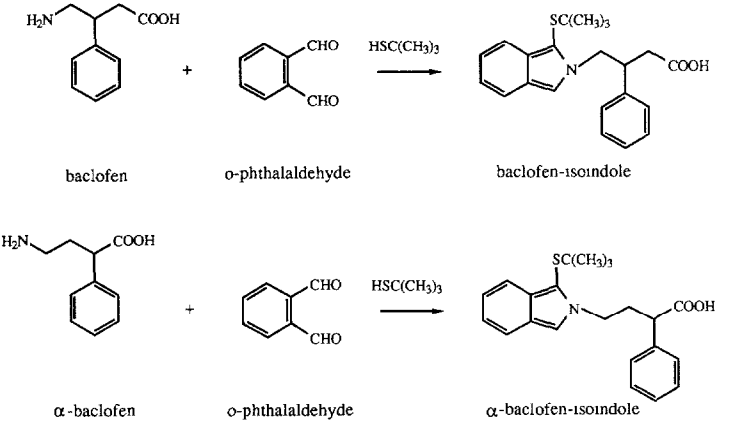

Fig. 1. Derivatization reaction between the amino acids, baclofen and α-baclofen, and *o*-phthalaldehyde–*tert.*-butyl thiol reagent.

ml of acetonitrile followed by 2×1 ml of 0.1% (v/v) orthophosphoric acid (85%) solution. Then, 1 ml of sample (liver homogenate supernatant or urine containing analytes) spiked with internal standard was passed through the column, followed by 1 ml of 0.1% orthophosphoric acid (85%) solution. After rinsing off the stainless-steel needles the analytes were eluted with 1.5 ml of 0.05 *M* potassium dihydrogenphosphate–acetonitrile (75:25, v/v). A 100-μl aliquot of this eluate was used for derivatization as described below.

The 'working OPA–*tert.*-butyl thiol reagent' was prepared fresh daily by mixing 75 mg OPA, 5.0 ml methanol, 50 μl *tert.*-butyl thiol, and 5.0 ml borate solution. The borate solution, 0.18 *M*, (observed pH 9.3), was prepared by dissolving 9.5 g sodium tetraborate decahydrate in 250 ml distilled water. For derivatization, a 100-μl test sample (column eluate described above) was treated with 25 μl working reagent and injected into the HPLC–AMP system within 1–2 min. The derivatization reaction for baclofen and α-baclofen is shown in Fig. 1.

Calibration

A calibration sample of 1 μg per 1.5 ml baclofen, α-baclofen, and the internal standard was prepared fresh daily using distilled water. To prepare the calibration sample, 100 μl of a 10 μg/ml stock solution of each compound in distilled water was placed in a vial and diluted to 1.5 ml with distilled water. A 100-μl aliquot of the calibration sample was placed in a vial with 25 μl working reagent, and 50 μl of this solution were injected. The calibration sample gave a 50–75% response on the AMP detector. If less response was obtained, the glassy carbon electrode was polished (about once every three or four weeks). A typical chromatogram showing the derivatives of baclofen, α-baclofen, and internal standard in a calibration sample appears in Fig. 2.

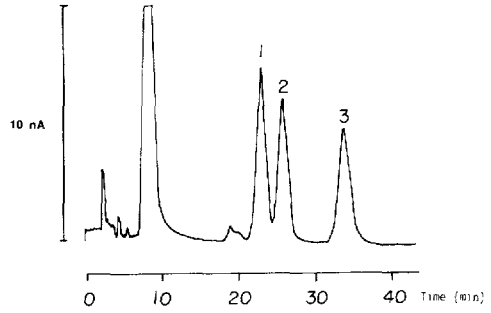

Fig. 2. Chromatogram of derivatives of baclofen (1), internal standard (2), and α-baclofen (3) (1.0 μg per 1.5 ml each) in distilled water. Mobile phase, 62% (v/v) methanol–0.1 *M* phosphate buffer. AMP detector, +0.6 V vs. Ag/AgCl, 10 nA full scale.

RESULTS AND DISCUSSION

Chromatograms of the derivatives of baclofen, α-baclofen, and the internal standard representing drug concentrations of 1 μg/ml in liver homogenate (Fig. 3A) and urine (Fig. 4B) were found to be free from interfering peaks in the corresponding blank samples of liver homogenate supernatant (Fig. 3B) and urine (Fig. 4C) which were derivatized in the same manner. Retention times for the derivatives of baclofen, α-baclofen, and the internal standard were 22, 25 and 32 min, respectively, using a mobile phase composition of 62% (v/v) methanol–0.1 *M* phosphate buffer.

The choice of derivatization with *tert.*-butyl thiol instead of mercaptoethanol was made for two reasons. Jacobs [16] has reported that the *tert.*-butyl thiol derivatives of a variety of amino acids were stable for several hours or more while the derivatives produced with mercaptoethanol were stable for only a few minutes. Secondly, *tert.*-butyl thiol derivatives were reported to yield greater AMP response than the corresponding mercaptoethanol derivatives [16]. Following the selection of *tert.*-butyl thiol as the optimal reagent, preliminary studies in these laboratories showed that AMP detection yielded five times greater sensitivity for *tert.*-butyl thiol derivatives of baclofen and α-baclofen and better signal-to-noise ratios as compared to fluorescence (AMP signal-to-noise ratio = 46; fluorescence signal-to-noise ratio = 9.7 for a 1 μg/ml water standard).

In most of the reported OPA derivatization procedures, large excesses of both OPA and *tert.*-butyl thiol were employed with little interference from the excess reagent. However, in the present study, it was found that the retention times for the derivatives were very close to the retention time of the OPA–thiol reagent peak (18 min, Fig. 3B). Consequently, much greater care had to be used in selecting the amount of derivatization reagent. Too little OPA and/or *tert.*-butyl thiol in the reagent gave variable analyte peaks due to exhaustion

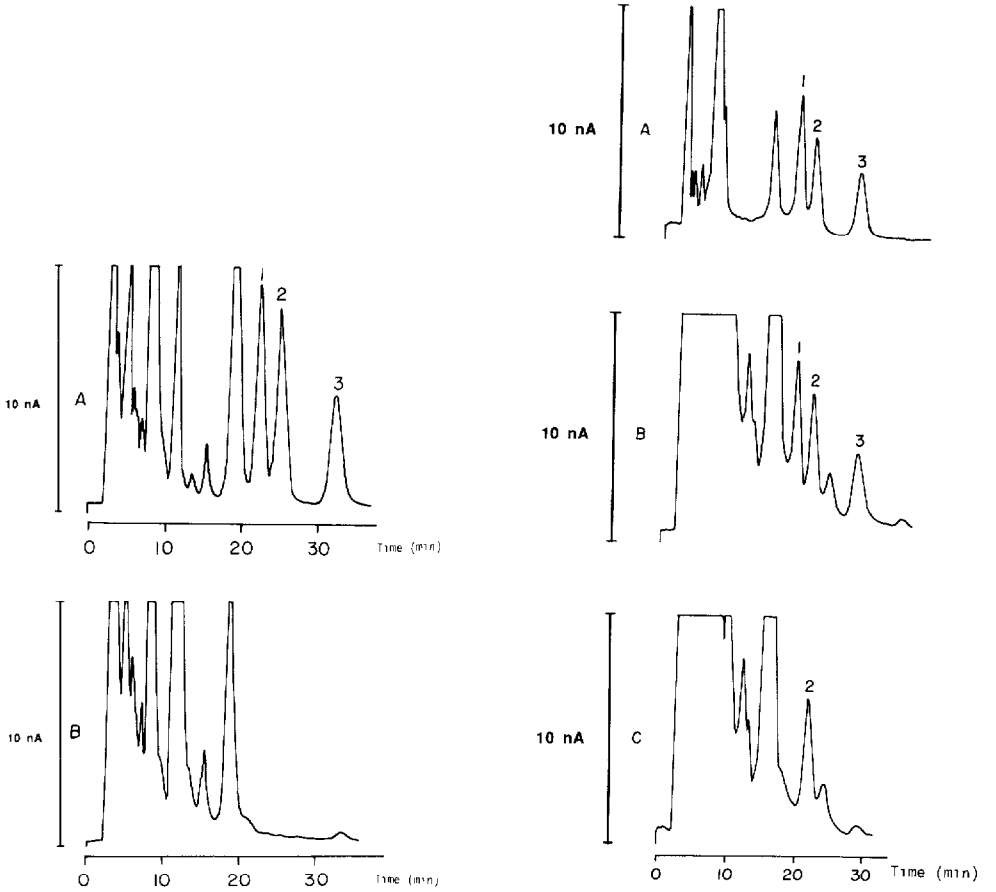

Fig. 3. (A) Chromatogram of baclofen (1), internal standard (2), and α-baclofen (3) in liver homogenate (1.0 μg/ml each). (B) Chromatogram of liver homogenate negative control. AMP detector, +0.6 V vs. Ag/AgCl, 10 nA full scale.

Fig. 4. (A) Chromatogram of baclofen (1), internal standard (2), and α-baclofen (3) in water (1.0 μg per 1.5 ml). (B) Chromatogram of baclofen (1), internal standard (2), and α-baclofen (3) in human urine (1.0 μg/ml). (C) Chromatogram of urine negative control containing internal standard (2) (1.0 μg/ml). AMP detector, +0.6 V vs. Ag/AgCl, 10 nA full scale.

of reagent by reaction with natural constituents in the biological samples. An excess of OPA and *tert.*-butyl thiol was desired to compensate for the natural interferences of the biological samples, but low enough to avoid a large reagent peak at 18 min (Fig. 3B). To optimize the concentrations of OPA and *tert.*-butyl thiol in the working reagent, a series of experiments were performed. First, the AMP response to derivatives of baclofen, α-baclofen, and internal standard in a liver homogenate extract was monitored at various concentrations of OPA (10–100 mg per 10 ml reagent solution) while holding the *tert.*-

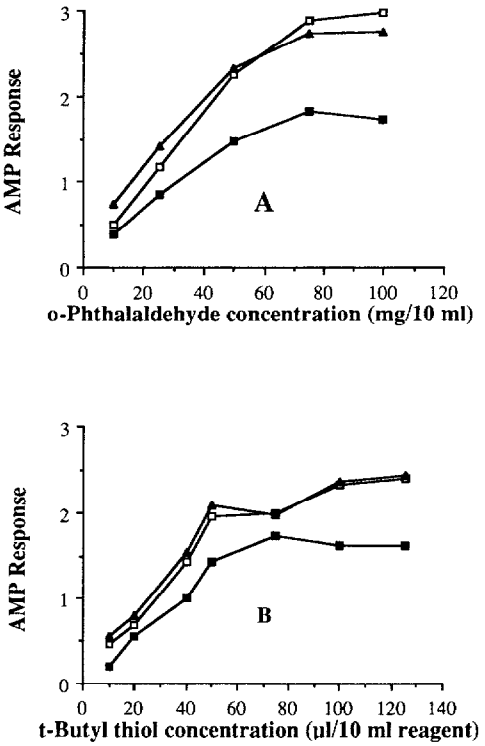

Fig. 5. (A) Optimization of *o*-phthalaldehyde concentration in reagent solution. (B) Optimization of *tert.*-butyl thiol concentration in reagent solution. Baclofen (□), α-baclofen (■), internal standard (▲) all at 1.0 µg/ml in a liver homogenate extract.

butyl thiol level constant (25 µl) (Fig. 5A). Secondly, the AMP response was monitored at various concentrations of *tert.*-butyl thiol (10–125 µl per 10 ml reagent solution) while holding the OPA level constant (75 mg per 10 ml reagent solution (Fig. 5B). From these experiments, the reagent composition of 75 mg OPA and 50 µl pure, liquid *tert.*-butyl thiol in 10 ml methanol–0.18 *M* borate solution (1:1, v/v), as described in the standard assay procedure, was established.

Upon addition of the reagent, the solutions remained clear and colorless (no visible change occurred) for several hours, after which they decomposed, turning an olive-green color. To determine the half-lives of these derivatives, a solution of the three analytes in water was derivatized and repeatedly analyzed by HPLC–AMP over a period of several hours to observe the AMP response. Half-lives were determined to be 4.6 h for the baclofen derivative, 2.7 h for the α-baclofen derivative, and 5.2 h for the derivative of the internal standard (Fig. 6).

Absolute recoveries of baclofen, α-baclofen, and internal standard from liver homogenate were determined by comparing AMP peak areas of reference stan-

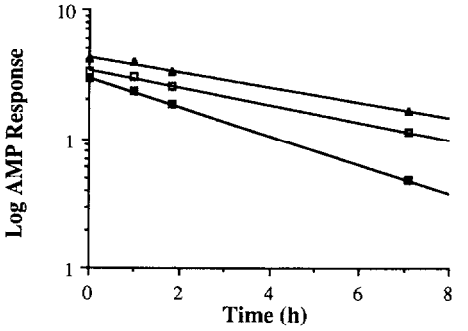

Fig. 6. Half-lives of derivatized baclofen (□), α-baclofen (■), and internal standard (▲).

TABLE I

ABSOLUTE EXTRACTION RECOVERIES FOR BACLOFEN, α-BACLOFEN AND INTERNAL STANDARD FROM LIVER HOMOGENATE

Concentration (μg/ml)	Recovery[a] (%)		
	Baclofen	α-Baclofen	Internal standard
0.1	67.5	85.4	61.5
0.5	99.1	106.3	79.9
1.0	86.4	90.8	89.7
5.0	77.8	84.9	79.3
10.0	67.5	77.6	75.8
Mean	79.7	88.9	77.3
S.D.	12.0	9.6	9.1
C.V. (%)	15.0	10.8	11.8
n	5	5	5

[a]Average recovery for the extraction of three separate liver homogenates.

dards in water to the peak areas of spiked biological samples subjected to extraction followed by derivatization. It was found that the wash and elution steps were critical to recovery. Harrison et al. [12] utilized a 0.5-ml wash (same dilute phosphoric acid solution) and a 0.3-ml elution (same phosphate buffer–acetonitrile) to obtain an average 42% recovery. In this study, recoveries were improved over two-fold by using 1-ml wash and 1.5-ml elution steps. Mean recoveries over the concentration range 0.1–10 μg/ml for baclofen, α-baclofen, and internal standard ranged from 77.3 to 88.9% (Table I).

Accuracy and precision

In order to determine the precision of the measurements resulting from the derivatization and chromatographic steps (without the additional errors of the

extraction process), replicate samples of baclofen and α-baclofen were spiked with the internal standard, derivatized, then chromatographed. The results of these preliminary experiments (Table II) indicated that data scatter associated with the derivatization and peak measurements were relatively small (coefficient of variation (C.V.) = 0.36 and 0.91% for the two drugs).

For the evaluation of the precision and accuracy of the assay of the biological samples, two types of reference standards containing baclofen, α-baclofen, and internal standard were initially investigated: (1) a simple standard prepared in water and (2) a spiked liver blank. In the first case, analytes in distilled water (1 μg per 1.5 ml) were derivatized (no extraction) and assayed. In the second case, blank liver homogenate (containing no analytes) was solid-phase-extracted, spiked with the analytes (1 μg per 1.5 ml), derivatized, and assayed. Since the ratios of both baclofen/internal standard and α-baclofen/internal standard prepared by method 1 were within 7% of the ratios obtained using standards prepared by method 2, determined for both types of standards, subsequent evaluations of the accuracy and precision of the method were conducted using the simple aqueous reference standards.

To determine the accuracy and precision, three liver samples were run through the standard assay procedure. Liver homogenate samples containing baclofen and α-baclofen at 1.0 μg/ml were spiked with the internal standard, solid-phase-extracted, derivatized, and assayed. The results of replicate runs (Table III) showed that the calculated amount of the two solutes were slightly lower than the true values, but the accuracy would be satisfactory for most pharmacokinetic studies. The intra-assay coefficient of variation for baclofen was found to be 4.7 and 4.9% for α-baclofen at 1.0 μg/ml.

TABLE II

INTRA-ASSAY PRECISION OF THE ANALYSIS OF REPLICATE SAMPLES PREPARED IN WATER

Sample	Sample No.	Mean peak-height ratio		Mean peak-area ratio	
		Baclofen/I.S.[a]	α-Baclofen/I.S.	Baclofen/I.S.	α-Baclofen/I.S.
Water standard[b]	1	1.0792	0.5782	0.9380	0.6947
	2	1.0803	0.5645	0.9345	0.6731
	3	1.0723	0.5759	0.9305	0.6847
	4	1.0718	0.5743	0.9295	0.6816
Mean		1.0759	0.5732	0.9331	0.6835
S.D.		0.0039	0.0052	0.0034	0.0077
C.V. (%)		0.36	0.91	0.36	1.13

[a]I.S. = internal standard.

[b]Baclofen and α-baclofen in distilled water (1 μg per 1.5 ml), no extraction prior to derivatization.

TABLE III

ACCURACY AND PRECISION OF THE ANALYSIS OF REPLICATE LIVER HOMOGENATE SAMPLES

Sample No.[a]	Concentration (μg/ml)	
	Baclofen	α-Baclofen
1	0.960	1.028
2	0.964	0.925
3	0.869	0.939
Mean	0.931	0.934
Error (%)	−6.9	−6.7
S.D.	0.044	0.046
C.V. (%)	4.7	4.9

[a]Liver homogenate containing baclofen and α-baclofen (1 μg/ml) run through the standard assay procedure. The method was calibrated using simple aqueous standards of baclofen, α-baclofen, and the internal standard in water with no extraction of the calibration samples.

Dynamic range and detection limits

To determine accuracy over a wide range of analyte concentrations, spiked liver homogenate samples containing various quantities of baclofen and α-baclofen were run through the standard assay procedure. Samples containing 0.1, 0.5, 1, 5, and 10 μg/ml baclofen and α-baclofen in liver homogenate were prepared. Each sample was spiked with the internal standard at 1 μg/ml (100 μl of a 10 μg/ml stock solution) prior to extraction. The samples were solid-phase-extracted, derivatized, and injected onto the HPLC–AMP system. Analytes were quantitated using the formula:

$$\text{concentration analyte} = \text{concentration standard} \times \frac{\text{peak-area ratio of analyte/internal standard in liver sample}}{\text{peak-area ratio of analyte/internal standard in calibration sample}}$$

The method was found to be linear over this concentration range (Table IV). Standard curve regression analyses were determined: concentration of baclofen (μg/ml) = 0.627 (baclofen peak area/internal standard peak area) + 0.377, $r = 0.97$ ($n = 5$); concentration of α-baclofen (μg/ml) = 0.684 (α-baclofen peak area/internal standard peak area) + 0.180, $r = 0.98$ ($n = 5$).

To determine the sensitivity of the method, samples were spiked with varying concentrations of baclofen and α-baclofen, extracted, derivatized, chromatographed, and then the signal-to-noise ratio of each of the peaks was determined. The observed signal-to-noise ratio versus concentration curves were extrapolated to a signal-to-noise ratio of 3.0 giving detection limits of 3.8 ng/ml for baclofen and 6.0 ng/ml for α-baclofen. The detection limit was not

TABLE IV

DYNAMIC RANGE OF THE STANDARD ASSAY PROCEDURE AS APPLIED TO LIVER HOMOGENATE SAMPLES

A single sample was prepared for each concentration. Values in parentheses are percentage error.

Actual concentration (μg/ml)	Observed concentration (μg/ml)	
	Baclofen	α-Baclofen
0.1	0.0702 (−29)	0.118 (18)
0.5	0.545 (9)	0.483 (−3)
1.0	1.049 (5)	1.030 (3)
5.0	4.963 (−1)	6.713 (34)
10.0	6.53[b] (−34)[a]	9.587 (−4)
Mean error[b] (%)	16	12

[a]A large baclofen peak carries the I.S. peak as a shoulder and complicates integration.
[b]Mean of |observed − true| × 100.

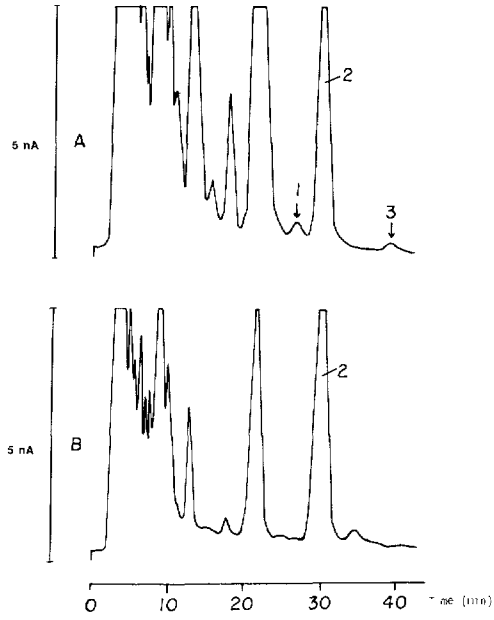

Fig. 7. Detection limits of the HPLC-AMP method. (A) Liver homogenate spiked with 10 ng/ml baclofen (1) and 10 ng/ml α-baclofen (3) with 1.0 μg/ml internal standard (2). (B) Liver homogenate containing only 1.0 μg/ml internal standard (2). AMP detector, +0.6 V vs. Ag/AgCl, 5 nA full scale.

usually governed simply by the signal-to-noise ratio, but rather by the proximity of the reagent peak [at 18 min using 62% (v/v) methanol–0.1 *M* phosphate buffer] which interfered with the baclofen derivative at these low levels (10 ng/ml baclofen and α-baclofen shown in Fig. 7). To minimize this interference, the detection of levels less than 50 ng/ml was performed using a solvent composition of 58–60% (v/v) methanol–0.1 *M* phosphate buffer while 60–62% methanol was used for levels greater than 50 ng/ml (adjustment of mobile phase was necessary due to aging of the column).

CONCLUSION

It was found that OPA and *tert.*-butyl thiol afforded derivatives of baclofen, α-baclofen, and the internal standard that were stable for several hours which was considerably more stable than the mercaptoethanol type of derivative that was previously reported for baclofen (stable only for several minutes). Also for these *tert*-butyl thiol derivatives, AMP detection gave much better signal-to-noise ratios than those obtained with the fluorescence method of detection, which has been commonly utilized.

A solid-phase extraction procedure was developed for these polar, amphoteric compounds that gave nearly quantitative recovery from complex biological samples. After optimization of the OPA and *tert.*-butyl thiol reagent concentrations to compensate for the side-reactions that occur in these biological samples, concentrations of baclofen and α-baclofen in the low ng/ml range in biological samples could be measured (3.8 ng/ml baclofen and 6.0 ng/ml α-baclofen at a signal-to-noise ratio of 3.0) and the method was found to give good linearity ($r=0.97$ and 0.98) up to 10 μg/ml.

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