Journal of Chromatography, 420 (1987) 212–216
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3740

Note

Rapid simultaneous determination of baclofen and its γ-hydroxy metabolite in urine by high-performance liquid chromatography with ultraviolet detection

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(First received February 16th, 1987; revised manuscript received April 3rd, 1987)

We have previously developed a method for the determination of the skeletal muscle relaxant baclofen (Fig. 1) in plasma and urine by reversed-phase high-performance liquid chromatography (HPLC) with fluorimetric detection [1]. This assay required cation-exchange extraction, pre-column derivatisation with *o*-phthaldialdehyde (OPA) and on-column concentration. Although many samples have since been measured in this way, a simpler method was needed in order to speed up the analysis. A rapid HPLC assay for baclofen in plasma was developed by Harrison et al. [2]. This paper describes a rapid reversed-phase HPLC method with UV detection for the determination of baclofen in urine. With this method it is also possible to measure the γ-hydroxy metabolite of baclofen (Fig. 1). No separation of the enantiomers is obtained, however. Recently, we published some preliminary results of an enantioselective assay [3].

EXPERIMENTAL

Chemicals

Baclofen and its γ-hydroxy metabolite (sodium salt) were kindly supplied by Ciba-Geigy (Basle, Switzerland). β-Glucuronidase was obtained from Sigma (St. Louis, MO, U.S.A.). Hydrochloric acid, sodium hydroxide, sodium acetate, acetic acid, methanol and tetrahydrofuran were of analytical-reagent grade (Merck, Darmstadt, F.R.G.) and were used without further purification.

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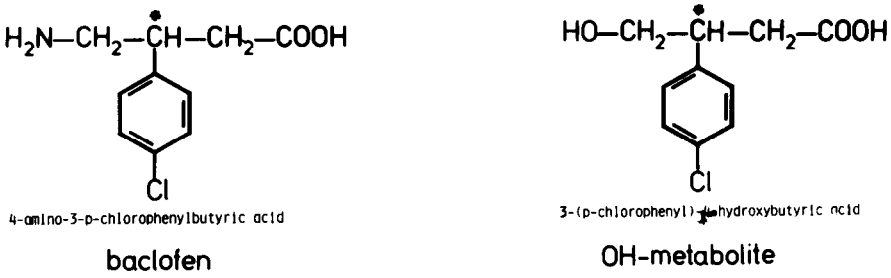

Fig. 1. Structures of baclofen and its γ-hydroxy metabolite.

Apparatus

A Model 9208 liquid chromatograph (Kipp Analytica, Delft, The Netherlands) with a variable-wavelength detector (Schoeffel SF 770 Spectroflow, GM 770 Monochromator; Kratos, Rotterdam, The Netherlands) was used. The analytical column (25 cm×4.6 mm I.D.) was packed with Cp-Spher C_8 reversed-phase material, particle size 8 μm (Chrompack, Middelburg, The Netherlands). A guard column (7.5 cm×2.1 mm I.D.) (Chrompack) was filled with pellicular reversed-phase material (Chrompack).

Procedure

Urine samples were diluted 1:3 with water and subsequently introduced into the HPLC system by means of a loop (50 μl). The mobile phase consisted of methanol–tetrahydrofuran–sodium acetate (0.02 *M*) (10:5:85, v/v/v). The flow-rate was 1 ml/min on average (pressure approximately 10 MPa). The substances were detected at 220 nm, and the peak heights were measured.

Deglucuronidation of urine samples was performed in three ways: enzymatically with β-glucuronidase and hydrolytic with hydrochloric acid or sodium hydroxide. In the enzymatic procedure, urine (0.3 ml) was incubated for 18 h at 37°C with 25 μl of β-glucuronidase (5000 U/ml) and 0.3 ml of a 0.2 *M* acetate buffer (pH 5.0). In the hydrolytic procedure, (a) urine (0.3 ml) was incubated for 2 h with 0.1 ml of 1 *M* hydrochloric acid at 95°C, then neutralised with 1 *M* sodium hydroxide, or (b) urine (0.3 ml) was incubated for 1 h with 0.1 ml of 1 *M* sodium hydroxide at 50°C, then neutralised with 1 *M* hydrochloric acid.

Urine samples

Baclofen was administered to dogs and human volunteers in different dosages. Urine was collected and stored at −20°C until analysis. More detailed results will be published elswhere.

Dog experiment with the metabolite

A female beagle dog of 11 kg body weight was anaesthetised with pentobarbitone sodium (30 mg/kg) and subsequently given 2 mg/kg γ-hydroxy metabolite as an intravenous (i.v.) infusion in 3 h. Urine samples were collected by means of a catheter for the first 7 h and subsequently spontaneously voided urine was used.

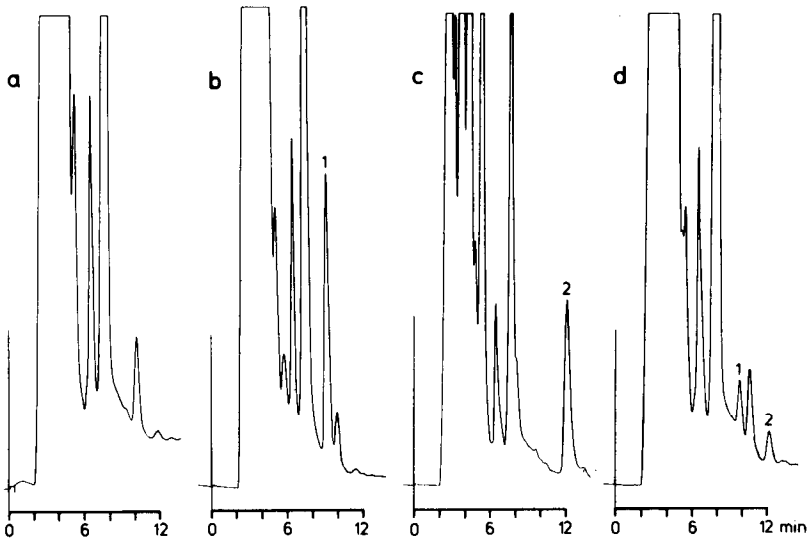

Fig. 2. Chromatograms of baclofen and its γ-hydroxy metabolite in urine. (a) Urine blank; (b) urine sample from a dog administered the γ-hydroxy metabolite i.v., containing 150 mg/l γ-hydroxy metabolite; (c) urine sample from a dog administered baclofen i.v. containing 84 mg/l baclofen; (d) urine spiked with 10.5 mg/l γ-hydroxy metabolite and 11.0 mg/l baclofen. Peaks: 1 = metabolite; 2 = baclofen.

RESULTS AND DISCUSSION

Typical chromatograms of baclofen and its γ-hydroxy metabolite are shown in Fig. 2. A blank dog urine did not show interfering substances (Fig. 2a). However, depending on the nature of the samples, small day-to-day variations in the mobile phase and flow-rate may be necessary. Urine samples from a dog after i.v. administration of baclofen (Fig. 2c) and after i.v. administration of its γ-hydroxy metabolite (Fig. 2b) are shown. The detection limit in spiked urine was approximately 50 ng for both baclofen and its metabolite. The capacity ratios (k') were 3.5 and 4.5 for the metabolite and baclofen, respectively. For both substances calibration graphs of blank urine, spiked with 4.0–100 mg/l and measured after diluting 1:3 with water, showed good linearity between peak height and concentration ($r^2 > 0.99$). The precision of the determinations in water and in urine was measured for three different concentrations within the same range ($n = 4$). The coefficients of variation were always less than 4% for both baclofen and its metabolite. Comparison of the direct UV method with the *o*-phthaldialdehyde (OPA) fluorimetric determination for baclofen showed a good correlation (Fig. 3). The correlation coefficient (r^2) based on fourteen samples was 0.99.

According to Faigle et al. [4], following the administration of ^{14}C-labelled baclofen 80–90% of the ^{14}C dose was excreted in the urine in the dog and in man. In man 90% of the total radioactivity was accounted for by the unchanged drug and 7% by 3-(*p*-chlorophenyl)-4-hydroxybutyric acid, the γ-hydroxy metabolite (Fig. 1). In the dog these values were 63 and 8%, respectively. In our experiments with unlabelled baclofen the recovery of the unchanged drug in the urine was

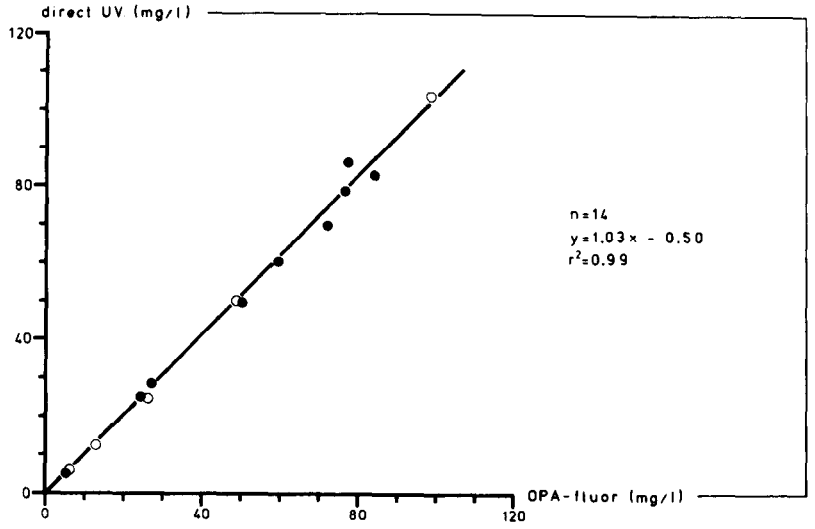

Fig. 3. Correlation of the direct UV method with the OPA fluorimetric method for the determination of baclofen in urine. o, Spiked samples; •, samples from dog urine following i.v. administration of baclofen.

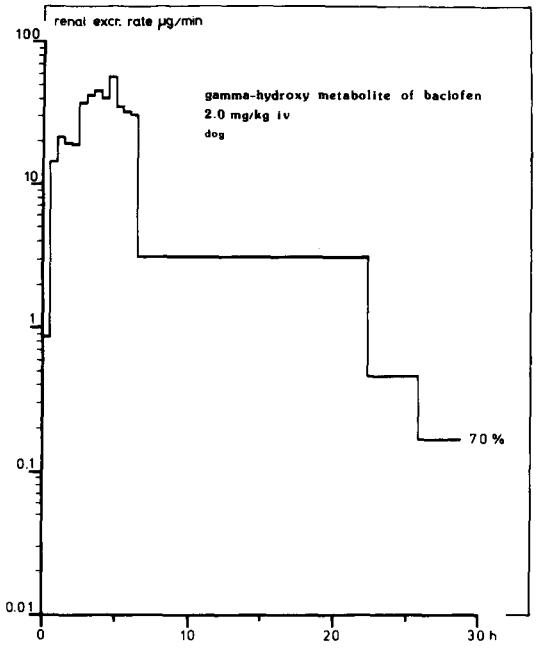

Fig. 4. Renal excretion rate-time profile of the γ-hydroxy metabolite of baclofen, administered as the parent compound to a dog. Dose 2 mg/kg as i.v. infusion. % = percentage of the dose excreted unchanged in the urine.

variable both in man and in the dog, with values ranging from 40 to 90% of the dose administered.

With the direct UV method the urine samples could also be screened for the presence of the γ-hydroxy metabolite. The earlier described OPA fluorimetric method could not be used as the metabolite lacks an NH_2 group, which is needed for derivatisation. As theoretically the metabolite (and also baclofen itself) could have been present as the corresponding glucuronide, the samples were analysed prior to and after enzymatic and hydrolytic deglucuronidation.

In urine samples from both dogs and human volunteers following i.v. administration of baclofen, however, no metabolite was found. Given the detection limit of the method, this means that in 48 h, depending on the urinary flow-rate, 2–10% of a dose at the most could have been present as the γ-hydroxy metabolite, without having been detected. No glucuronides were found. Because of the urinary pH values (5–9) it is unlikely that the γ-hydroxy metabolite in the urine samples analysed would have been present as the corresponding lactone, which is not detected with this method.

When the γ-hydroxy metabolite was administered to a dog as the parent compound, 70% was recovered unchanged in the urine in 30 h (Fig. 4). This means that the described assay is suitable for the measurement of the γ-hydroxy compound if present in sufficiently high concentrations. Apparently, the incomplete mass balance of baclofen is mainly due to other causes.

With this simple reversed-phase HPLC method, baclofen can be measured in urine samples. For low concentrations (less than 4 mg/l), the more lengthy OPA fluorimetric method should be used. The γ-hydroxy metabolite can also be measured in urine by the direct UV method if present at concentrations higher than 4 mg/l. For separation of the enantiomers other methods are required.

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