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Direct high-performance liquid chromatography determination of diastereomeric oxprenolol glucuronides

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Abstract

The β-blocking agent oxprenolol is used therapeutically as the racemate. In humans and animals it is metabolized i.a. to ether glucuronide diastereomers. A stereoselective HPLC assay was developed to determine directly, without hydrolysis to their parent enantiomers, the oxprenolol glucuronides in biological samples. The glucuronide standards for this direct assay are prepared by incubation of rabbit liver microsomes with *RS*-oxprenolol. The glucuronides obtained are purified and concentrated with solid-phase extraction, and their concentration is measured by an indirect method, i.e. HPLC assay of the oxprenolol enantiomers after enzymatic hydrolysis with β-glucuronidase. The direct assay involves separation by HPLC using a C_{18}-reversed-phase column, with UV detection at 274 nm; nalorphine is used as internal standard. On injection onto the column, without previous hydrolysis, the limit of detection is 20 ng for both glucuronides. The assay is sensitive, accurate and reproducible. The method is suitable for the assay of glucuronides in liver microsomal incubates and plasma.

Keywords: Oxprenolol; Oxprenolol glucuronides; enantioselectivity

1. Introduction

The non-selective β-blocking agent oxprenolol is used therapeutically as the racemate. In humans and animals it is metabolized i.a. to ether glucuronide diastereomers [1–3]. There are marked pharmacodynamic and pharmacokinetic differences between the enantiomers [2–4], and we found also a marked enantioselectivity of the renal excretion of the oxprenolol glucuronides [3]. Multiple reverse isotope dilution and HPLC have been used for the assay of the oxprenolol enantiomers [2,5,6] and their oxidative metabolites [2,5]. The oxprenolol glucuronides can be assayed after enzymatic hydrolysis to their parent enantiomers [2,3], but for further study of the glucuronidation process, e.g. in liver microsomal incubates, a direct assay is needed. Such an assay has not been described, and we, therefore, developed a HPLC method for the direct determination of the oxprenolol glucuronide diastereomers.

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2. Experimental

2.1. Materials

RS-Oxprenolol-HCl, Brij-58, bovine serum albumin, β-glucuronidase and uridine 5′-diphosphoglucuronic acid (UDPGA) were purchased from Sigma (St. Louis, MO, USA). *R*(+)- and *S*(−)-oxprenolol were gifts from Ciba-Geigy (Basel, Switzerland). Tris and perchloric acid were obtained from Merck (Darmstadt, Germany). Acetonitrile, dichloromethane and methanol were of HPLC grade and

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were purchased from Labscan (Dublin, Ireland). All other solvents were of analytical grade.

2.2. Chromatographic conditions

The HPLC system consisted of a Varian 9010 solvent delivery system (Varian, Sunnyvale, CA, USA), an automatic injector with a 100-μl loop (Merck AS 2000 A, Darmstadt, Germany), a Model 484 tunable UV detector (Waters, Milford, MA, USA) and a HP 3395 A integrator (Hewlett Packard, Avondale, PA, USA). The oxprenolol glucuronides and the internal standard were separated using a 25 cm×4 mm Lichrospher C_{18} column (Merck, Darmstadt, Germany) with a 5-μm particle size. A mixture of phosphate buffer (0.1 *M*, pH 6.6)–acetonitrile–tetrahydrofuran (83:13:4, v/v) was used as mobile phase at a flow-rate of 1.0 ml/min. Detection was performed by UV absorption at 274 nm. The maximum wavelengths (273 and 275 for *R*- and *S*-oxprenolol glucuronide, respectively) were established using a diode-array detector (Perkin Elmer LC 235, Norwalk, CT, USA). Nalorphine was used as internal standard. Valproic acid, morphine-3-glucuronide, morphine-6-glucuronide and phenobarbital were also tested as internal standards, but were found to be less suitable because of their long elution times.

2.3. Standards

The standard solution of the oxprenolol glucuronides is obtained by incubation of rabbit hepatic microsomes with *RS*-oxprenolol. The liver microsomes of male rabbits were prepared according to Amar-Costesec et al. [7], and stored at −80°C. Protein content was determined by the method of Lowry [8], with bovine serum albumin as standard. The microsomal incubation mixture (250 μl) contained 8 mg/ml protein, 6 m*M* *RS*-oxprenolol, 50 m*M* Tris buffer (pH 7.4) and 5 m*M* $MgCl_2$. The mixture was preincubated for 2 min at 37°C, and the reaction was started by addition of 1 μmol UDPGA. After incubation for 3 h at 37°C, the reaction was stopped by addition of 25 μl 4 *M* $HClO_4$. After centrifugation at 16 000 *g*, 250 μl of the supernatant was alkalinized with sodium hydroxide, and excess oxprenolol was extracted with 5 ml dichloromethane. The aqueous layer was buffered (Tris buffer, 0.5 *M*, pH 7.4) and applied to Bakerbond C_{18} cartridges which had been conditioned with methanol (3×2 ml), acetonitrile–phosphate buffer (25:75, pH 2.1, 3×2 ml) and water (3×2 ml). After application of the samples, the cartridges were washed with 3 ml buffer (5 m*M* ammonium sulphate buffer, pH 5.9) and 3 ml water. Finally, the glucuronides were eluted with 2 ml 2% ammonia solution in methanol, and pooled. The resulting solution was evaporated partially at room temperature under nitrogen.

The glucuronide concentrations in the stock solution were determined after enzymatic hydrolysis with β-glucuronidase as described previously [3]: 10 μl of a 1/10 dilution of the stock solution was incubated with 250 units of β-glucuronidase. After hydrolysis (24 h, 37°C) the oxprenolol enantiomers were determined by HPLC as described previously [3]. The results of five incubations with β-glucuronidase were averaged. This stock solution contained 14.0 μg/ml *R*- and 17.5 μg/ml *S*-glucuronide and was stored at −20°C.

2.4. Calibration curves, accuracy and precision

The calibration curves were constructed by spiking blank biological samples (microsomal suspensions or plasma) with oxprenolol glucuronide standards as described (see above). Accuracy and precision were determined for microsomal samples: for each glucuronide a quality control sample (3.0 μg *R*- and 3.7 μg *S*-glucuronide) was analyzed in fivefold with a calibration curve consisting of seven standards (1.0 to 6.0 μg *R*- and 1.2 to 7.5 μg *S*-glucuronide). The samples were analyzed after addition of the internal standard (5 μg) and peak-height ratios were used to construct the calibration curve. The calibration curve was tested for linearity over the concentration range used.

2.5. Application of the assay

The method was applied to determine the in vitro formation rates of the glucuronides in rabbit liver microsomes, and the glucuronide concentrations in rabbit plasma.

The liver microsomes were incubated in the

presence of increasing concentrations (0–6 m*M*) of *RS*-oxprenolol. The incubation mixture contained 4 mg/ml protein, 4 mg/ml Brij, 5 m*M* $MgCl_2$, 50 m*M* Tris buffer and 5 m*M* UDPGA. After incubation for 40 min in a shaking waterbath at 37°C, 5 μg internal standard and 25 μl 4 *M* $HClO_4$ were added. The precipitated proteins were removed by centrifugation at 16 000 *g* for 10 min. The supernatant was alkalinized with NaOH, and excess oxprenolol was extracted with dichloromethane. The aqueous layer was adjusted to pH 7.0 before injection into the HPLC system.

Plasma concentrations of oxprenolol glucuronides were determined at different times after oral administration of 50 mg/kg *RS*- oxprenolol to a rabbit. The samples were stored at −20°C until assay. The plasma samples were analyzed twice, once after enzymatic hydrolysis of the samples [3], and once directly, without hydrolysis. Plasma samples (500 μl) were deproteinized with 50 μl $HClO_4$ (4 *M*) after addition of 5 μg of the internal standard nalorphine. The precipitated proteins were removed by centrifugation at 16 000 *g* for 10 min and the supernatant was alkalinized with 50 μl NaOH (5 *M*). After extraction of the unchanged oxprenolol enantiomers with dichloromethane, the aqueous layer was adjusted to pH 7 with phosphoric acid, and 50-μl aliquots were injected into the HPLC system.

3. Results and discussion

3.1. Chromatography

Fig. 1 shows representative chromatograms obtained after incubation of rabbit liver microsomes with *RS*-oxprenolol. In the presence of UDPGA two peaks are apparent, which are not present when UDPGA is omitted. These peaks correspond to the glucuronides, and this is confirmed by their disappearance after incubation with β-glucuronidase (results not shown). The elution order of the enantiomers was checked by incubation experiments with pure *R*- and *S*-oxprenolol. The capacity factors for *R*- and *S*-oxprenolol glucuronide and for the internal standard nalorphine were 4.6, 6.3 and 10.8, respectively.

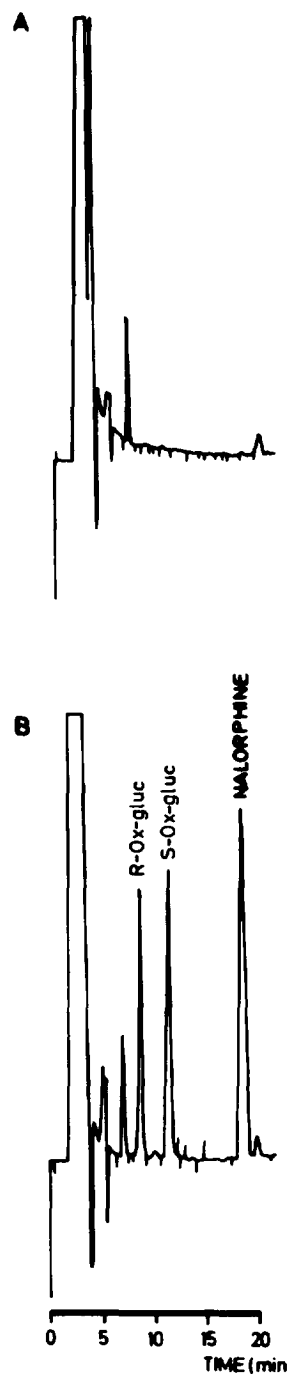

Fig. 1. Representative chromatograms obtained after incubation of rabbit liver microsomes with *RS*-oxprenolol. The microsomal suspensions are incubated with *RS*-oxprenolol for 40 min in the absence (A), and in the presence (B) of UDPGA.

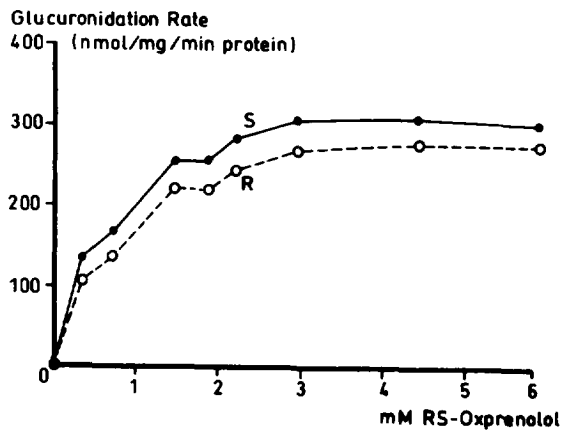

Fig. 2. Formation rates of *R*- and *S*-oxprenolol glucuronide after incubation during 40 min of a rabbit liver microsome preparation in the presence of UDPGA, as a function of the concentration of *RS*-oxprenolol.

3.2. Detection limit

The limit of detection of the assay, defined as a signal-to-noise ratio of 3:1, was for both diastereomers 20 ng.

3.3. Calibration curves, accuracy and precision

The calibration curves in microsomes were linear in the concentration range from 0.5 to 6 μg *R*-glucuronide and from 0.6 to 7.5 μg *S*-glucuronide. The intra-day coefficients of variation were 6.2 and 7.4%, and the deviations of nominal value were 9.0 and 8.8% for *R*- and *S*-glucuronide, respectively.

3.4. Application of the assay

Fig. 2 shows the formation rate of both glucuronides after incubation of rabbit liver microsomes with increasing concentrations of *RS*-oxprenolol for 40 min in the presence of UDPGA. Glucuronidation followed Michaelis-Menten kinetics. The formation rates were slightly stereoselective in favour of *S*-oxprenolol glucuronide (*S*/*R* ratio: 1.23).

The plasma concentrations obtained after oral administration of *RS*-oxprenolol to a rabbit were measured with the direct method as well as after enzymatic hydrolysis (Fig. 3). For both glucuronides, the correlation coefficient between the results obtained with both methods was 0.997 (n=6). The slopes and the intercepts were, respectively, 0.98 and −0.12 for the *R*-glucuronide, and 0.97 and −0.01 for the *S*-glucuronide. Therefore a good correlation and a lack of systematic error exist between both methods.

4. Conclusion

A sensitive, reproducible and accurate HPLC method has been developed to measure directly,

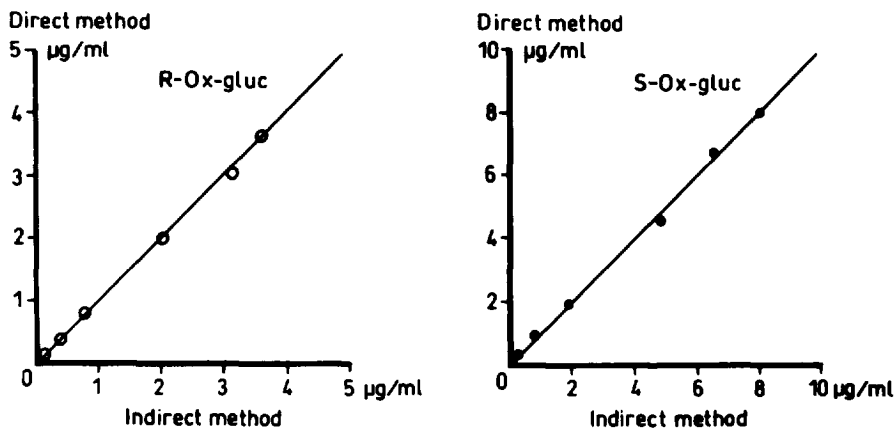

Fig. 3. Correlation between the concentrations of *R*- and *S*-glucuronide measured in rabbit plasma by the direct HPLC method, and those obtained by measuring the oxprenolol enantiomers after enzymatic hydrolysis. The concentrations are expressed as oxprenolol equivalents. The calculated correlation coefficient for both glucuronides is 0.997. The diagonal line represents perfect correlation.

without hydrolysis, the oxprenolol glucuronides. It allows the study of the in vitro glucuronidation of the oxprenolol enantiomers in liver microsomal incubates; plasma concentrations can likewise be assayed.

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