Journal of Chromatography, 497 (1989) 313–318
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4991

Note

Measurement of underivatised metoprolol enantiomers in human plasma by high-performance liquid chromatography with a chiral stationary phase

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(First received May 17th, 1989; revised manuscript received July 25th, 1989)

Although the β-adrenoceptor antagonist, metoprolol, is used clinically as a racemate its therapeutic efficacy resides predominantly in the *S*-isomer. Furthermore, the metabolism of this compound is stereoselective and is linked to the debrisoquine oxidation phenotype [1,2]. Thus, in phenotypically extensive metabolisers (EMs) significantly higher plasma concentrations of the *S*(−)-enantiomer are observed after administration of the racemate, whereas the opposite is observed in poor metabolisers (PMs) [2].

Previous methods for the analysis of metoprolol enantiomers in plasma have involved either the addition of chiral ion-pairing agents such as (+)-1-camphorsulphonic acid to the mobile phase [3] or the formation of diastereomers with L-leucine [1,4], 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate [5] or *S*(−)-phenylethyl isocyanate [6], followed by chromatography on an achiral high-performance liquid chromatographic (HPLC) system. Another method has involved the derivatisation of metoprolol with phosgene and separation by chiral capillary gas chromatography [7]. Separation of metoprolol enantiomers in solution has also been achieved using β-cyclodextrin-bonded [8] and α_1-acid glycoprotein-bonded [9] columns.

Okamoto and co-workers [10,11] have recently reported the development of a new HPLC chiral stationary phase based upon cellulose tris(3,5-dimethylphenylcarbamate) coated on macroporous silica which is capable of re-

0378-4347/89/$03.50

solving the enantiomers of metoprolol in solution [12]. We now report the direct assay of underivatised metoprolol enantiomers in human plasma using a combination of solvent extraction and solid-phase extraction followed by HPLC with this new stationary phase.

EXPERIMENTAL

Chemicals

Racemic metoprolol tartrate and alprenolol hydrochloride were obtained from Hässle (Molndal, Sweden) and *R*(+)- and *S*(−)-metoprolol were gifts from Ciba-Geigy (Basel, Switzerland). Hexane and isopropanol were of HPLC grade (Rathburn, Walkenburn, U.K.) and general laboratory-grade triethylamine (BDH, Poole, U.K.) and HPLC-grade methanol (Rathburn) were glass-distilled before use.

Instrumentation and conditions

The chromatographic system comprised a solvent delivery system (Model 6000A, Waters Assoc., Milford, MA, U.S.A.), an injector (Model 7125, Rheodyne, Cotati, CA, U.S.A.), cellulose tris(3,5-dimethylphenylcarbamate) polymer bonded to macroporous silica commercially prepacked in a 25 cm × 4.6 mm I.D. stainless-steel column (Chiralcel OD column, Daicel Chemical Industries, Tokyo, Japan), an in-line guard column (5 cm × 4.6 mm I.D.) packed with the same material as the analytical column (Daicel Chemical Industries) and a Schoeffel fluorimetric detector (Model 970, Kratos, Manchester, U.K.) equipped with a quartz photomultiplier tube window operating at an excitation wavelength of 220 nm with a 280-nm emission cut-off filter. The mobile phase was hexane–isopropanol–diethylamine (90:10:0.01, v/v) and was pumped at 1 ml/min at ambient temperature.

Extraction procedure

Plasma (1.0 ml), internal standard (alprenolol hydrochloride, 5 μg/ml, 50 μl) and sodium hydroxide (0.1 ml, 1 *M*) were shaken gently with dichloromethane (4 ml for 10 min). After centrifugation (1200 *g*, 15 min) the upper aqueous layer was removed. The organic phase (3–4 ml) was applied to a 1 ml capacity CN Bond Elut column (Harbor City, CA, U.S.A.) that was previously wetted with 1 ml of methanol and 2 ml of dichloromethane. The column was washed with 1 ml of dichloromethane and then eluted with 2 ml of 1% (v/v) triethylamine in methanol. The eluent was collected in a 10-ml conical tube and evaporated to dryness in a Buchler vortex evaporator (Baird and Tatlock, Romford, U.K.). At no stage was the CN Bond Elut column allowed to dry out. After reconstitution of the residue in 200 μl of mobile phase, the sample was transferred to a 0.3-ml glass vial (Model 03-CVG, Chromacol, London, U.K.) within a 1.5-ml microfuge tube. After centrifugation for 2 min in a microfuge

(Model 5414S, Eppendorf, F.R.G.), 50–150 μl of the sample were injected into the chromatograph.

Application

The assay was applied to the measurement of plasma samples from a patient known to be an EM of debrisoquine. After administration of a 200-mg oral dose of racemic metoprolol, blood was sampled at 0.25, 0.5, 1, 2, 3, 4, 6, 8 and 12 h. Plasma was separated and stored at −20°C until assay.

RESULTS AND DISCUSSION

Assay performance

Under the chromatographic conditions employed, the enantiomers of both metoprolol and the internal standard alprenolol were well resolved (Fig. 1). *R*-Metoprolol and *S*-metoprolol eluted with retention times of 7.8 and 16.2 min, respectively ($\alpha=3.1$, $R=2.8$); the retention times for the alprenolol enantiomers were 5.8 and 13.0 min ($\alpha=4.6$, $R=3.2$). The chromatogram from blank plasma was free from interference at the retention times of the drug peaks of

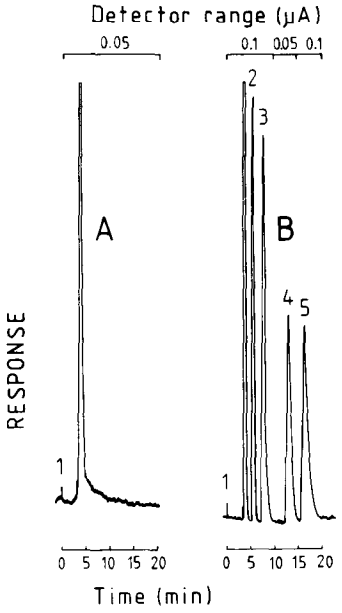

Fig. 1. Chromatograms of (A) a blank human plasma extract and (B) a plasma extract from a subject containing *R*-metoprolol (concentration=228 ng/ml) and *S*-metoprolol (concentration=242 ng/ml). The peaks are the injection event (1), first alprenolol enantiomer (2), *R*-metoprolol (3), second alprenolol enantiomer used as the internal standard (4) and *S*-metoprolol (5).

interest. Because one of the alprenolol enantiomers eluted very close to the solvent front, the other alprenolol enantiomer was used as the internal standard.

Calibration curves (concentrations of each enantiomer were 12.5, 25, 50, 100 and 200 ng/ml) were linear and the recovery of both metoprolol isomers at 100 ng/ml was 90%. Standards prepared by spiking blank plasma with known amounts of the drugs were included in each assay. The equations and coefficients of correlation for the calibration curves of *R*- and *S*-metoprolol were $y=0.017x+0.00052$ ($r=0.9999$) and $y=0.0079x-0.016$ ($r=0.9995$), respectively. The intercepts on the *y*-axis were not significantly different from zero for *R*-metoprolol ($p>0.5$) and *S*-metoprolol ($p>0.2$) indicating that the calibration curves passed through the origin.

The minimum assayable concentration (defined as giving a peak height three times baseline noise) was 3 ng/ml for *R*-metoprolol and 6 ng/ml for *S*-metoprolol. Intra-assay coefficients of variation for the assay of *R*- and *S*-metoprolol at 12.5 ng/ml were 5.3 and 8.6%, respectively ($n=6$); the respective values at 200 ng/ml were 3.7 and 3.6% ($n=6$). The β-adrenoceptor antagonist or agonist compounds acebutolol, atenolol, labetalol, nadolol, pamatolol, pindolol, propranolol and salbutamol (each as the racemate), and *S*-prenalterol and *S*-timolol, did not interfere in the assay.

Optimisation of the extraction procedure

The present assay involves an organic solvent extraction of alkalinised plasma followed by further sample clean-up using a CN Bond Elut column. In the initial assay development, attempts were made to analyse the dichloromethane plasma extract without the sorbent extraction step. However, this resulted in the appearance of interfering peaks in the chromatograms from both blank and spiked plasma samples. This was overcome by additional sample clean-up of the organic dichloromethane plasma extract with the use of the Bond Elut column. In contrast, two recent reports [13,14] have described assays of propranolol enantiomers using Chiralcel OD column chromatography after only a single organic solvent extraction step. It is possible that a less rigorous sample clean-up is sufficient to analyse propranolol enantiomers because of the different detector wavelength required for the fluorescence detection of propranolol. Although the present method is more laborious than those described for propranolol isomers, the more rigorous sample clean-up step may help to extend the column life. This is an important consideration in view of the expense of chiral columns.

Extraction using Bond Elut solid-phase extraction alone was not attempted. The sequential solvent/solid-phase extraction method was employed to help rigorously exclude the presence of water in extracted samples, which in turn minimised the possibility of aqueous contamination of the Chiralcel normal-phase column.

Several other points regarding the present assay method deserve further

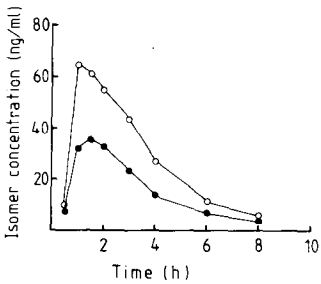

Fig. 2. Plot of plasma *R*-metoprolol (●) and *S*-metoprolol (○) concentrations versus time in an EM patient after single oral dose (200 mg) of racemic metoprolol.

comment. During wetting, loading and eluting the Bond Elut column, it was essential not to allow the column to run dry or to introduce air bubbles into the packing material. The presence of 1% (v/v) triethylamine in the methanol elution step was also essential to ensure good assay reproducibility and drug recovery during the Bond Elut extraction step. Furthermore, particularly clean solvents and chemicals for the Bond Elut column elution were required to avoid chromatographic interference, hence the use of distilled methanol and triethylamine in the method. A further observation was that if the reconstituted residue from the Bond Elut column was injected directly into the chromatograph, a gradual build up of column back-pressure developed after repeated injections. This appeared to be due to particulate matter originating from the Bond Elut column and was overcome by centrifuging the reconstituted residue prior to injection. The reconstituted residue was also best centrifuged in a 0.3-ml Chromacol glass tube rather than in a standard microfuge tube because the organic solvent mobile phase extracted material from the latter which caused chromatographic interference.

Application

Following administration of 200 mg racemic metoprolol tartrate to one subject (Fig. 2) the *S*/*R* ratio of the area under the plasma drug concentration versus time profile from 0 to 8 h was 1.81, confirming previous observations of stereoselectivity in the kinetics of metoprolol in man [1].

ACKNOWLEDGEMENTS

This work was supported by the Swann Morton Foundation of Sheffield. M.S. Ching is a Neil Hamilton Fairley Fellow supported by the National Health and Medical Research Council of Australia. We thank Professor H.F. Woods for provision of laboratory facilities.

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