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Sensitive chiral high-performance liquid chromatographic assay for labetalol in biological fluids

Joanna M. Dakers, David W. Boulton[1], J. Paul Fawcett*

School of Pharmacy, University of Otago, P.O. Box 913, Dunedin, New Zealand

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Abstract

The four stereoisomers of the combined α- and β-adrenoceptor antagonist labetalol were separated and quantified at therapeutic concentrations by normal-phase high-pressure liquid chromatography using a chiral stationary phase and fluorescence detection. Drug in plasma or urine was recovered by solid-phase extraction with 83±5% efficiency. Limits of detection from biological samples (3 ml) were between 1.5–1.8 ng ml^{-1}. Intra-day and inter-day variation at 25 ng ml^{-1} were ≤2.7% and ≤5.80% respectively for all stereoisomers. The assay was applied to an examination of the disposition of labetalol stereoisomers after a single oral dose of racemate to a human volunteer. Labetalol appears to undergo enantioselective metabolism leading to relatively low plasma concentrations of the pharmacologically active enantiomers.

Keywords: Enantiomer separation; Diastereoisomer separation; Labetalol; β-Blockers

1. Introduction

Labetalol was the first antihypertensive drug with combined α- and β-adrenoceptor blocking properties [1]. It is now one of a number of ''hybrid'' β-blockers which reduce blood pressure by interfering with normal compensatory responses to adrenoceptor blockade [2]. Labetalol has been widely used for the past two decades and is generally regarded as a safe and effective drug although orthostatic hypotension due to α-blockade limits the use of higher doses [3]. It undergoes extensive pre-systemic and systemic metabolism leading to low oral bioavailability (about 30%) although inter-individual variation is large [4]. The low bioavailability and high volume of distribution lead to relatively low plasma concentrations after a therapeutic dose (maximum plasma concentration (C_{max}) of ''total'' labetalol of around 100 ng ml^{-1}) [5].

Labetalol contains two chiral centers and is currently marketed as a racemic mixture of four stereoisomers (Fig. 1). The combined α/β-adrenoceptor

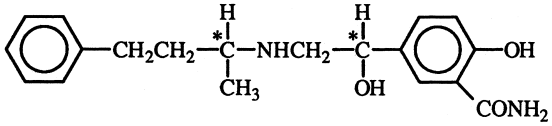

Fig. 1. Chemical structure of labetalol (* denotes chiral centres).

*Corresponding author.

[1]Present address: Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC 29425, USA.

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antagonism of labetalol results from different receptor selectivity of these component stereoisomers [2]. The (*R*,*R*)-stereoisomer is principally a β_1-antagonist responsible for virtually all the β-blocking properties of labetalol but in addition it exerts some α_1-antagonism and β_2-agonist activity [6,7]. The (*S*,*R*)-stereoisomer is highly selective for α_1-adrenoceptors and is responsible for most of the α-blocking activity although a small contribution is made by (*S*,*S*)-labetalol. (*R*,*S*)-labetalol appears to be pharmacologically inactive. A single enantiomer formulation of (*R*,*R*)-labetalol was licensed in Japan and Portugal for a short time in 1990 (Dilevalol, for a review see Ref. [8]) but was withdrawn due to an association with hepatotoxicity [9]. Interestingly, this may suggest the other stereoisomers exert a protective effect on the liver when administered in the racemate.

Non-chiral high-performance liquid chromatography (HPLC) can be used to analyse the two diastereoisomers of labetalol due to their different physicochemical properties [10,11]. During the 1980s, advances in chiral separation technology provided the means to separate all stereoisomers of labetalol using chiral stationary phases. Two HPLC assays employing α_1-acid glycoprotein (AGP) columns and fluorescence detection have been applied to biological samples following administration of racemate to humans [12] and sheep [13].

The study of labetalol stereoisomer disposition in humans involved oral administration of racemate and provided the first evidence of stereoselective presystemic metabolism of labetalol [12]. Although the limited sensitivity of the assay restricted quantification to maximum plasma concentrations of enantiomers, the β-blocking (*R*,*R*)-stereoisomer was found to have a significantly lower plasma concentration than the other three stereoisomers. The study in sheep found no evidence of stereoselective disposition following intravenous infusion of racemic labetalol but the assay employed had an even lower limit of detection [13]. Clearly, there is a need for a sensitive chiral assay for labetalol to examine the pharmacokinetics of this important, archetypal drug.

We report the development and validation of a chiral HPLC assay for labetalol in plasma and urine using a Pirkle-type column with fluorescence detection. A solid-phase extraction (SPE) procedure for clean-up and concentration of biological samples was also developed. To demonstrate the suitability of the assay for enantioselective pharmacokinetic studies, we have applied it to a pilot study of oral administration of racemate to a single human volunteer.

2. Experimental

2.1. Materials

Racemic labetalol hydrochloride was purchased from Sigma (St. Louis, MO, USA). Pure stereoisomers of labetalol were a gift from Glaxo Wellcome Research and Development (Hertfordshire, UK). Analytical grade absolute ethanol was supplied by Ajax (Auburn, NSW, Australia). HPLC-grade 1,2-dichloroethane and trifluoroacetic acid (TFA) and analytical grade sodium hydroxide were obtained from BDH Chemicals (Poole, UK). HPLC-grade hexane was purchased from J.T. Baker (Phillipsburg, NJ, USA). Distilled, deionized water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). SPE cartridges (3 ml, C_{18} Extract-Clean) were purchased from Alltech Associates (Deerfield, IL, USA).

2.2. Instrumentation and conditions

The HPLC system consisted of a Jasco 880-PU pump (Japan Spectroscopic, Tokyo, Japan), a manual injector (Rheodyne 7125, Cotati, CA, USA) fitted with a 100 μl stainless-steel loop, a Chirex 3022 30×4.0 mm stainless-steel guard column (Phenomenex, Torrance, CA, USA), a Chirex 3022 250×4.0 mm stainless-steel analytical column (Phenomenex) and a fluorescence detector (Jasco FP-920). The detector excitation and emission wavelengths were set at 220 nm and 412 nm, respectively with corresponding excitation and emission slit widths of 18 nm and 40 nm, respectively. The mobile phase of hexane–1,2-dichloroethane–ethanol–TFA (55.75:35:9:0.25) was filtered through a 0.45 μm PTFE filter and degassed by sonication under vacuum before use. The flow-rate was 0.6 ml min^{-1} and the system was operated at ambient temperature. Chromatographic data were acquired and analysed with a computerized integration system

(Delta Chromatography Data System v4.02, Digital Solutions, Margate, Queensland, Australia).

2.3. SPE of biological samples

SPE cartridges were mounted on a vacuum manifold box maintained at −3 mm Hg for loading and at −15 mm Hg for drying (1 mm Hg=133.322 Pa). Cartridges were conditioned with methanol (3 ml) followed by deionized water (2×3 ml). The pH of samples (3 ml plasma or urine) was adjusted to 10.5 with 0.2 *M* sodium hydroxide (approximately 0.3 ml) prior to application to cartridges. After drying, cartridges were washed with deionized water (4 ml) and dried again before labetalol was eluted into clean sample tubes with 5 ml aliquots of methanol previously adjusted to an apparent pH of 10 with sodium hydroxide. Extracts were placed in a Speed Vac concentrator with a −106°C trap (SVC 200H, Savant Instruments, Farmingdale, NY, USA) and evaporated to dryness under reduced pressure at 1725 rpm (approximately 2 h). Samples were reconstituted for analysis by vortexing for 1 min in mobile phase (150 μl) immediately prior to injection. The recovery of extraction was assessed using spiked plasma standards prepared as described in Section 2.4.

2.4. Assay validation

Standards were prepared by freeze-drying appropriate aliquots of stock solutions (1 μg ml^{-1}) of racemic labetalol or pure stereoisomers and reconstituting in 150 μl of mobile phase. Spiked plasma standards were prepared by reconstitution in 3 ml of drug-free plasma. Spiked plasma standards of 2.5, 12.5, 25, 37.5, 75 and 125 ng ml^{-1} of each stereoisomer were used to construct calibration curves. Intra-day variation of the assay at 25 ng ml^{-1} of each stereoisomer was assessed by injecting three independently prepared standards three times each on the same day. Inter-day variation was determined by injecting one of the standard solutions a further three times on two subsequent days. Spiked standards (25 and 125 ng ml^{-1}) were stored at −80°C and used to determine the slopes of the calibration curves on each assay day.

To determine the order of stereoisomer elution, solutions of pure stereoisomers in mobile phase (125 ng ml^{-1}) were injected into the HPLC system and their retention times compared with those of peaks obtained for the racemate.

2.5. Pharmacokinetic study

A healthy non-smoking Caucasian male (age 26 years, weight 85 kg) was recruited for the study. The volunteer fasted for 8 h before and for 4 h following drug administration after which a meal was served. The subject had a cannula inserted into a forearm vein and the bladder was emptied immediately prior to dosing. A tablet containing 200 mg *rac*-labetalol as the hydrochloride salt (Trandate, Duncan Flockhart, Palmerston North, New Zealand) was administered with a glass of water. Blood samples (10 ml) were collected into heparinized sample tubes (Vacutainer, Becton Dickinson, Rutherford, NJ, USA) pre-dose and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12 h following drug administration. Samples were centrifuged, plasma aspirated and stored in plastic tubes at −80°C until analysis. Urine was collected cumulatively between 0–4, 4–8, 8–12, 12–24 and 24–48 h after administration and stored in plastic specimen bottles at −80°C until analysis. The study protocol was approved by the Southern Regional Health Authority Ethics Committee (Otago) and the volunteer gave written informed consent prior to the study.

2.6. Data analysis

Calibration curves were assessed by linear regression analysis (SigmaStat version 1.0, Jandel, San Rafael, CA, USA). A two-tailed Student's *t*-test was used to test for differences between the *y*-intercepts of regression lines and zero. The limit of detection was defined as the concentration at which peak height-to-noise ratio was 3:1. Intra-day and inter-day variation are reported as coefficients of variation (C.V.s).

For the pharmacokinetic study, the values and times of peak plasma concentrations (C_{max} and t_{max}) for each stereoisomer were noted directly from the concentration versus time profiles. Areas under the plasma concentration versus time profiles (AUCs) were determined by the linear trapezoid rule to C_{max} and by the log trapezoid rule from C_{max} thereafter.

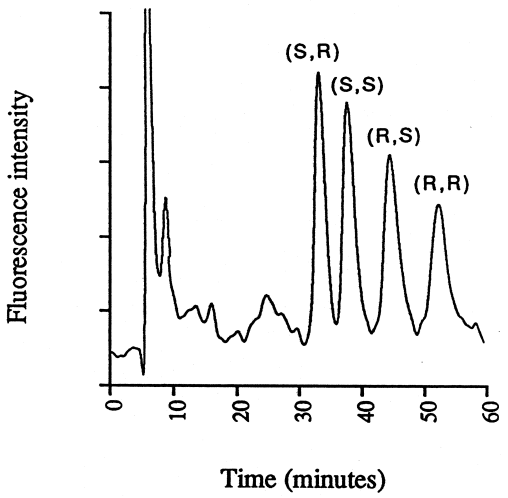

Fig. 2. HPLC chromatogram of a 3 ml drug-free plasma sample spiked with 100 ng ml^{-1} *rac*-labetalol following SPE. See Sections 2.2 and 2.3 for conditions.

3. Results

3.1. Assay

Chromatograms of a drug-free plasma sample spiked with 100 ng ml^{-1} *rac*-labetalol is shown in Fig. 2. Retention times for (*S*,*R*)-, (*S*,*S*)-, (*R*,*S*)- and (*R*,*R*)-labetalol were 33, 37, 43 and 51 min, respectively. The run time of 60 min could be reduced by increasing the concentration of ethanol in the mobile phase albeit with some loss of resolution. The SPE procedure gave a recovery of labetalol of 83±5% (n=12) from plasma samples. A number of drugs were evaluated as internal standards for the assay but neither β-blockers (timolol, atenolol, alprenolol and nadolol) nor β-agonists (terbutaline and isoprenaline) were suitable due to either instability, coelution on the HPLC column or inadequate retention on the SPE column under the conditions that were optimal for labetalol.

Calibration curves for each stereoisomer were linear over the concentration range 2.5–125 ng ml^{-1} (r>0.98). In all cases, the y-axis intercepts of the regression lines of best fit were not statistically different from zero. The limits of detection for (*S*,*R*)-, (*S*,*S*)-, (*R*,*S*)- and (*R*,*R*)-labetalol were 1.5, 1.6, 1.6 and 1.8 ng ml^{-1}, respectively (signal-to-noise, 3:1). The intra-day and inter-day C.V.s were ≤2.7 and ≤5.80%, respectively for all stereoisomers.

3.2. Pharmacokinetic study

The plasma concentration versus time profiles for the stereoisomers of labetalol were clearly different as shown in Fig. 3a. The practically inactive (*S*,*S*) stereoisomer was present in the largest concentration throughout the sampling period while the β-blocking (*R*,*R*)-stereoisomer was present in the smallest concentration. The C_{max} values for (*S*,*S*)-, (*S*,*R*)-, (*R*,*S*)-

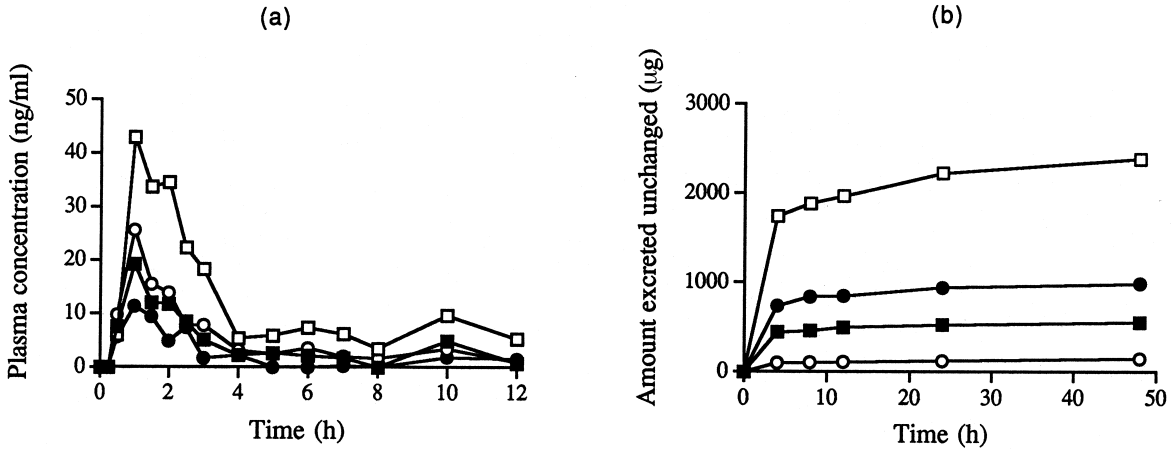

Fig. 3. Plasma concentration–time profiles (a) and cumulative urinary excretion–time profiles (b) for (*S*,*S*)- [□] (*S*,*R*)- [■], (*R*,*S*)- [○] and (*R*,*R*)-labetalol [●] following 200 mg of *rac*-labetalol by the oral route to a healthy male volunteer.

and (*R*,*R*)-labetalol were 43, 19, 26 and 11 ng ml^{-1}, respectively with t_{max} values of 60 min for all stereoisomers. The C_{max} for the sum of the stereoisomers was consistent with values reported from non-chiral studies [5]. The AUCs as percentages of the total AUC over the 12 h of the study were 41, 25, 25 and 9% for (*S*,*S*)-, (*S*,*R*)-, (*R*,*S*)- and (*R*,*R*)-labetalol, respectively.

The cumulative urinary excretion of unchanged labetalol stereoisomers is shown in Fig. 3b. Only 2% of the total dose was excreted over 48 h but, as with plasma data, clear differences were observed in the amounts of stereoisomers excreted in the urine. The practically inactive (*S*,*S*)-stereoisomer was predominant in all urine samples and accounted for 58% of the total amount of unchanged labetalol excreted in 48 h. The (*S*,*R*)-, (*R*,*S*)- and (*R*,*R*)-enantiomers accounted for 14, 4 and 24%, respectively of the total amount excreted over this time.

4. Discussion

The four stereoisomers of labetalol have previously been separated on α_1-AGP columns but the assays lacked the sensitivity required for detection in blood at concentrations associated with therapeutic doses [11–13]. Our HPLC system utilized a ''Pirkle'' (Type I) chiral column which has a higher sample ''loading'' capacity than α_1-AGP columns. Good separation of the four stereoisomers required a runtime of 60 min similar to that required for resolution on an α_1-AGP column. The limit of detection of 1.8 ng ml^{-1} or less for each stereoisomer based on a 3 ml sample makes this assay suitable for pharmacokinetic studies in humans following a therapeutic dose of *rac*-labetalol.

Although fluorescence detection provides a sensitive and specific method for analysis of drugs in biological fluids, its use is not always compatible with the organic-based mobile phases involved in normal-phase HPLC. The Jasco FP-920 fluorescence detector was capable of providing the low excitation wavelength of 220 nm needed to detect labetalol stereoisomers at therapeutic levels but at this wavelength the ethanol in the mobile phase produced a relatively high background fluorescence. The use of other polar modifiers with lower native fluorescence such as methanol or 1-propanol did not provide the level of chiral resolution of labetalol stereoisomers attained with ethanol. Thus the ability to suppress the high background fluorescence of ethanol using the adjustable slit width and gain settings available on the Jasco FP-920 was another important factor in the development of this assay.

The results of the pharmacokinetic study clearly showed the relative predominance of the practically inactive (*S*,*S*)-stereoisomer in both plasma and urine. The two therapeutically active stereoisomers, (*R*,*R*)- and (*S*,*R*)-labetalol, were present in plasma at lower concentrations. Labetalol has previously been shown to undergo significant pre-systemic Phase II metabolism (glucuronidation) [14] and a smaller amount of Phase I metabolism (N-dealkylation) in the liver and possibly the gut wall [15]. Interestingly, the relative proportion of the (*R*,*S*)- and (*R*,*R*)-stereoisomers in plasma (22 and 9%, respectively) was markedly different from the relative proportion found in urine (4 and 24%, respectively) which suggests labetalol may be subject to stereoselective renal clearance.

Lalonde et al. [12] also found a relatively smaller concentration of the (*R*,*R*)-stereoisomer in C_{max} plasma samples. In contrast to our results, their study found equal levels of the other three stereoisomers but there were limitations in their assay sensitivity. Nevertheless, the clinical implications of the more extensive metabolism of the β-blocking (*R*,*R*)-stereoisomer relative to that of the α-blocking (*S*,*R*)-stereoisomer require further investigation. The possibility that there is little difference in labetalol enantiomer disposition following an intravenous dose, as has been observed in sheep [13], also requires confirmation.

This study reports the development and validation of a chiral HPLC assay for labetalol enantiomers in plasma and urine. The assay is reproducible and sensitive enough to quantify stereoisomer concentrations at therapeutic levels following administration of racemate. The assay procedure has been applied to a pilot study of stereoisomer disposition following a single oral dose of racemate to a healthy volunteer. Results of this study suggest extensive stereoselective distribution of labetalol with high relative metabolism of the two most active stereoisomers. The application of this assay to further in vivo and in

vitro studies will significantly enhance our understanding and rational use of labetalol in the future.

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