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Determination of pindolol enantiomers in human plasma and urine by simple liquid–liquid extraction and high-performance liquid chromatography

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Abstract

A simple method for the measurement of pindolol enantiomers by HPLC is presented. Alkalinized serum or urine is extracted with ethyl acetate and the residue remaining after evaporation of the organic layer is then derivatised with (*S*)-(−)-α-methylbenzyl isocyanate. The diastereoisomers of derivatised pindolol and metoprolol (internal standard) are separated by high-performance liquid chromatography (HPLC) using a C_{18} silica column and detected using fluorescence (excitation λ: 215 nm, emission λ: 320 nm). The assay displays reproducible linearity for pindolol enantiomers with a correlation coefficient of $r^2 \geq 0.998$ over the concentration range 8–100 ng ml^{-1} for plasma and 0.1–2.5 μg ml^{-1} for urine. The coefficient of variation for accuracy and precision of the quality control samples for both plasma and urine are consistently <10%. Assay parameters are similar to those of previously published assays for pindolol enantiomers, however this assay is significantly easier and cheaper to run. Clinically relevant concentrations of each pindolol enantiomer can readily be measured.

Keywords: Enantiomer separation; Pindolol

1. Introduction

Pindolol ((±)-4-(2-hydroxy-3-isopropyl-amino-propoxy)-indole) [Fig. 1] is a β-adrenergic antagonist with intrinsic sympathomimetic activity. Pindolol (Visken®), which is available for the clinical treatment of angina and hypertension, is produced as the racemic mixture. It has one chiral centre and two optical isomers which are eliminated stereoselectively [1]. (−)-Pindolol is the more pharmacologically active [1]. Therefore, it is essential that the bioanalysis be enantioselective.

Recent articles describing the measurement of pindolol enantiomers by high-performance liquid chromatography (HPLC) have utilised chiral col-

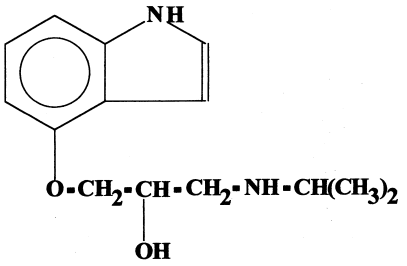

Fig. 1. Pindolol.

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umns [2]. It can be difficult to obtain symmetrical peak shape for both enantiomers and internal standard using these columns. As the later peaks broaden integration can be difficult and sensitivity becomes a problem as the limit of detection is approached [3]. Solid-phase columns are often used for screening methods for β-blocking agents from biological specimens [2,4]. However, where greater sensitivity is required selectivity may be compromised due to interfering compounds which remain following the gentle wash steps and increased elution volumes.

Liquid–liquid extractions involving pH controlled extractions and then back extractions have been employed for effective sample clean up in some studies [5,6]. This can be time consuming for a routine analysis. Methods involving column switching have also been used for β-blocking agents to divert endogenous compounds in large concentrations to increase selectivity, and where direct injections are used to overcome sensitivity problems [7,8]. However, column switching requires specialised equipment and a more advanced knowledge of HPLC [3,9].

This report describes a sensitive and specific method for the analysis of pindolol enantiomers involving a single extraction followed by simple derivatisation and measurement by HPLC with fluorescent detection. Quantitation is achieved using an internal standard.

2. Method

2.1. Reagents and chemicals

Racemic pindolol, the tartrate salt of racemic metoprolol and the derivatising agent, (*S*)-(−)-α-methylbenzyl isocyanate were purchased from Sigma (Sydney, Australia). Methanol (BDH-HiperSolv™), chloroform (BDH-HiperSolv™) and ethyl acetate (Mallinkrodt-ChromAR®) were all HPLC grade. Anhydrous sodium sulphate which was used to dry the chloroform, sodium hydrogen carbonate and disodium carbonate (anhydrous) were all Ajax Univar® analytical grade chemicals. All water used was deionised at 18 MΩ. Drug free human plasma was obtained from the blood bank (Brisbane, Australia) and the blank urine was obtained from volunteer laboratory personnel.

Carbonate buffer was prepared by titrating 1 *M* disodium carbonate and 1 *M* sodium hydrogen carbonate to pH 9.3±0.05. Derivatising reagent was prepared daily by adding 2 μl (or 10 μl for the urine assay) of (*S*)-(−)-α-methylbenzyl isocyanate to 5 ml chloroform which was previously dried with sodium sulphate.

2.2. Standard preparation

A stock solution of racemic pindolol in methanol was prepared at a concentration of 1 mg ml^{-1}. The working standard for plasma (1 μg ml^{-1}) and urine (10 μg ml^{-1}) were both prepared by a serial dilution of the stock solution with methanol. The stock solution of racemic metoprolol (internal standard) was prepared at a concentration of 1 mg ml^{-1} and this was serially diluted with methanol to give a working internal standard solution for plasma (5 μg ml^{-1}) and urine (20 μg ml^{-1}). Stock solutions and standards were all stored in glass amber vials at −20°C.

2.3. HPLC

The column used was a Beckman C18, 5 μm, 4.6 mm×25 cm (Beckman, Sydney, Australia) which was protected by an in-line filter (2 μm) (Alltech, Brisbane, Australia). The HPLC system consisted of a Shimadzu LC-10AD pump (Shimadzu, Kyoto, Japan) set at 1.3 ml min^{-1} and a Shimadzu SIL-10AXL autoinjector (Shimadzu, Kyoto, Japan). The Shimadzu RF-10A spectrofluorometric detector (Shimadzu, Kyoto, Japan) was set at 215 nm excitation, 320 nm emission. The mobile phase (62% methanol–H_2O) was prepared by adding 380 ml deionised water to 620 ml methanol and filtering through 4.5 μm filter membrane.

2.4. Sample analysis

Standard solutions were prepared over the concentration range 16–200 ng (±)-pindolol ml^{-1} for plasma, and 0.2–5.0 μg (±)-pindolol ml^{-1} for urine by adding the appropriate working standard solution to a clean 10 ml pyrex screw cap tube, followed by

the addition of the specified working internal standard solution (50 μl for plasma; 100 μl for urine). This was dried under nitrogen gas with low heat (~35°C). Then 1 ml plasma or 0.5 ml urine, 0.5 ml carbonate buffer (1 *M*, pH 9.3) and 6 ml ethyl acetate was added prior to capping and mixing by gentle inversion for 4 min.

Following centrifugation (717×*g* for 6 min), the organic layer was transferred to a clean pyrex screw cap tube and dried down under nitrogen gas with moderate heat (~45°C). When the samples were thoroughly dried a 50 μl aliquot of derivatising reagent was added and the sample was capped and vortexed for 1 min. The chloroform was dried off under nitrogen gas with low heat (~35°C) and the sample residue was then reconstituted in 100 μl of mobile phase and vortexed for 10 s. An 80 μl aliquot was injected on to the column.

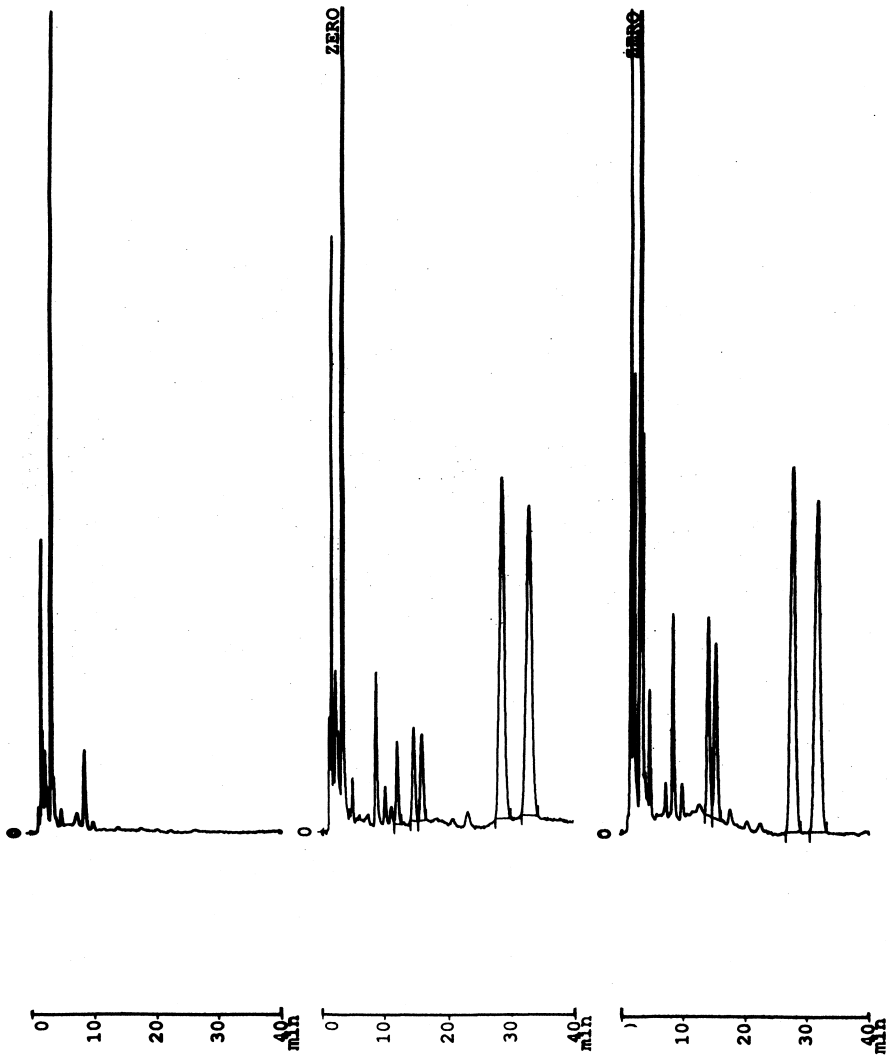

Fig. 2. (a) Chromatogram of blank plasma, (b) Chromatogram of pindolol low QC, (+)- 22.7 ng ml^{-1} (14.6 min) and (−)- 23.6 ng ml^{-1} (15.8 min), metoprolol (I.S.) 250 ng ml^{-1} *rac* (28.6*, 32.8 min), (c) Chromatogram of a subject sample at t=1.0 h, (+)- 46.1 ng ml^{-1} and (−)- 47.9 ng ml^{-1}. *I.S. peak used for quantitation.

2.5. Assay validation

Peak area ratios of (+)-pindolol and (−)-pindolol to internal standard generated from a duplicate seven point standard curve were used on each day of analysis. Triplicate quality controls at three concentrations were included with each calibration curve to assess intra- and inter-day variability. Recovery was determined by comparing extracted to non-extracted (+)-pindolol and (−)-pindolol peak area ratios with non-extracted internal standard. Specificity was established with ≥6 independent sources of drug free plasma. Stability was tested on samples subjected to two and three freeze–thaw cycles and samples stored at room temperature for 24 h. The limit of quantitation is evaluated by analysing six replicates of the lowest calibration standard.

2.6. Quantitation

Duplicate calibration standards in human plasma from three separate assay occasions were used to calculate linearity over the concentration range 16–200 ng (±)-pindolol ml^{-1} plasma using linear regression analysis. Precision and accuracy were determined by using back calculated concentrations of spiked plasma samples in triplicate at three different concentrations (25, 50, 150 ng (±)-pindolol ml^{-1} plasma) on each assay occasion. Each day of routine analysis includes a single seven point standard curve and duplicate quality controls at three concentrations. Criteria for acceptance of a run included that the correlation coefficient (r) must be ≥0.995, the slope of a regression line must be consistent with past slopes. Back-calculated values for calibration standards must be within 10% (LOQ within 15%) and four of the six quality controls must be within 15% of the theoretical concentration (where each quality control concentration must be represented).

3. Results and discussion

3.1. Plasma

Derivatives of both (±)-pindolol and (±)-metoprolol were well separated on the C_{18} column. There were no interfering substances in plasma as shown in Fig. 2. The approximate retention times for (+)-pindolol and (−)-pindolol were 14.6 min. and 15.8 min. respectively. Retention times for the (±)-metoprolol tartrate derivatives were approximately 28.6 min and 32.8 min. The linearity for the duplicate standard curves of each pindolol enantiomer at the concentrations 8, 25, 38, 50, 65, 75, and 100 ng ml^{-1} plasma was calculated using peak area ratios of derivatised (+)- and (−)-pindolol to metoprolol. The linear regression calculations consistently produced an r^2 value ≥0.998. Table 1 shows the accuracy of the back calculated peak area ratios. Accuracy of the quality control plasma samples spiked with (±)-pindolol at 50, 100 and 150ng ml^{-1} was 7.9, 4.5 and 4.0% for (+)-pindolol and 8.6, 3.6 and 4.2% for (−)-pindolol at the respective concentrations. Precision of the quality control plasma samples spiked with (±)-pindolol at 50, 100 and 150 ng ml^{-1} was, respectively, 7.0, 3.8 and 2.7% for (+)-pindolol and 8.1, 4.7 and 3.4% for (−)-pindolol. The recovery of

Table 1
Measured plasma concentrations of pindolol enantiomers over three assay occasions (n=2 at each concentration on each occasion)

Back Calculated Plasma Concentrations				
Standard concentration ng ml^{-1}	(+)-pindolol (n=6) ng ml^{-1}	S.D. ±	(−)-pindolol (n=6) ng ml^{-1}	S.D. ±
8	7.82	0.936	7.56	1.30
25	25.6	0.999	25.7	1.12
38	37.5	1.14	38.4	1.10
50	50.4	0.609	49.9	0.947
65	65.0	1.60	64.6	1.97
75	75.0	1.22	75.1	1.39
100	99.9	0.903	99.9	0.971

(+)-pindolol and (−)-pindolol were determined to be 89.9% ±5.6 and 90.3% ±8.9 respectively when calculated from spiked plasma samples at 25, 50 and 75 ng ml^{-1} (n=4 at each concentration). The recovery of the (±)-metoprolol diastereoisomer used as the internal standard is 108.0%±5.4. The efficiency of the derivatisation and the identification of the diastereoisomers have been previously determined [5].

Pindolol was found to be stable in plasma following a comparison of three sets of samples compared to a control group. Stability test samples at 25, 50

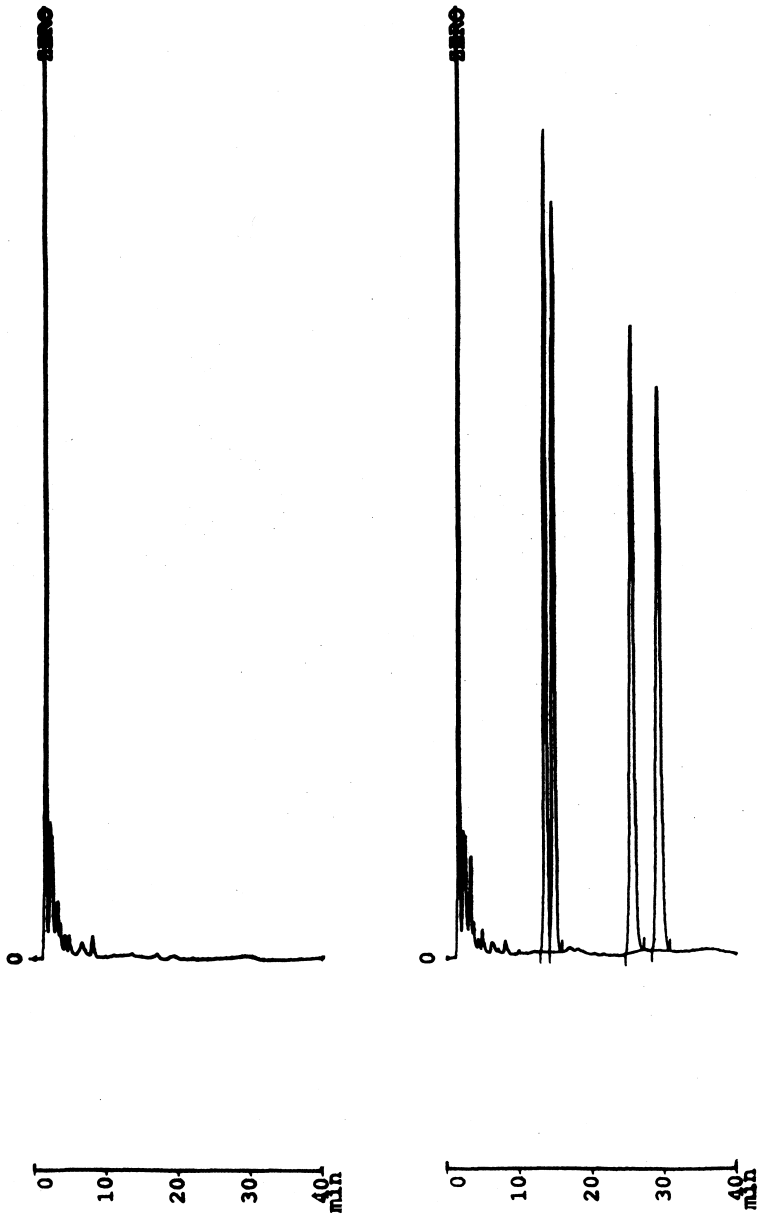

Fig. 3. (a) Chromatogram of blank urine, (b) Chromatogram of pindolol calibration standard, (+)- 2.063 μg ml^{-1} (13.3 min) and (−)- 2.058 μg ml^{-1} (14.5 min), metoprolol (I.S.) 2.0 μg ml^{-1} *rac* (25.3, 29.0* min). *I.S. peak used for quantitation.

and 75 ng ml^{-1} (n=3 at each concentration) were subjected to two and three cycles of freezing and thawing and the third set was stored at room temperature (22°C) for 24 h prior to analysis. There was no significant difference in measured concentrations when compared with the control samples.

3.2. Urine

The urine standard curve for pindolol enantiomers at the concentrations 0.1, 0.4, 0.8, 1.0, 1.5, 2.0 and 2.5 μg ml^{-1} demonstrated linearity with the coefficient of determination (r^2) consistently greater than 0.999 for (+)-pindolol and (−)-pindolol. The intra-day precision of the quality controls was 5.2, 4.5, and 0.22% for (+)-pindolol and 2.6, 3.9, and 0.71% for (−)-pindolol at the corresponding concentrations of 0.2, 0.5 and 1.0 μg ml^{-1} urine (n=3 at each concentration). The accuracy was calculated to be 3.5, 3.1 and 1.3% for (+)-pindolol and 4.2, 2.7 and 0.73% for (−)-pindolol at 0.2, 0.5 and 1.0 μg ml^{-1} urine (n=3 at each concentration). There were no endogenous compounds in urine which co-eluted with the peaks of interest as shown in Fig. 3.

4. Conclusion

This report presents a sensitive and specific method for the analysis of pindolol enantiomers in plasma or urine which combines a single extraction step with a one minute derivatisation at room temperature. The simplicity of this extraction method results in a greatly reduced assay time while sensitivity, linearity and reproducibility are maintained. An internal standard is used to enhance the robustness of the assay and the use of fluorescent detection is sufficient to provide selectivity without the use of column switching or multi-step liquid–liquid extractions. The chromatograms displayed well resolved peaks for (+)- and (−)-pindolol and the internal standard without using a chiral column. The method is effective over the range 8–100 ng ml^{-1} for pindolol enantiomers in plasma and 100–2500 ng ml^{-1} for pindolol enantio-

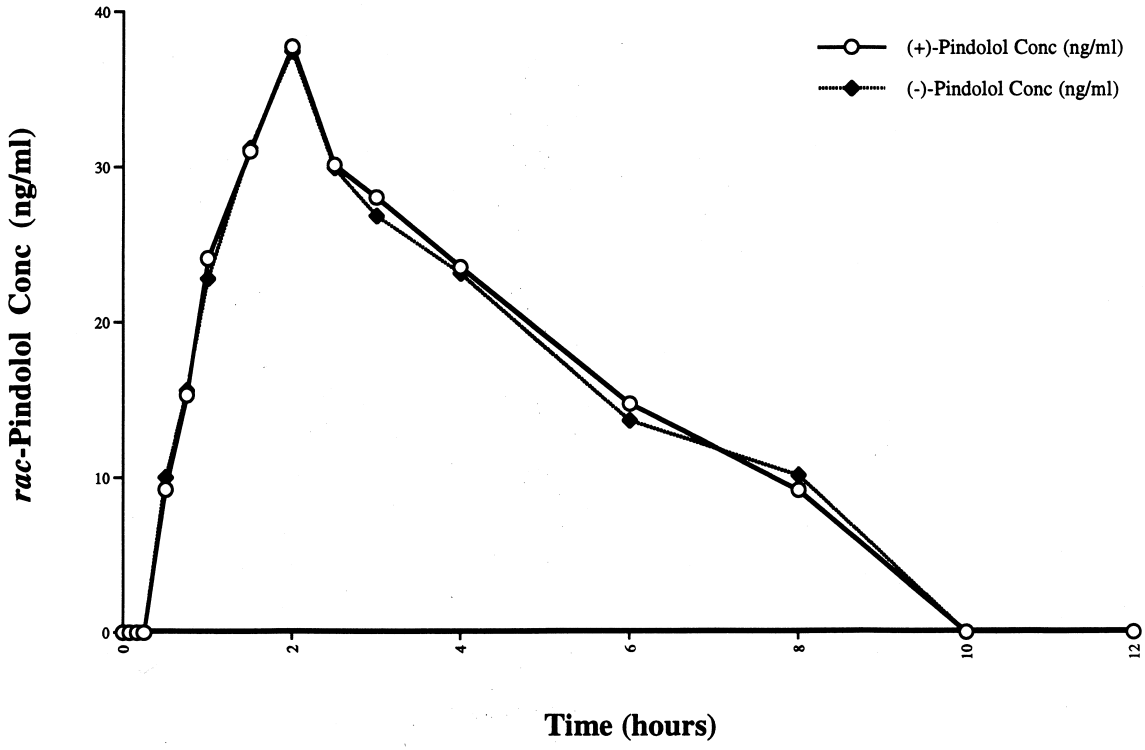

Fig. 4. Concentration-time profile in one volunteer following the administration of *rac*-pindolol.

mers in urine which is therapeutically relevant following an oral dose of 15 mg of (±)-pindolol as shown in Fig. 4. The assay will now be used as part of a larger study designed to investigate stereoselective renal elimination processes in the immunocompromised.

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References

[1] P.-H. Hysu, K.M. Giacomini, J. Clin. Invest. 76 (1985) 1720.
[2] H. Zhang, J.T. Stewart, M. Ujhelyi, J. Chromatogr. B. 668 (1995) 309.
[3] G. Egginger, W. Lindner, C. Vandenbosch, D. Luc Massart, Biomed. Chromatogr. 7 (1993) 277.
[4] M.S. Leloux, E.G. De Jong, R.A.A. Maes, J. Chromatogr. 488 (1989) 357.
[5] P.-H. Hysu, K.M. Giacomini, J. Pharm. Sci. 75 (1986) 601.
[6] H.T. Smith, J. Chromatogr. 415 (1987) 93.
[7] T. Ohta, S. Niida, H. Nakamura, J. Chromatogr. B. 675 (1996) 168.
[8] W.D. Hooper, P.V. Baker, J. Chromatogr. B. 672 (1995) 89.
[9] C.L. Davies, J. Chromatogr. 531 (1990) 131.