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Note

Sensitive high-performance liquid chromatographic method for the determination of labetalol in human plasma using fluorimetric detection

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Since 1977, labetalol {2-hydroxy-5-[1-hydroxy-2-(1-methyl-3-phenyl propylamino)ethyl] benzamide hydrochloride} has been in use as an antihypertensive agent. Its pharmacological effect is based on the fact that it antagonizes both α- and β-adrenoceptors [1, 2]. The effective plasma levels are reported to be 100—300 ng/ml, but inter-individual variations are considerable in this respect [3].

Several methods are at present available for monitoring labetalol levels [4—6]. Detection limits ranging from 40 to 80 ng per sample were reported, which means that at least 1—2 ml of plasma are required for a single analysis. Some fluorimetric methods do not include a chromatographic separation and are therefore expected to be less reliable. Detailed pharmacokinetic studies using such methods will be troublesome.

In the recent literature there is growing support for the usefulness of labetalol in the treatment of pregnancy-induced hypertension [7—9]. No detrimental effects to the mother or foetus have been observed. Additionally, there are indications that labetalol enhances early foetal lung maturation. For pharmacokinetic studies during the perinatal period a sensitive and reliable method for the determination of labetalol could be useful.

The present method, which involves separation by ion-pair reversed-phase high-performance liquid chromatography (HPLC) and fluorimetric detection of labetalol and the internal standard chloroquine after on-line post-column buffering of the eluent, meets these sensitivity and selectivity requirements.

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EXPERIMENTAL

Chemicals

All aqueous solutions were prepared with double-distilled water. Labetalol hydrochloride was kindly supplied by Glaxo (Hoofddorp, The Netherlands); chloroquine sulfate was obtained from Nogepha (Alkmaar, The Netherlands). All other chemicals were obtained from E. Merck (Darmstadt, G.F.R.) and were of analytical reagent grade, except for the trimethylammonium chloride, (TMA) which was of synthetical reagent grade. All chemicals were used as received.

Standard aqueous solutions were prepared of labetalol (10.0 mg/l, of the base) and of chloroquine (2.0 mg/l, of the base). These solutions were slightly acidified with formic acid (± 0.01%) for better preservation. The carbonate buffer (pH 9) contained 90 g of $NaHCO_3$ and 32 g of K_2CO_3 per liter. The borate buffer (pH 10.3), which was used for buffering the eluent post column, contained 110 g of Borax and 17 g of sodium hydroxide per liter. A mixture of ethylene dichloride—diethyl ether—isopropanol (45:45:10) was used as the extraction solvent.

Apparatus

The eluent was delivered by a Kipp 9208 HPLC pump (Kipp Analytica, Emmen, The Netherlands). Samples were injected with a Rheodyne 7120 (Berkeley, CA, U.S.A.) injection valve, equipped with a 100-μl loop. The borate buffer was delivered by a 60-ml syringe infusion pump (Dascon B.V., Uden, The Netherlands) and mixed with the eluent in a T-union (15 MTA; Inacom, Veenendaal, The Netherlands) with the column effluent inflow opposite to that of the infusion pump's effluent. A Schoeffel FS 970 LC fluorometer (Schoeffel, Westwood, NJ, U.S.A.) was used as the detector with an excitation wavelength of 335 nm and emission wavelength of 370 nm. An Aminco-Bowman spectrophotofluorometer was used for scanning the fluorescence spectra.

Chromatographic system

Separations were performed on LiChrosorb 10 RP-18 or Nucleosil 10 C-18 obtained as prepacked columns (250 × 4.6 mm) from Chrompack (Middelburg, The Netherlands). The particle size of both columns is 10 μm. The composition of the eluent was water—acetonitrile (68:32) containing 40 g of $NaClO_4$, 40 g of trimethylammonium chloride and 4 g of sodium acetate per liter. The pH of the eluent was about 4.5. The system was operated at ambient temperature with a flow-rate of 1.0 ml/min. The borate buffer was added post-column with a flow-rate of 0.23 ml/min, adjusting the pH of the eluent to about 9.3.

Sample preparation

In a glass-stoppered tube 1.0 ml of plasma sample was mixed with 25 μl (50 ng) of the internal standard solution, and 200 μl of the carbonate buffer (pH 9) were added. If less than 1.0 ml of plasma was used, this needed to be diluted

with water to that volume. Extraction was carried out with 5.0 ml of the extraction mixture by shaking for 10 min.

After centrifugation the organic layer was evaporated under nitrogen at about 40°C. The residue was then redissolved in 120 μl of eluent and 50 μl of this solution were injected into the liquid chromatograph. Calibration curves using labetalol concentrations in the range 12.5—250 ng/sample were obtained by adding 25 μl of the (appropriately diluted) standard solution to 1.0 ml of blank human plasma and utilizing the same procedure.

RESULTS

The results presented in this section were obtained on a LiChrosorb 10 RP-18 column. Chromatograms of plasma samples are shown in Fig. 1. In most cases the baseline was hardly disturbed by a front, even at high detector sensitivity. A representative plot of peak height ratio vs. labetalol concentration was described by the equation $y = 0.0084x + 0.0234$. The coefficient of correlation of this calibration curve was 0.9992. The within-day coefficient of variation, calculated from fifteen determinations of a plasma sample spiked with 25 ng of labetalol, was found to be 7.2%. In the same experiment the recovery of labetalol was 95.3 ± 10.6%. The day-to-day coefficient of variation was calculated from duplicate measurements for the concentration ranges 8—25 ng/ml and 25—250 ng/ml and was found to be 8.08% ($n = 7$) and 2.22% ($n = 8$), respectively. The recovery of the internal standard was 96.0 ± 7.7% ($n = 5$). Although we did not measure concentrations below 8.0 ng/ml, the signal-to-noise ratio indicates a detection limit down to 1 ng/ml.

Interference with the method was excluded for the following antihypertensive and diuretic drugs: clonidine, diazoxide, hydralazine, propranolol, chlorthalidone, chlorthiazide, frusemide and triamterene. Interference with the method by prazosin can be circumvented by choosing an alternative eluent

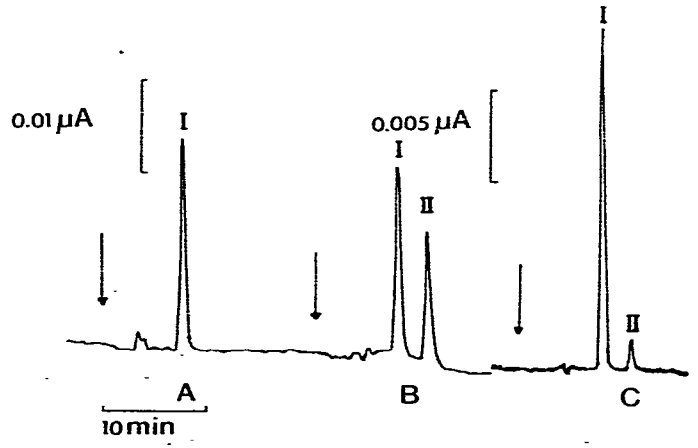

Fig. 1. Chromatograms of plasma samples from the same patient as in Fig. 2. I = internal standard, II = labetalol. (A) Blank plasma with internal standard; (B) 33 ng/ml labetalol, 0.5 ml of plasma used; (C) 8 ng/ml labetalol, 1.0 ml of plasma used.

composition as follows: 27.5% of acetonitrile instead of 32% and 1.5% $NaClO_4$ instead of 4%. Under the latter conditions, however, triamterene does interfere with the method.

In Fig. 2 a plasma concentration—time curve is shown for a patient who received 100 mg of labetalol orally. Plasma samples of 0.5 ml were used for analysis, except for the last four observations when 1.0-ml samples were used.

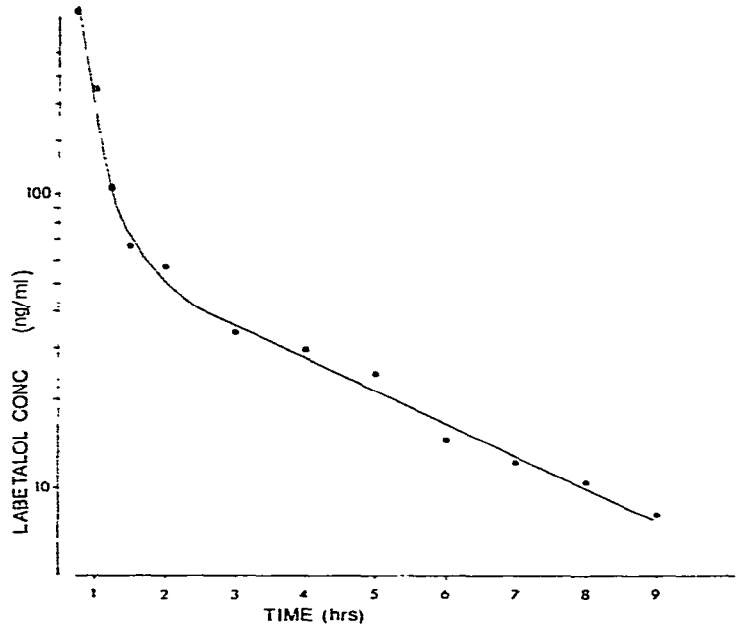

Fig. 2. Semilogarithmic plot of plasma labetalol concentration vs. time after oral administration of 100 mg of labetalol to a patient. The line represents that of the best visual fit.

DISCUSSION

Chloroquine was chosen as the internal standard because its fluorescence properties are very similar to those of labetalol, although it belongs to quite another therapeutic class of drugs. The precision of the method was not significantly improved by the use of the internal standard; however, its use was maintained out of considerations of practicality.

The extraction of labetalol and chloroquine has been investigated by other workers [5, 6, 10]. Both compounds require a rather polar extraction solvent. The composition of our solvent was adjusted in order to obtain about the same recovery for both compounds, and to get a floating organic layer. Raising the extraction pH beyond a value of 9.0 caused a decrease of the recovery of labetalol due to its phenolic hydroxyl group, whereas the recovery of chloroquine was not further improved.

When chloroquine was eluted on a LiChrosorb 10 RP-18 column with buffered acetonitrile—water or methanol—water mixtures it gave extremely

broad bands. We observed an increase in retention time and tailing with decreasing pH, indicating that particularly the protonated form of chloroquine interacts strongly with the unshielded residual silanol groups of the LiChrosorb RP-18 [11]. As we associated this effect with the tertiary amino group in the chloroquine molecule [12], we examined the influence of trimethylammonium chloride (TMA) as a possible adsorption competitor. Addition of this compound to the eluent indeed caused a dramatic improvement in peak shape as well as a decrease in the elution volume and plate height. With increasing TMA concentration the asymmetry factor [13] and the plate height, along with the elution volume, did approach a constant value, giving support to the idea of adsorption competition (Fig. 3). As it is a well-known fact that RP columns of the same type but from different producers can differ strongly in this respect [11], we investigated whether the same effect could be obtained with a Nucleosil 10 C-18 column. The results of this experiment are demonstrated in Fig. 4. When no TMA was added to the eluent, only a rather broad labetalol band could be discerned in the case of the LiChrosorb whereas a good separation is obtained on Nucleosil. However, when TMA was added to the eluent, the chromatograms on LiChrosorb and Nucleosil became very comparable. The efficiency of the Nucleosil column seems somewhat better. In this particular case the Nucleosil column in combination with an eluent without TMA seems preferable. On the other hand, these observations suggest that if our method is used with other octadecyl RP columns an eluent with TMA should be used. The unpleasant odour of trimethylamine can be neutralized by eluent efflux into dilute acetic acid.

Due to ion-pair formation [14] the capacity factor of both labetalol and chloroquine increases when sodium perchlorate is added to the eluent. As this effect was stronger in the case of chloroquine, it was utilized to adjust the resolution between the chloroquine and labetalol bands. Changes in pH between 2 and 5 appeared not to influence the separation. With sodium acetate

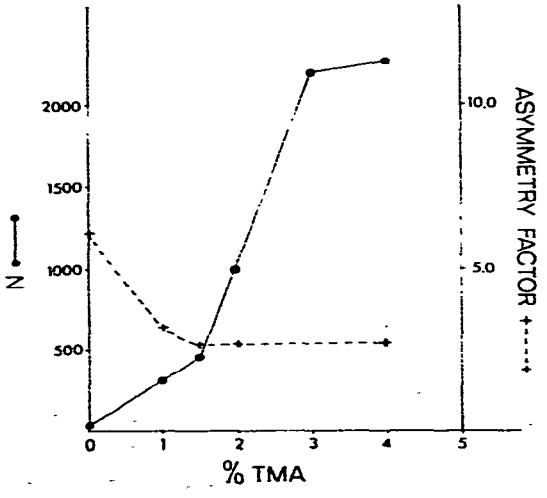

Fig. 3. A plot of plate number (N) and asymmetry factor vs. TMA concentration in the eluent (calculated for the chloroquine peak on the LiChrosorb 10 RP-18 column).

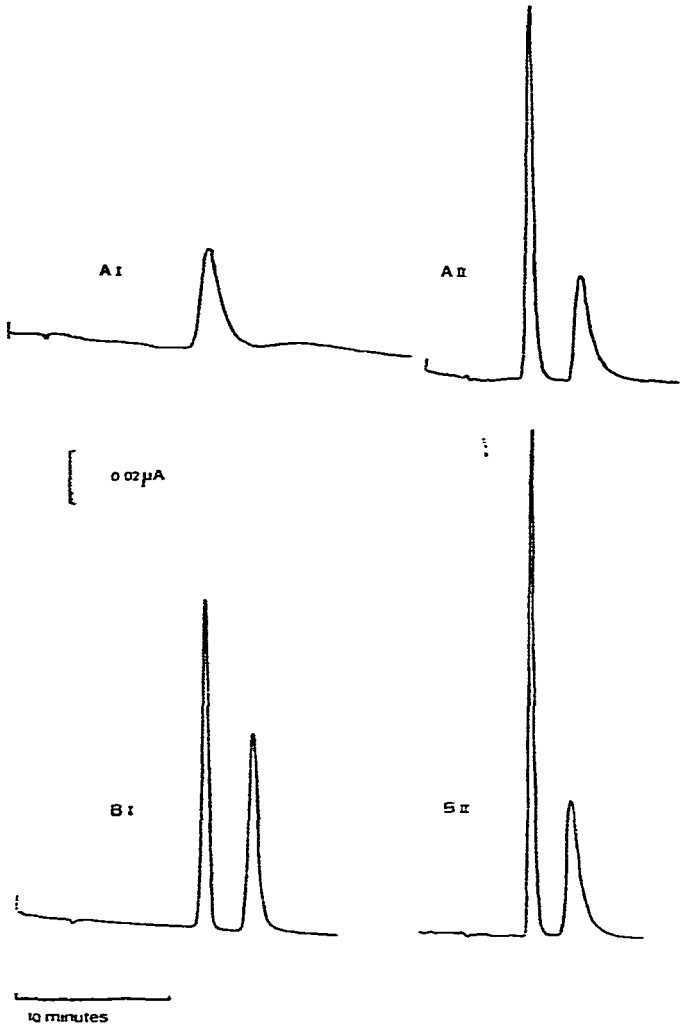

Fig. 4. Chromatograms of a standard sample containing 125 ng of labetalol and 100 ng of chloroquine on a LiChrosorb 10 RP-18 column (A) and a Nucleosil 10 C-18 column (B). In the chromatograms on the left (AI, BI) TMA was omitted from the eluent (pH corrected with HCl). In the chromatograms on the right (AII, BII) the eluent contained 4% TMA.

the pH of the eluent was adjusted to 4.5. The analytical column showed no important loss of performance during six months of intensive use.

Optimum conditions for the fluorescence detection of labetalol were investigated by Martin et al. [6]. Under our conditions there was an excitation maximum at 340 nm and an emission maximum at 415 nm.

We examined the influence of pH and composition of the post-column buffer mixture on the fluorescence yield of both labetalol and chloroquine. Our observations did not confirm the advantage of ammonium hydroxide over buffer solutions [6]. Optimum pH values were less critical for labetalol

($9 < pH < 10$) than for chloroquine ($9.2 < pH < 9.3$). The fluorescence yield of labetalol was almost identical with the different buffer mixtures we tested, but for chloroquine it was optimum with the borate buffer in comparison to a carbonate or a glycine buffer.

The fluorimetric detection under the above conditions appears to improve the selectivity of the method [15, 16], which is particularly important for antihypertensive agents which are frequently given in combination.

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