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Note

Determination of pindolol in plasma and urine by thin-layer chromatography*

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Pindolol, DL-4-(2-hydroxy-3-isopropylaminopropoxy)indole, is a widely used non-cardioselective β-adrenoceptor blocking agent with intrinsic sympathomimetic activity. As the doses administered are usually very low and the plasma half-life is comparably short, sensitive methods are necessary for the determination of the drug in biological materials after single or chronic administration. A method for the fluorimetric determination of pindolol in plasma and urine was described by Pacha [1]. This procedure involves extraction from biological material and subsequent derivatization with *o*-phthaldialdehyde to a product that was described to have a 10^4 times higher fluorescence than the parent molecule. This method, however, does not include the separation of the pindolol derivatization product from metabolites or plasma and urine constituents, i.e., the fluorescence is measured in solution without previous chromatography.

Guerret [2] and Guerret et al. [3] suggested a gas- liquid chromatographic method for the determination of pindolol. This method involves a re-extraction step and a derivatization procedure, both of which are time-consuming. This also holds for a high-performance liquid chromatographic (HPLC) method described by Bangah et al. [4]. Mohamed et al. [5] proposed a spectrophotometric method for the determination of pindolol. This method evaluates pindolol in tablets, and therefore no extraction and separation procedures were suggested. Another method, described by Lefebvre et al. [6], is based on HPLC

*Part of the dissertation of M. Prinoth (Frankfurt/M.).

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separation and measurement of UV absorbance. The detection limit of this method is not sufficient for the determination of plasma levels.

A test for monitoring antihypertensive drug compliance with β-blockers was suggested by Jack and co-workers [7, 8]. This method involves an extraction step, which reduces the applicability of the test, and in addition a relatively large volume of urine is necessary.

In view of the above shortcomings, a qualitative test for pindolol that is simple, reliable and easy to apply was developed.

The quantitative method consists of an extraction step, thin-layer chromatographic (TLC) separation and densitometric evaluation of the intrinsic fluorescence. We also investigated whether treatment of the TLC plate after chromatography with a solution of *o*-phthaldialdehyde [9, 10], which has also been proposed as a TLC spray reagent, leads to a marked fluorescence enhancement and thus lowers the detection limit significantly.

EXPERIMENTAL

Chemicals and materials

Solvents (analytical-reagent grade) and pre-coated silica gel 60 TLC plates without fluorescence indicator (20 × 20 and 5 × 10 cm) were obtained from E. Merck (Darmstadt, F.R.G.). Pindolol and its metabolite 4-(2-hydroxy-3-isopropylaminopropoxy)oxindole (23-179) were supplied by Sandoz (Basle, Switzerland) and nadolol (internal standard) by Squibb (Princeton, NJ, U.S.A.).

Instruments

The TLC plates were scanned with a KM 3 chromatogram-spectrophotometer (Carl Zeiss, Oberkochen, F.R.G.) and a Model 56 recorder (Perkin-Elmer, Überlingen, F.R.G.). Solutions were applied on to TLC plates using a Linomat III (Camag, Muttenz, Switzerland).

Extraction from plasma samples

The extraction is performed in screw-capped glass centrifuge tubes, in which 0.5 g of sodium chloride, 1 ml of 5 *M* sodium hydroxide solution, 20 μl of a methanolic solution of nadolol (20 μl = 200 ng of nadolol) and 5 ml of methylene chloride—diethyl ether (20:80) are added to 2 ml of plasma. After shaking (15 min), using a mechanical shaker, the tubes are centrifuged (10 min, 4000 *g*) to separate the layers, then 4 ml of the organic phase are transferred into another tube and evaporated to dryness under a stream of nitrogen at 50°C.

Extraction from urine samples

A 50-μl volume of 1 *M* sodium hydroxide solution, 0.5 g of sodium chloride and 6 ml of *n*-butyl acetate are added to 0.5 ml of urine. The mixture is extracted for 20 min and, after centrifugation at 4000 *g* for 5 min, 5 ml of the organic layer are transferred into another glass tube and evaporated to dryness. The evaporation process is carried out in a vacuum centrifuge at 30°C (Speed Vac Concentrator; Bachofer Laboratoriumswerke, Reutlingen, F.R.G.).

Thin-layer chromatography

A 50-µl volume of methanol is added to the residue of the plasma extract and 40 µl of the resulting solution are then applied to a TLC plate in a 5-mm strip. A volume of 50 µl of *n*-butyl acetate is added to the residue of the urine extract, then 35 µl of this solution are applied to a TLC plate in a 5-mm strip.

To determine the concentration of unknown samples, three extracts from urine and plasma standards are also applied so that a calibration graph is included for each plate. Plasma standard concentrations are 50, 100, and 200 ng/ml and urine standard concentrations are 100, 300 and 500 ng per 500 µl.

The plate is developed at room temperature in an unlined glass tank (Desaga) containing 100 ml of chloroform–methanol–acetic acid (75:20:5). After developing the plate (12 cm, ambient temperature), it is air-dried. The R_F values of pindolol and nadolol are 0.45 and 0.24, respectively.

Fluorescence enhancement

(A) After drying, the TLC plate is dipped into a mixture of 4% paraffin liquid in cyclohexane.

(B) After complete drying (the acetic acid must be removed), the plate is sprayed with an *o*-phthaldialdehyde reagent solution [100 mg of *o*-phthaldialdehyde and 200 µl of mercaptoethanol in ethanol–buffer (pH 11) 1:1)] and heated at 40–50°C for 10 min.

Densitometric evaluation of the chromatogram

The spectrophotometer was operated in the fluorescence mode (arrangement: monochromator–sample). The light source was an ST 41 mercury lamp. For measuring the intrinsic fluorescence of the compounds an excitation wavelength of 265 nm was chosen. Emission was filtered with an M 313 monochromatic filter. The slit was 1 × 6 mm.

For measuring the fluorescence after *o*-phthaldialdehyde treatment, the excitation wavelength was 365 nm and an FL 43 filter was used to filter the emitted light (slit: 1 × 6 mm).

Unknown concentrations were measured by calculating the peak areas of the standards and the samples. If an internal standard was used, the peak area ratio was calculated.

Metabolite and drug interferences

The pindolol metabolite and several drugs and their metabolites were tested for interference with the assay. The substances tested belonged to the following groups of drugs: (a) antirheumatic/analgesic drugs (acetylsalicylic acid and the metabolites salicylic acid and salicyluric acid, azapropazone, phenylbutazone and oxyphenbutazone); (b) other β-adrenoceptor-blocking agents (propranolol, metoprolol and atenolol); and (c) quinidine.

Qualitative test for the urine samples

Depending on the dosage of pindolol, different volumes of urine (3–10 µl) are directly applied to a TLC plate (silica gel 60, 5 × 10 cm) with a disposable ring-marked micro-pipette. As a reference, 20 µl of a methanolic solution containing 10 mg of pindolol per 100 ml are also applied to the plate. The plate is developed in a glass tank containing chloroform–methanol (95:5; ammonia

atmosphere) at ambient temperature. After development, the plate is dried with a hair-dryer until the ammonia is completely evaporated.

For the detection of pindolol, approximately 50 μl of *p*-dimethylaminobenzaldehyde reagent (prepared by dissolving 1 g of *p*-dimethylaminobenzaldehyde in 100 ml of ethanol and adding 10 ml of concentrated hydrochloric acid) are applied to the area of the R_F value (R_F = 0.33), also using a micro-pipette. Pindolol turns blue immediately after the treated area dries.

RESULTS AND DISCUSSION

Fluorescence enhancement and detection limit

The detection limit for pindolol is 2- 4 ng per spot. This can be improved by dipping the plate into paraffin—cyclohexane (A); the fluorescence intensity is then 3.4 times higher and a detection limit of 0.5- 1 ng per spot results. The detection limit in plasma samples is about 2 ng/ml after paraffin—cyclohexane treatment. We tried to improve the sensitivity by spraying the plates with *o*-phthaldialdehyde solution (B), but better results could not be obtained; the detection limit after this treatment was 2.5 ng/ml if a 2-ml sample was used. This is due to the fact that in samples with low concentrations the fluorescence intensity of partially interfering peaks increases to the same extent as that of pindolol. Therefore, method A was used to enhance the fluorescence intensity. Using HPTLC plates we were able to detect 1 ng/ml in plasma samples. For the determination of pindolol in urine, 20 ng per 500 μl was the lowest limit using silica gel 60 plates (20 × 20 cm).

The investigations showed that the plates must be measured immediately after chromatography and not be exposed to light and air, as pindolol decomposes on the TLC plate (see Table I). The fluorescence was more intense and the detection limit, if the pure substance was used, increased after treatment of the plate with *o*-phthaldialdehyde solution. However, if pindolol

TABLE I

CHANGES IN THE FLUORESCENCE INTENSITY OF PINDOLOL (1000 ng PER SPOT) WITH TIME

(A) During storage of the TLC plates in the dark and under vacuum conditions; (B) while exposed to light and air.

Time (h)	Fluorescence intensity (%)		Intensity of violet colour of the decomposition product*	
	A	B	A	B
0.0	100	100	—	—
0.5	100	69	—	+
1.0	100	60	—	+
1.5	100	52	—	++
2.0	100	40	—	+++
5.0	90	28	(+)	++++

*—, No colour, (+), weakly visible; +, visible (the number of +-signs indicates the intensity of the colour).

was extracted from plasma samples, the detection limit was not improved, as the fluorescence of plasma constituents also increased. Further, the deviations between different plates were high, because trace amounts of acetic acid on the plate disturbed the reaction with *o*-phthaldialdehyde (although buffer of pH 11 was added). Hence the determination of the parent compound was more reliable.

Recovery and linearity

Recovery studies were performed by extracting and analysing spiked plasma and urine samples and comparing the resulting peaks with those of a methanolic solution of pindolol. Mean recoveries of 93% for plasma and 71% for urine were obtained. The calibration graphs for plasma and urine were linear up to 350 ng/ml and 2000 ng per 500 μl, respectively.

The coefficient of variation for plasma was 3.7% at a concentration of 50 ng/ml (n = 10) and 5.5% at a concentration of 10 ng/ml (n = 8) with nadolol as an internal standard. For urine the coefficient of variation was 5.5% at a concentration of 200 ng per 500 μl (n = 6).

Drug and metabolite interferences

Plasma constituents do not interfere with pindolol or nadolol, even at low concentrations, as can be seen in Fig. 1A and B, whereas urine constituents influence the nadolol peak (Fig. 1C). This is why nadolol cannot be used as an internal standard in urine samples.

No interference by the metabolite tested is observed.

If triamterene and hydrochlorothiazide are present at very high concentrations there might be interactions. All the other drugs tested do not interfere with pindolol or the internal standard.

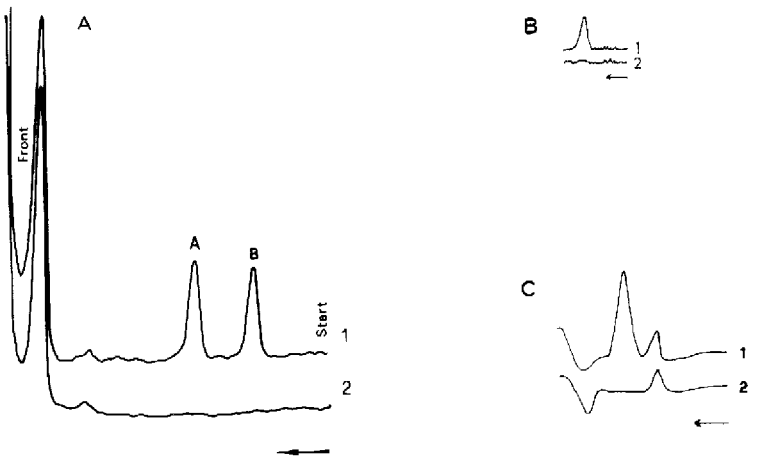

Fig. 1. (A) Thin-layer chromatograms of extracts from a plasma standard containing 50 ng/ml of pindolol (1) and from blank plasma (2). Peaks: A = pindolol; B = internal standard (nadolol). (B) Thin-layer chromatograms of extracts from a plasma standard containing 5 ng/ml pindolol (1) and from blank plasma (2) without an internal standard. (C) Thin-layer chromatograms of extracts from a urine sample obtained 8 h after oral application of 5 mg of pindolol (1) and from blank urine (2). Concentration of pindolol: 178 ng per 500 μl.

Determination of plasma levels

Fig. 2A and B show plasma levels of pindolol with time for samples obtained from two volunteers (multiple oral dosage, plasma levels after application of 10 ng of pindolol during 12 or 24 h). These data show the applicability of the method to pharmacokinetic studies.

Determination of urine levels

The applicability of the method was also tested by analysing urine samples from volunteers who took pindolol at different therapeutic concentrations (2.5, 5 and 10 mg) (Fig. 3). The concentrations found ranged from 20 to 2500 ng per 500 μl.

Urine samples from these volunteers were also analysed by the qualitative method. The value above which every test was positive was 3 ng per spot. This means that, after normal dosing, pindolol is detectable over a period of at least 8 h and consequently allows the assessment of patient compliance for every single day.

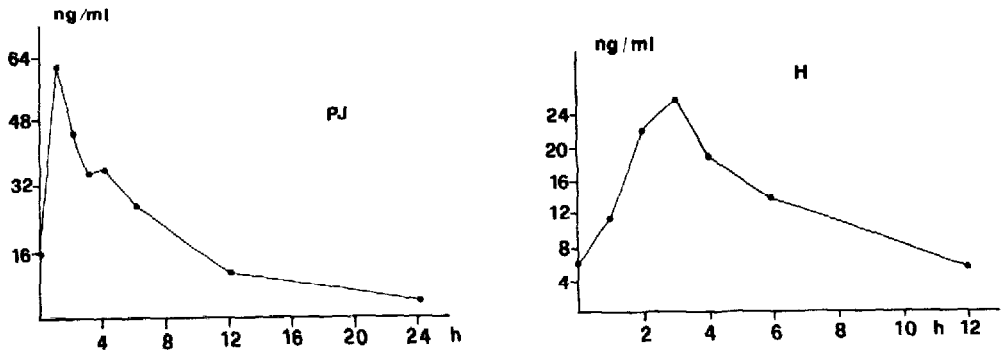

Fig. 2. Plasma levels of pindolol with time for samples obtained from two volunteers (PJ and H, after multiple oral administration of 10 mg of pindolol twice daily) on the seventh day of treatment after the morning dose (10 mg).

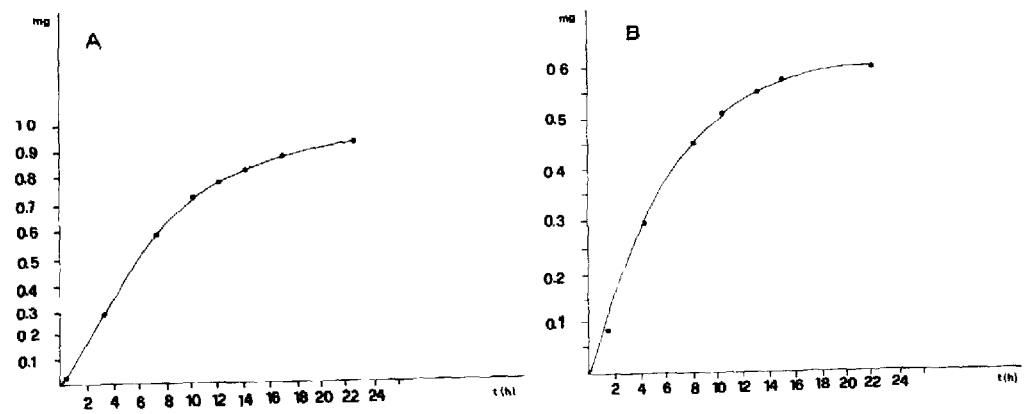

Fig 3. Cumulative urinary excretion of pindolol in one volunteer after oral administration of (A) 5 mg and (B) 2.5 mg of pindolol.

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