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Note

Determination of metipranolol and desacetylmetipranolol in aqueous humor of rabbit eye by gas chromatography with electron-capture detection

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Metipranolol is a β-adrenoceptor blocking drug, recently used in France as eye drops for the treatment of glaucoma. Desacetylmetipranolol is the most important metabolite with major pharmacological activity. Few pharmacokinetic data about the ocular administration of this drug have been published. Moreover, the evaluation of its concentration in aqueous humor requires sensitive trouble-free techniques. The method described by Endele et al. [1] employed a chemical ionization mass spectrometry detection system that is not readily available.

Several techniques have been described for the determination of other β-blocking drugs, which demonstrated satisfactory selectivity and sensitivity by gas chromatography (GC) with electron-capture detection (ECD) after derivatization with trifluoroacetic anhydride (TFAA) or heptafluorobutyric anhydride (HFBA) [2—4]. Recent papers have proposed the determination of β-blocking agents using liquid chromatography [5], but this method is not sensitive enough to apply to very small samples (100—200 μl) of aqueous humor. We propose a simultaneous determination of metipranolol and its desacetylated metabolite with sufficient selectivity and sensitivity.

EXPERIMENTAL

Chemicals

Sodium hydroxide, sodium chloride, dipotassium hydrogen phosphate (RP Normapur, Prolabo, France); heptafluorobutyric anhydride, pyridine (Pierce, Rockford, U.S.A.); diethyl ether, dichloromethane, *n*-hexane, acetone

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(Pestipur, SDS, Peypin, France) were used. The extraction solvent was diethyl ether—dichloromethane—2-propanol (59:40:1). The water used was filtered in a Milli-Q Waters purification system. Metipranolol, desacetylmetipranolol were supplied by Dulcis Labs. (Monaco) and the internal standard, propranolol, was kindly supplied by ICI Pharma (France).

All extraction tubes had glass-stoppered tops and were rinsed with diethyl ether before use. Derivatization was carried out in conical-bottomed glass tubes, which were rinsed three times with methanol and dried to ensure better cleaning.

Standard solutions

Amounts of 10 mg (expressed in base equivalent) of metipranolol, desacetylmetipranolol and propranolol (hydrochloride) powders were accurately weighted into three 10-ml volumetric flasks. An acetone—hexane (20:80) mixture was added to 10 ml and sonicated to facilitate dissolution. These solutions were stored up to three weeks at 4°C. Working dilutions were prepared just before analysis.

Extraction conditions and work-up procedure

In order to obtain a high extraction efficiency of metipranolol and its desacetyl metabolite, various solvents and pH conditions were investigated. The most efficient pH value for the compounds was found to be 10. A mixture of diethyl ether—dichloromethane—2-propanol (59:40:1) was used for extraction.

A 100—200 μl volume of aqueous humor sample was transferred into the extraction tube, and the internal standard (400 μl of 0.1 *M* sodium hydroxide) and 400 μl of a sodium chloride saturated aqueous solution were also added. The aqueous phase was extracted by 8 ml of the solvent extraction mixture. The tubes were shaken for 15 min, then centrifuged at 1500 *g* for 5 min. The organic supernatant was transferred into a ground-glass-stoppered conical-bottomed tube and removed with a stream of nitrogen in a 37°C water-bath.

Derivatization

When it had been ascertained that all the solvent had been removed, 3 μl of pyridine, 500 μl of *n*-hexane and 15 μl of HFBA were added. The tube was vortexed for 15 s, and allowed to react at 0°C for 30 min. The derivatization was stopped by adding 1 ml of a dipotassium hydrogen phosphate saturated aqueous solution, as described in the Pearce derivatization catalogue. The tube was vortexed for 30 s and centrifuged for 5 min at 1500 *g*. The supernatant (2—4 μl) was injected into the chromatograph.

Chromatography

A Varian 3300 gas chromatograph fitted with an 8-mCi ^{63}Ni electron-capture detector and a glass column (2.5 m × 4 mm I.D.) packed with 3% OV 17 on Gas-Chrom Q (80—100 mesh) was used. The instrument parameters were as follows: injector temperature, 270°C; oven temperature, 192°C; detector temperature, 280°C. The carrier gas was nitrogen at a flow-rate of 75 ml/min.

Calculation

To calculate the level of metipranolol and desacetylmetipranolol in experimental samples, a calibration graph of peak-height ratio versus the concentration of known standard was plotted for each one.

RESULTS

Retention time

Under these conditions, the retention time was 2 min 15 s for desacetylmetipranolol, 5 min 31 s for internal standard and 6 min 59 s for metipranolol. The total run-time for one sample before the next injection was 8 min (Fig. 1).

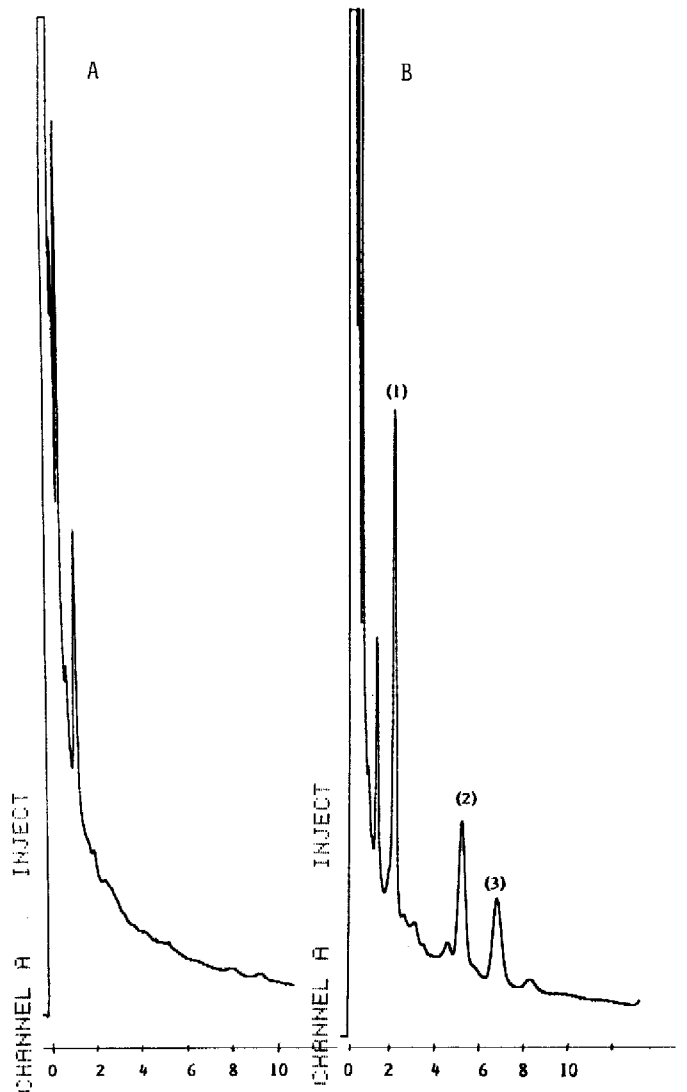

Fig. 1. (A) Chromatogram obtained from an aqueous humor blank; (B) chromatogram obtained from a rabbit eye aqueous humor containing 250 ng/ml desacetylmetipranolol (1), 500 ng/ml metipranolol (3) and propranolol (2) added as internal standard.

Calibration

The correlation coefficient was good for metipranolol in the range 0—800 ng/ml (r = 0.932) and excellent for desacetylmetipranolol in the range 0—4000 ng/ml (r = 0.975).

Analytical recovery, sensitivity and reproducibility

Recoveries of metipranolol and desacetylmetipranolol were determined by comparing the peak-height ratios between extracted aqueous humor samples containing known amounts of metipranolol and of its desacetyl metabolite and untreated samples supplemented with the same amounts of the two molecules. The recoveries of metipranolol and desacetylmetipranolol were 60 and 90%, respectively. The sensitivity of this method was evaluated by analysing aqueous humor samples after addition of metipranolol and desacetylmetipranolol at concentrations reaching almost the sensitivity limit. The parameters of the method remained consistent to 30 ng/ml for desacetylmetipranolol.

The reproducibility was evaluated after extraction of several aqueous humor samples with given concentrations of the β-blocking agent and its metabolite (inter-assay) or after repeated injections of the same extract (intra-assay). The respective results for metipranolol and desacetylmetipranolol expressed by the coefficient of variation were 5 and 7% (inter-assay) and 4 and 6% (intra-assay).

Application of the method

A pharmacokinetic study of the metipranolol and its desacetylated metabolite was carried out on albino rabbits after ocular instillation of Betanol 0.6% (metipranolol 0.6%) in several aqueous humor samples. Results are shown in Fig. 2.

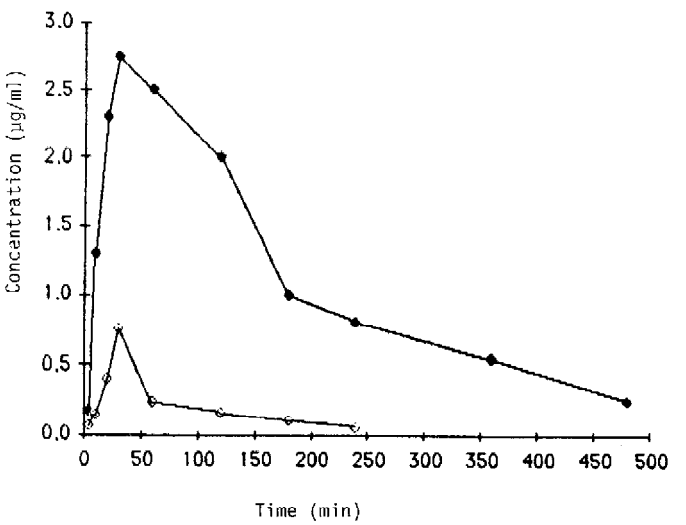

Fig. 2. Concentration—time curve of metipranolol (◇) and desacetylmetipranolol (◆) in rabbit eye aqueous humor after a one-drop instillation of 0.6% metipranolol.

DISCUSSION AND CONCLUSION

Several methods have been described for the determination of various β-blocking agents and each of them used a different extraction solvent. Degen and Riess [6] used diethyl ether—dichloromethane (4:1) and Kinney [4] used this mixture with double the proportion of dichloromethane. The choice of the solvent polarity was not easy because of the great differences in the solubility properties of metipranolol and its metabolite. Using only diethyl ether, the extraction coefficient was 75% for metipranolol, but only 40% for desacetylmetipranolol. Increasing the mixture polarity by adding dichloromethane and isoamylic alcohol significantly improved the efficacy of desacetylmetipranolol but lowered the coefficient for metipranolol. As the biological activity is essentially supported by the metabolite, this was not considered to be an impediment.

In agreement with Endele et al. [1], the addition of saturated sodium chloride solution was found to enhance the recovery. The occurrence of an in vitro conversion of metipranolol into desacetylmetipranolol during the preparation and conservation of standard solution or after extraction was tested. There was no evidence for this transformation.

In conclusion, a one-step extraction method is proposed with electron-capture detection after derivatization. It is simple, reproducible and sensitive enough to allow pharmacokinetic studies even when available samples are very small.

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