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Report

Radioreceptor Assay of Metoprolol in Human Plasma: Comparison with an Enantiospecific High-Performance Liquid Chromatographic (HPLC) Procedure

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Plasma concentrations of metoprolol after acute and repetitive administration of *R/S*-metoprolol to healthy volunteers were measured by a β-adrenoceptor subtype-specific radioreceptor assay (RRA) and by an enantiospecific high-performance liquid chromatographic (HPLC) method. In the RRA, *R/S*-metoprolol showed a 20-fold β_1-subtype selectivity: the *S*-(−)-enantiomer was 35-fold more potent than the *R*-(+)-enantiomer. A comparison between *S*-(−)-metoprolol concentrations detected in the plasma samples by HPLC and those detected by RRA yielded a 1/1 relationship, indicating that active metabolites are not present to a significant extent. These results were independent of the widely scattering metabolic clearance of metoprolol (with the potential of differences in the rate and extent of formation of active metabolites) in the volunteers. In general, HPLC methods can be validated by comparison with RRA in order to clarify whether active metabolites are present and—on the basis of the K_i value from RRA—whether the detection limit of the physicochemical procedure is sufficient to cover the therapeutically relevant range.

KEY WORDS: β-adrenoceptor antagonist; metoprolol; enantiospecific assay; radioreceptor assay.

INTRODUCTION

Measurement of the effective plasma concentrations of a drug including active metabolites in order to correlate drug level and therapeutic effect represents a major aim of pharmacokinetics (1). For several groups of drugs, namely, those for which receptor preparations as well as radioligands are available, the radioreceptor assay (RRA) has turned out to be a very efficient and reliable analytical method. Further, the correlation of the obtained antagonist plasma concentrations from RRA with the observed clinical effect was significant, e.g., for the β-adrenoceptor antagonists (2,3).

All compounds with a suitably high affinity to this receptor, including active metabolites, are quantified as they competitively displace the radioligand. In the case of β-adrenoceptor antagonists, the affinity to receptors differs significantly between enantiomers. However, most of them are administered as racemates.

Apart from the different activities of the enantiomers, differences in the disposition (4) were found between the enantiomers. Thus, measurement of the total concentrations *(S+R)* applying physicochemical methods may be misleading, as the *S/R* ratio can change versus time, between individuals, and between different compounds. Enantiospecific methods are therefore needed (5), which have the advantage of not utilizing radioactive material and may include the detection of possible active metabolites.

The β-adrenoceptor antagonist metoprolol {*R/S*-(±)-1-isopropylamino-3-[4-(2-methoxyethyl)phenoxy]-2-propanol} has been investigated extensively with respect to a possible metabolic polymorphism, including stereoselective effects (6,7). The aim of the present study was to establish concentration/*in vitro* effect curves of metoprolol enantiomers on the basis of their receptor binding parameters and to compare the results obtained with RRA and with an enantiospecific assay method for the parent drug with physicochemical detection. Displacement of the radioligand from the binding sites of the receptor was correlated with the concentrations of the metoprolol enantiomers in plasma samples from different volunteers, including slow metabolizers, after single and repetitive oral administration.

METHODS

Biological Samples

The samples were taken from a previously performed study on enantioselective drug disposition of metoprolol dependent on the sparteine clearance (8).

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The investigation was performed in five healthy volunteers, from which two had been identified as non- or moderate metabolizers: one volunteer excreted 98.8% and another 45.8% of the administered sparteine dose unchanged in urine, whereas the urinary excretion in the other volunteers was lower than 29% (8). The correlation of total metoprolol clearance and polymorphic sparteine oxidation was described previously (9).

The present study consisted of two parts. In the first part, metoprolol, 100 mg (as tartrate; Lopresor), was given once as a single oral dose to two volunteers; and in the second part, twice daily for 7 days and in the morning of day 8 to three volunteers. After single-dose administration or for multiple-dose administration on day 8, blood samples were taken before and 1, 2, 3, 4, 6, 12, 24, and 48 hr after dosing. Plasma was obtained by centrifugation of heparinized blood samples, immediately frozen in two separate portions and stored at −20°C until analysis. One portion was used for the RRA; the other, for the enantiospecific high-performance liquid chromatographic (HPLC) assay. To avoid inaccuracies caused by changes in protein binding of the enantiomers, samples were thawed once only for the RRA.

Compounds Used

(−)-^{3}H-CGP 12177 (sp act, 40–50 Ci/mmol) was from Amersham–Buchler (Braunschweig, F.R.G.). Racemic metoprolol tartrate and the enantiomers (*R*-metoprolol as hydrochloride and *S*-metoprolol as tartrate) were kindly provided by Astra Chemicals (Wedel, F.R.G.). Acetylphenyl hydrazine was obtained from Schuchardt (Munic, F.R.G.).

Receptor Binding Studies

Membrane Preparations

Membrane preparations were used to measure the affinity of β-adrenoceptor antagonist enantiomers to β-adrenoceptors *in vitro*. The β_1/β_2 selectivity of racemic metoprolol was measured with two membrane preparations, which contain either β_1- or β_2-adrenoceptors only. The preparation of the membrane fractions was performed as described earlier (10). Briefly, salivary gland membranes were prepared from salivary glands of untreated White Wistar rats. After their excision they were homogenized in ice-cold 155 m*M* sodium phosphate buffer, pH 7.4. The 12,000*g* precipitate was washed twice with pH 7.4 buffer, resuspended, and stored at −40°C. To obtain reticulocyte membranes, rats were treated with acetylphenyl hydrazine (40 mg/kg, 3 days) to induce reticulocytosis. On the seventh day the rats were exsanguinated, and their blood was heparinized and centrifuged at +4°C at 1500*g* (10 min). The supernatant was removed and the precipitate washed twice with 155 m*M* phosphate buffer, pH 7.4. After cell lysis, the lysate was centrifuged at 12,000*g*. The membrane fraction was washed twice with phosphate buffer, then reconstituted in buffer and stored at −40°C.

Preparations from rat reticulocytes exhibit a high density of β_2-adrenoceptors, whereas membrane preparations of the rat salivary gland were the source for β_1-adrenoceptors, as they contain only a β_1-subpopulation. The nonselective antagonist ligand (−)-^{3}H-CGP 12177 [(−)-4-(3-t-butylamino-2-hydroxypropoxy)-5,7-^{3}H benzimidazole-2-one] served as a radiolabel at the adrenoceptors.

Membranes (50–100 μg of protein) were incubated with 20 μl of radioligand (~1 n*M*), 30 μl of unlabeled competitor (0.1 n*M*–1 m*M*), and 200 μl of human plasma from a drug-free period, at 25°C for 1 hr. To detect antagonist present in unknown samples, 200 μl of plasma from the verum period was used, and the competitor was replaced by buffer solution. Membrane-bound radioligand was separated by a rapid filtration of the total sample volume through glass-fiber filters (AP15, Millipore, Dreieich, F.R.G.). Retained radioactivity was detected by liquid scintillation counting (for further details see (11)).

The samples were run at least in triplicate. The values used were the arithmetical means.

Data Analysis

The following equation was fitted to the receptor binding data by a nonlinear least-squares fitting procedure [GIP package (2)]:

$$B = B_{max} \times [^3H]/\{[^3H] + K_{D^3H}(1 + [i]/K_i)\} + {}^3H \times nsb$$

where B is bound radioligand, B_{max} the maximal capacity of binding, [^{3}H] the concentration of the radioligand and K_{D^3H} its equilibrium dissociation constant, [i] the concentration of inhibitor (metoprolol), K_i the respective equilibrium dissociation constant of the inhibitor, and nsb the slope of nonspecific binding of the radioligand. From this equation binding parameters of the inhibitor and the amount of β-adrenoceptor antagonist present in plasma samples (as metoprolol equivalents) were calculated using a Hewlett–Packard 9826 desktop calculator (11).

The pharmacokinetic model applied for the metoprolol concentration time curve is based on a Bateman function:

$$i_t = C_o \times k_a/(k_a - k_e) \times [\exp(-k_e \times t) - \exp(-k_a \times t)]$$

where i_t is the concentration of antagonist at time t, C_o is the apparent concentration at zero time, and k_a and k_e are absorption and elimination rate constants. The corresponding half-lives ($t_{1/2}$) were then calculated as ln2/k.

Furthermore, a lag time was included ($t' = t - t_{lag}$). These procedures had been validated earlier in studies of kinetic, receptor binding, and effect data of other β-adrenoceptor antagonists in man (2).

HPLC Measurement of Metoprolol Enantiomers

S-(−)- and *R*-(+)-metoprolol were assayed after extraction from the biological samples with diisopropyl ether, and chiral derivatization was performed with *R*-(−)-phenylethyl isocyanate. The diastereomeric urea derivatives of *R*- and *S*-metoprolol were quantified after reversed-phase HPLC (ODS column, methanol–water as mobile phase) by measuring the intrinsic fluorescence of metoprolol (12). The detection limit was ~2 ng/ml per enantiomer. The coefficients of variation were between 7 and 8%.

RESULTS AND DISCUSSION

In Vitro Characterization of the Affinity of Metoprolol to β-Adrenoceptors

As depicted in Fig. 1A metoprolol has an affinity to β_1 and β_2 receptors. The K_i values are 67 n*M* for β_1 and 1390 n*M* for β_2. Thus, the β_1/β_2 selectivity factor is about 20. Comparison of the two metoprolol enantiomers at the β_1 receptor (Fig. 1B) resulted in an enantioselectivity *S*-(−)/*R*-(+) of 33 to 50 not taking into account possible enantiomeric impurities, which, if present in the *R*-(+)-enantiomer, can strongly influence its apparent K_i. The K_i values for the *S*-(−)-enantiomer and the *R*-(+)-enantiomer were 85 ±15 n*M* and 4 μ*M*, respectively. Hence, the observed effect at the β-adrenoceptor was caused largely by the *S*-(−)-enantiomer. For the RRA the coefficient of variation was below 8%, and the detection limit was 1–2 ng equiv./ml. The degree of nonspecific binding was below 10% (3–8%).

Comparison of RRA and HPLC

The receptor assay and enantiospecific HPLC (*S*-enantiomer concentrations) gave congruent results in single- and multiple-dose studies with slow and rapid metabolizers. In Fig. 2 (the arrows mark the K_i value), the respective data are shown for two volunteers, an extensive metabolizer and a nonmetabolizer with a very low metabolic sparteine clearance, using either RRA (Fig. 2A) or HPLC (Fig. 2B). The concentrations in the nonmetabolizer were above K_i (concentration, at which 50% of the receptors are occupied) significantly longer than in the rapid metabolizer.

The half-lives obtained for the two volunteers either by linear regression analysis from the HPLC data or by application of the curve-fitting program for the RRA data are almost the same (poor metabolizer, 7.2 hr; extensive metabolizer, 4.0 hr). Drug concentrations determined by RRA [calculated as *S*-(−)-metoprolol equivalents] and stereospecific HPLC correlated well (Fig. 3; calculated regression line, $y = 1.17x + 6.4$; $r = 0.71$). The good correlation between RRA and physicochemical detection depicted in Figs. 2 and 3 argues against the presence of significant amounts of a metabolite of metoprolol active at β-adrenoceptors.

Validation of a Chemical Assay via RRA

From methods based on physicochemical detection alone—even if they are highly sensitive and/or enantiospecific—no conclusion is possible concerning duration of ac-

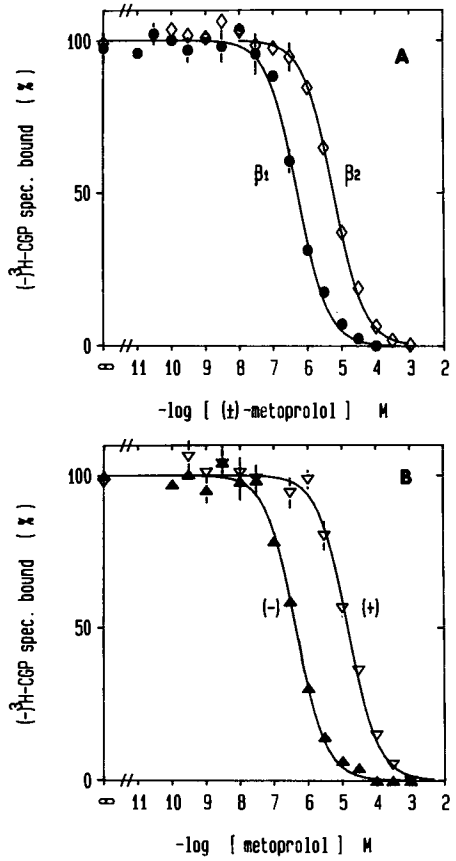

Fig. 1. Competition between metoprolol and (−)-^{3}H-CGP 12177 at the β-adrenoceptors from rat salivary gland (β_1) and reticulocytes (β_2), respectively (human plasma present). (A) *R/S*-(±)-Metoprolol at β_1- and β_2-adrenoceptors; (B) *S*-(−)- or *R*-(+)-metoprolol at β_1-adrenoceptors.

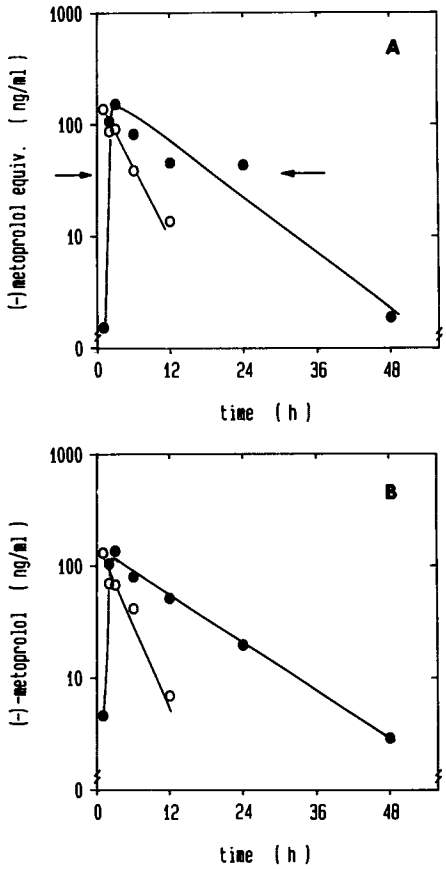

Fig. 2. Plasma concentrations within 48 hr detected in two volunteers with different sparteine clearances. Amount of unmetabolized sparteine excreted renally: (○) 22.5%; (●) 98.8%. (A) RRA [concentration in *S*-(−)-metoprolol equivalents]; (B) enantiospecific HPLC determination of *S*-(−)-metoprolol. The arrows give the K_i value.

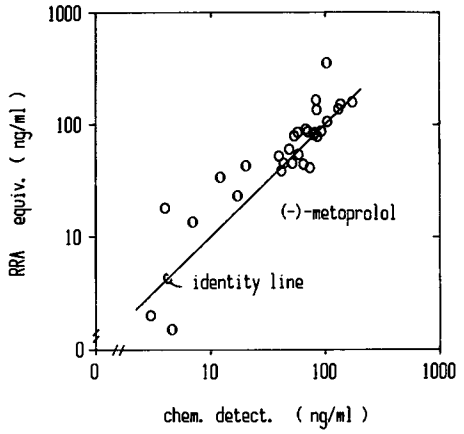

Fig. 3. Correlation between RRA and enantiospecific HPLC determination. *S*-(−)-Metoprolol equivalents from RRA correlate 1/1 with *S*-(−)-metoprolol from HPLC determination.

tion or potential toxicity. HPLC data alone do not define whether the measurable concentrations are in a therapeutically relevant range, i.e., the pharmacodynamic effect cannot be assessed, whereas RRA data make a prediction of β blockade possible. They allow for definition of the time interval during which a significant reduction in heart rate is to be expected and whether the administered dose is reasonable (2,13). A 50% β blockade in man coincides with plasma concentrations close to the K_i value of a β blocker, e.g., atenolol or propranolol (2). Regarding the duration of significant receptor occupancy, a clear difference can be observed between extensive and poor metabolizers. In the case of extensive metabolizers, metoprolol is eliminated faster and K_i is reached within 6 to 12 hr after drug administration, whereas the metoprolol concentrations found for the poor metabolizers are above K_i during a period of 24 hr after 100 mg of metoprolol p.o. This is in agreement with the findings of McGourty *et al.* (6) and Silas *et al.* (14), who were able to show a significantly longer β-adrenoceptor blockade in poor metabolizers. Therefore, metoprolol has to be administered twice daily in the extensive metabolizers but only once daily in the poor metabolizers.

On the basis of K_i receptor binding values, it is also possible to evaluate whether the sensitivity of a method is sufficient to cover the therapeutically relevant concentration range. From concentration effect considerations for competitive antagonists at membrane-bound receptors, a detection to at least 1/10 of the K_i value is useful, where the receptor occupancy is expected to be <10% (2). Comparison of the K_i value in the RRA and detectability by physicochemical assays of β-adrenoceptor antagonists indicates that this quality criterion is fulfilled for propranolol, atenolol, and bisoprolol [HPLC detection limit, ~0.1 × K_i (15)], while for other β blockers the physicochemical assay is not sensitive enough to cover the relevant range because of low K_i values [e.g., carteolol; LC detection limit, ~3 × K_i (11)]. The metoprolol HPLC assay used in the present paper is sufficiently sensitive as judged by metoprolol's β_1-receptor affinity.

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REFERENCES

1. H. Druckrey and K. Küpfmüller. *Pharmazie* 8 (Suppl. 1):514–645 (1949).
2. A. Wellstein, D. Palm, G. G. Belz, and H. F. Pitschner. *Drug Res.* 35:2–6 (1985).
3. A. Wellstein and D. Palm. *Eur. J. Clin. Pharmacol.* 29:293–300 (1985).
4. B. Testa. *Trends Pharmacol. Sci.* 7:60–64 (1986).
5. E. J. Ariens. *Eur. J. Clin. Pharmacol.* 26:663–668 (1984).
6. J. C. McGourty, J. H. Silas, M. S. Lennard, G. T. Tucker, and H. F. Woods. *Br. J. Clin. Pharm.* 20:555–566 (1985).
7. M. S. Lennard, G. T. Tucker, J. H. Silas, S. Freestone, L. E. Ramsey, and H. F. Woods. *Clin. Pharmacol. Ther.* 34:732–737 (1983).
8. G. Pflugmann, W. Kirch, E. E. Ohnhaus, H. Spahn, E. Mutschler, and M. Eichelbaum. Submitted for publication.
9. W. Kirch, E. E. Ohnhaus, C. Zekorn, M. Eichelbaum, H. Spahn, and E. Mutschler. *Arch. Pharmacol. Suppl.* 8:401–402 (1985).
10. A. Wellstein, D. Palm, J. H. Matthews, and G. G. Belz. *Meth. Find. Exp. Clin. Pharmacol.* 7:645–651 (1985).
11. A. Wellstein, D. Palm, G. Wiemer, M. Schäfer-Korting, and E. Mutschler. *Eur. J. Clin. Pharmacol.* 27:545–553 (1985).
12. G. Pflugmann, H. Spahn, and E. Mutschler. *J. Chromatogr.* 421:161–164 (1987).
13. A. Wellstein, D. Palm, G. G. Belz, and H. F. Pitschner. *Eur. J. Clin. Pharmacol.* 29:131–147 (1985).
14. J. H. Silas, J. C. McGourty, M. S. Lennard, G. T. Tucker, and H. F. Woods. *Eur. J. Clin. Pharmacol.* 28:85–88 (1985).
15. A. Wellstein, D. Palm, G. G. Belz, G. Leopold, K. V. Bühring, and J. Pabst. *J. Cardiovasc. Pharmacol.* 8 (Suppl. 11):S41–S45 (1986).