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STEREOSELECTIVE BINDING IN CARDIAC TISSUE OF THE ENANTIOMERS OF BENZETIMIDE, AN ANTIMUSCARINIC DRUG

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1 Benzetimide, possessing two stable enantiomers, dexetimide and levetimide, has been investigated in guinea-pig atria with respect to its atropine-like action and its tissue distribution.

2 The antagonistic potency of dexetimide was found to be over 6000 times higher than that of levetimide, the pA_2 values being 9.82 and 6.0 respectively.

3 The tissue accumulation was investigated for both isomers in the concentration range from 1.5×10^{-9} M to 10^{-6} M yielding tissue to medium ratios (T/M) of between approximately 50 and 10. The highest values were found for the lowest concentrations. At any concentration investigated, dexetimide exhibited a higher uptake than the levoisomer.

4 The rate of uptake and washout of dexetimide was extremely slow, that of levetimide being considerably faster at equimolar concentrations. The same pattern held true for the onset and decline of the antagonistic action.

5 The high accumulation was found to be almost entirely due to unspecific binding. Even in the case of dexetimide the relative size of the receptor compartment could not be determined. The unspecific binding sites displayed a certain stereoselectivity but to a much lesser extent than the specific receptor binding sites.

Introduction

Benzetimide is an atropine-like compound which acts in both the peripheral and central autonomic nervous systems. It has been shown in both intact organisms and isolated organs that the antimuscarinic action of benzetimide is significantly greater than that of atropine (Janssen & Niemegeers, 1967; Janssen, Niemegeers, Schellekens, Demoin, Lenaerts, van Nueten, van Wijngaarden & Brugmans, 1971). Like atropine, benzetimide exists chemically as a racemic compound, possessing two optical isomers. However, whereas in the case of atropine these isomers spontaneously interconvert, both dexetimide and levetimide, the enantiomers of benzetimide, are stable in this respect.

Chemical studies using X-ray diffraction and other methods have revealed the S-configuration of the highly active dextro-enantiomer. This conformation coincides closely with the trans-conformation of acetylcholine, which is thought to be responsible for the muscarinic activity of the molecule (Spek, Peerdeman, van Wijngaarden & Soudijn, 1971). For the sake of clarity the active groups of the benzetimide molecule which resemble atropine are heavily marked in the structural formula shown in Figure 1.

An earlier attempt was made to correlate the biological effect of atropine with its quantitative uptake and washout in guinea-pig atria. These investigations were hampered by the considerable degree of accumulation found in our radioactivity experiments, leading us to suspect the involvement of a large proportion of unspecific binding sites. However, these findings provided a possible explanation for the apparently paradoxical nature of atropine, with its competitive antagonistic action and its remarkably delayed uptake and washout. In order to see whether this kinetic behaviour held true for other antimuscarinic compounds, benzetimide has been subjected to similar investigations. We chose benzetimide since it seemed a particularly attractive tool: its enantiomers are not only stable but are also unusually dissimilar in their antagonistic potencies.

However, the results of our investigations led us in another direction. First, unusually high tissue to medium ratios (T/M) were obtained. Second, the unspecific binding sites appeared to be capable of exerting a certain stereoselectivity towards the two isomers. It is on these latter findings particularly that we wish to report in this paper.

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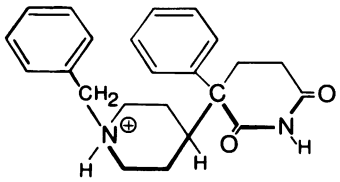

Mol.wt = 398,92
pA_2 = 9,82 Dexetimide
pA_2 = 6,0 Levetimide
pKa = 8,70

Figure 1 Structure of benzetimide. That part of the molecule, which resembles the active groups of atropine is accentuated (facing downwards). Mol. wt. = molecular weight; pA_2 values denote the atropine like potency of the two enantiomers; pK_a = dissociation constant of the tertiary nitrogen.

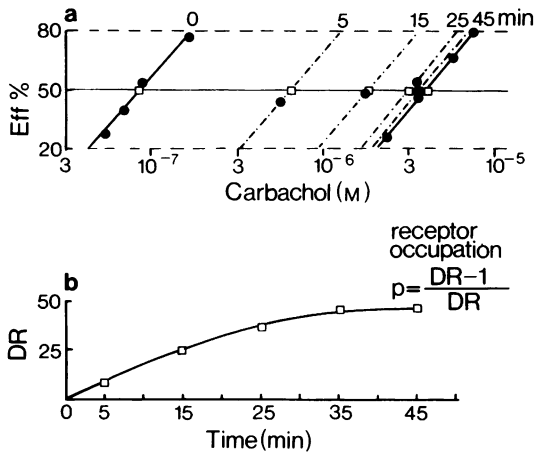

Figure 2 (a) Experimental procedure to demonstrate a parallel shift of the dose-response curve induced by levetimide (3×10^{-5} M) and the time course of onset of antagonistic action. (●) Experimental values; (□) calculated ED_{50}'s after addition of levetimide, as indicated on top of the curves. (b) Calculated dose-ratio values (DR) after adding the antagonist; for details see methods section.

Methods

Determination of the pharmacological effect of benzetimide

Electrically driven left auricles of guinea-pigs were used throughout this investigation. The following standard procedure was employed: after dissection the muscle was transferred to an organ bath containing Tyrode solution of the following composition (mmol/l): NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 12.0, NaH_2PO_4 0.21. The solution, with a resultant pH-value of 7.4, was gassed with 95% O_2 and 5% CO_2 and maintained at a constant temperature of 32°C. The stimulating pulses were of 4 to 5 ms duration, and 7 to 10 V, corresponding to double threshold; the frequency of stimulation was 180/minute. The contractions of each muscle were isometrically recorded via a standard transducer/amplifier/pen recorder system (SWEMA, Hellige 19).

After an equilibration period of at least 30 min the initial carbachol dose-response curve was determined. For this purpose, at least four different concentrations of carbachol, each eliciting an effect between 20 and 80% were applied, using the following procedure: (a) an equilibration period of 5 min in fresh Tyrode solution (b) exposure to a dose of carbachol for 3 min (c) a washout period of 2 minutes. This procedure having been carried out for each of the 4 concentrations of carbachol, the antagonist benzetimide was added to the Tyrode solution. Thereafter the time course of the onset of the antagonistic action was determined by applying test doses of carbachol at fixed time intervals. These doses were chosen to yield as closely as possible a 50% effect, as shown in Figure 2. After the attainment of final antagonistic equilibrium at which no further shift of the dose-response curve could be detected, a second complete dose-response curve for carbachol was determined. Fresh (benzetimide-free) Tyrode solution was then taken and the same procedure adopted to determine the disappearance, or decline of the antagonistic action.

Determination of the uptake and washout of ^3H-labelled benzetimide

Tritium-labelled dexetimide and levetimide were used, the specific activity amounting in both cases to 600 mCi/mmol. The radiochemical purity was determined as greater than or equal to 97% for dexetimide or less than or equal to 99% for levetimide. Throughout the experiments large organ baths containing electrodes capable of accommodating several auricles at once were used. After an equilibration period of 30 min in normal Tyrode solution the isolated auricles were transferred to a bath containing the radioactively labelled compound; 1.5×10^{-9} M was found to be the lowest concentration which could be reliably employed in practice, the Tyrode solution then yielding a disintegrations per minute (d/min) value of approximately 700/ml i.e. at least four times that of the background count. The auricles were removed from the bath after various fixed periods of time (5–180 minutes). Each was blotted according to a

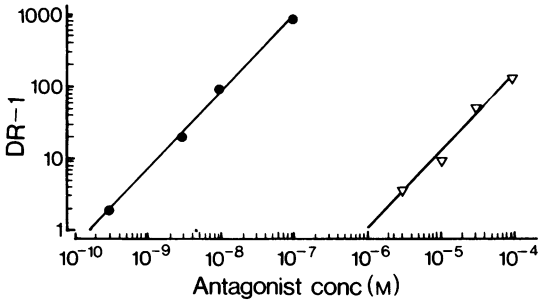

Figure 3 Double logarithmic plot (according to Arunlakshana & Schild, 1959) to demonstrate the competitive mode of antagonism, and the different antagonistic potencies of the two enantiomers. Dexetimide (●), $pA_2 = 9.82$. Levetimide (△), $pA_2 = 6.0$.

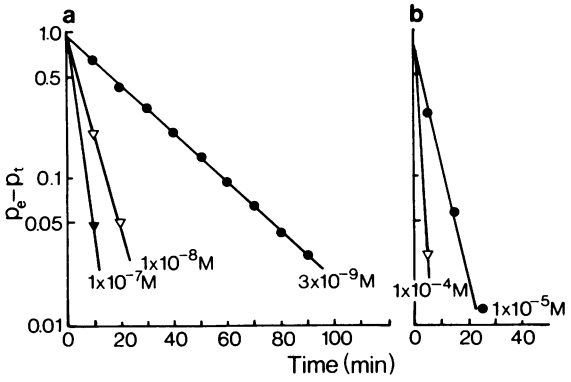

Figure 4 Onset of the antimuscarinic action of the two enantiomers in guinea-pig auricles. Ordinates: receptor occupation ($p_e - p_t$); abscissae: time of incubation. (a) Half-life times of the onset of dexetimide's action were 18, 5 and 2 min for the concentrations 3×10^{-9} M, 1×10^{-8} M and 1×10^{-7} M respectively. (b) The corresponding values for levetimide were 4 and 1 min at concentrations of 1×10^{-5} M and 1×10^{-4} M, respectively. The points represent means of at least 6 individual experiments, s.e. mean was less than 5%.

standard procedure, weighed and then dissolved in 1 ml of a tissue solvent (Soluene 50, Fa. Packard-Instruments). Aliquots from each sample of dissolved tissue were mixed with liquid scintillation fluid (Unisolve, Fa. Zinsser) neutralized by 0.1 M HCl, and counted in a scintillation counter (Packard Tri-Carb Liquid Scintillation Spectrometer, Model 544).

For the washout experiments, the auricles were loaded with the labelled benzetimide at different concentrations for 60 minutes. The tissue samples were then transferred to a bath containing 'benzetimide-free' solution and removed after various fixed periods of time. Thereafter the same procedure as that used for the uptake experiments was adopted.

The radioactivity counts were then expressed as d min^{-1} g^{-1} wet weight (w.wt.) or as tissue/medium ratios (d min^{-1} g^{-1} w.wt./d min^{-1} ml^{-1} of incubation medium).

A brief explanation of the dose-ratio (DR) technique (Paton, 1961)

Information concerning the initial slope of the curve and the concentration producing a 50% effect was obtained from the original dose-response curve. As shown in Figure 2 a parallel slope is evaluated at antagonistic equilibrium. From this fact we may assume a parallel shift of the dose-response curve throughout the onset of antagonistic action. Hence a theoretical ED_{50} can be calculated for the time of each test dose application. The ratio, ED_{50} at time t in the presence of antagonist/initial ED_{50}, is the dose-ratio (DR). According to Paton (1961) the receptor occupation, p, is defined as (DR−1)/DR. Using the p-values thus obtained, semilogarithmic plots can be made for the onset and decline of reactions as seen in Figures 4 and 5.

Results

Onset and decline of antagonistic action

When the dose-ratios obtained for the onset reaction were plotted against time a final equilibrium value was attained as shown in the lower part of Figure 2. The level of this final value was dependent upon the concentration of the antagonist used, that is to say the higher the concentration, the higher the level and the speed of its attainment.

As shown in Figure 3 a double logarithmic plot was then made of the equilibrium values (DR−1) versus the respective antagonistic concentrations. Straight-line graphs were obtained for both enantiomers. The negative logarithmic value of the abscissal cutting point of such a line is equal to the pA_2-value. The corresponding pA_2-value for dexetimide was found to be 9.82, that for levetimide 6.0. Thus the difference between the antagonistic potencies of the isomers is remarkably great, exceeding a factor of 6600 as already reported by Mitchelson (1975) and Soudijn, van Wijngaarden & Ariëns (1973).

In order to obtain information about the time course of the onset and decline of antagonistic action, graphs were plotted as shown in Figures 4 and 5. For onset (see Figure 4) receptor occupation at equilibrium, which is p_e, minus receptor occupation at time t which is p_t was plotted against time. In this way straight-line curves of negative slope could be

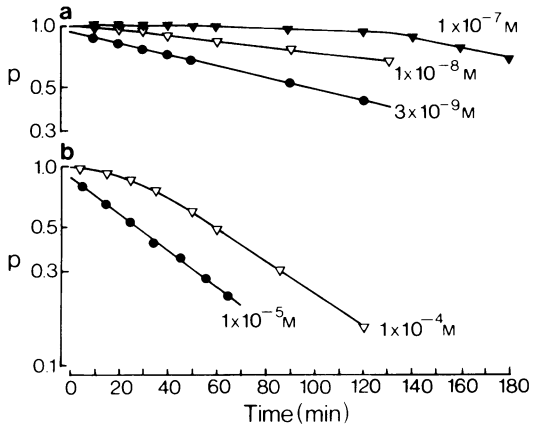

Figure 5 Decline of the antimuscarinic action of the two enantiomers in guinea-pig auricles: (a) dexetimide, (b) levetimide. Ordinates: receptor occupation (p); abscissae: time of washout of the compounds. Only the lowest concentrations of both enantiomers yielded straight lines with half-life times of 110 min for 3×10^{-9} M of dexetimide and 35 min for 1×10^{-5} M of levetimide, concentrations which are approximately equieffective with respect to the antagonistic action. The points represent means of at least 6 individual experiments; s.e. mean was less than 5%.

described, thus making possible the evaluation of half-life times for the time course of the onset reaction. The intercept with the ordinate represents the receptor occupation after the attainment of antagonistic equilibrium. For dexetimide it is 93% for a concentration of 3×10^{-9} M; 98% for 10^{-8} M and 99% for 10^{-7} M. For levetimide it is 90% for a concentration of 10^{-5} M, 98% at 10^{-4} M. Longer half-life times were found for lower concentrations. Nevertheless at equieffective concentrations of both isomers the half-life time of onset was considerably shorter in the case of levetimide.

To describe the decline of the reaction, graphs of receptor occupation (p) against time were plotted as shown in Figure 5. At low concentrations straight lines representing a monoexponential decline of the receptor occupation were found for both isomers. However, with increasing concentration a delay of the initial phase of the time course of decline became evident. This 'plateau' effect was most pronounced for dexetimide at the highest concentration used. This phenomenon will be discussed at a later stage. In comparing the decline curves of the two isomers at equieffective concentrations it could be seen that the decline in receptor occupation was significantly more rapid in the case of levetimide.

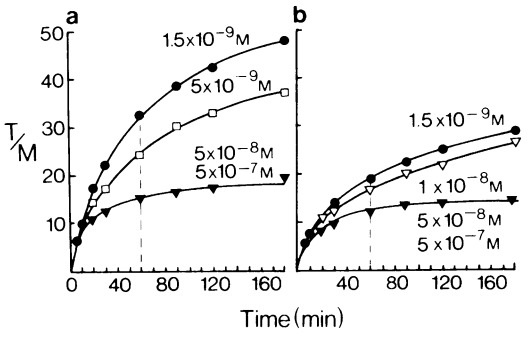

Figure 6 Accumulation of ^{3}H-labelled enantiomers in guinea-pig auricles: (a) dexetimide, (b) levetimide. Ordinates: concentration of the compounds expressed as tissue to medium (T/M) ratios. The points represent means of at least 8 individual auricles; s.e. mean was less than 5%. The dotted lines indicate the T/M values at which the washout experiments were started (see Figure 7).

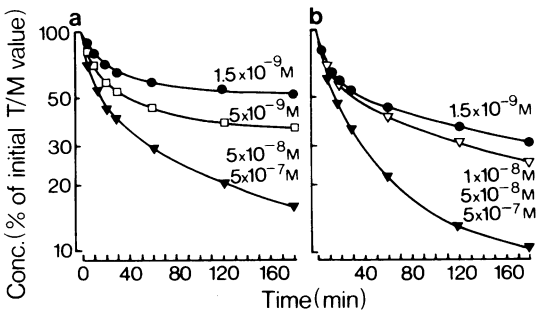

Figure 7 Release of ^{3}H-labelled enantiomers in guinea-pig auricles. Ordinates: concentration of drugs expressed as percentage of the initial T/M value after 60 min of incubation (see Figure 6); abscissae: time of washout. The points represent means of at least 8 individual auricles; s.e. mean was less than 5%.

Uptake and washout of labelled benzetimide

The uptake of the two isomers was investigated in the concentration range, 1.5×10^{-9} M to 10^{-6} M. As depicted in Figure 6 graphs of T/M ratios against time were plotted with the following results: first after 3 h of incubation in no case was a final equilibrium value attained; second the highest T/M ratios corresponded to the lowest concentrations of both isomers, e.g. T/M values of 48, 28 for 1.5×10^{-9} M dexetimide and levetimide respectively. These values reflect a surprisingly high accumulation capacity in the cardiac

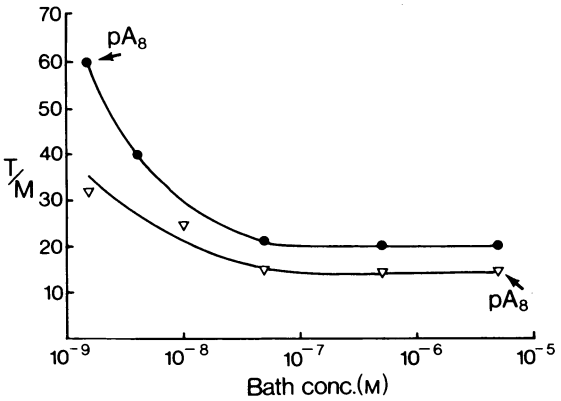

Figure 8 The final values of accumulation of the isomers are plotted versus the corresponding bath concentrations. (●) Dexetimide; (▽) levetimide. The antimuscarinic activity is marked by the pA_8-values for both enantiomers.

tissue. At higher concentrations the T/M values diminished and reached a minimum at a concentration of 5×10^{-8} M. Above this concentration no further decline in the T/M values was detectable (T/M values of 20, 15 for dexetimide, levetimide respectively). A more detailed analysis of the curves revealed that in no case was a simple monoexponential function obeyed. Furthermore, the rate of accumulation was proved to be consistently higher for levetimide.

The washout curves were plotted semi-logarithmically in relative values as shown in Figure 7. The T/M values after 60 min of incubation (see dotted lines in Figure 6) were taken as the 100% values or starting points for the washout process. In general the drug release process could be described in terms of an initial fast and second slower phase. With increasing concentration the process was accelerated. As can be clearly seen in Figure 7, at equal concentrations the washout of levetimide is quicker than that of the dextro-isomer.

Discussion

As has already been mentioned the advantages of using benzetimide in these kinetic studies stem from the stability of its enantiomers and the unusually large difference between their antagonistic potencies (see Figure 3). The combination of both these properties provides us with the possibility of distinguishing clearly between the two enantiomers in our investigations. This distinction may be applied to both static equilibrium values and kinetic data obtained. This is clearly demonstrated in respect of the former in Figure 8.

Differing final T/M values were found for the two isomers after a 3 h incubation period. It should be noted that no terminal equilibrium values were obtained. Figure 8 shows the relationship between the calculated equilibrium T/M ratios and the corresponding bath concentrations. This represents the equilibrium binding curves of both isomers over the entire concentration range investigated. On examination of the diagram the following become clear: (1) that the overall binding differs between the two isomers; (2) that below a concentration of 10^{-7} M a saturable compartment is evident in the case of both isomers (i.e. the curves show a negative gradient); (3) that above a concentration of 10^{-7} M a non saturable compartment is present (i.e. beyond this concentration the curves run parallel to the abscissa scale). These results should be analysed in conjunction with the kinetic data obtained from our onset/decline experiments and the corresponding uptake/washout investigations. Such an approach reveals the following: dexetimide attains approximately 100% receptor occupation by a concentration of 10^{-7} M (see Figure 4). In contrast, at such a concentration there is no measurable receptor occupation for levetimide ($pA_2 = 6{,}0$). In this case the total receptor occupation corresponds to a concentration exceeding 10^{-5} M. Relating this to the binding curves in Figure 8 an interpretation can be made as follows: whereas both enantiomers show an initial 'saturable' phase, different binding sites are involved at this stage. In the case of dexetimide this is the receptor compartment; in the case of levetimide we must deduce that an unspecific saturable binding compartment is responsible, displaying nevertheless a higher affinity for the drug than the specific compartment. Indeed when the receptor compartment of levetimide eventually comes into play it is overwhelmed by a predominant unspecific unsaturable compartment.

The difference between the initial phases of the two curves can thus be understood in terms of the presence of two distinct saturable binding sites.

During the later 'unsaturable' phase of accumulation, when physico-chemical properties are normally assumed to underly the distribution of a drug, one would expect the T/M values for the two isomers to reach the same final level. However, it should be noted that this is not the case. In addition the results of our radioactivity experiments reveal significantly differing rates of uptake and washout when comparing the behaviour of both isomers at equal concentrations. Combining these two results we may justifiably conclude that unspecific binding sites may also display stereoselective properties, being capable of distinguishing between left and right rotating enantiomers.

At present little information is available in the literature concerning the stereo-selectivity of unspecific binding sites. Different binding affinities of

plasma albumin have been demonstrated for the enantiomers of benzodiazepines by Müller & Wollert (1975) and for those of noradrenaline by Powis (1975). An investigation of the binding of the isomers of noradrenaline in connective tissue (Powis, 1973) and in heart and brain tissue (Iversen, Jarrott & Simmonds, 1971) disclosed a certain stereoselectivity also of the unspecific binding. With respect to benzetimide, the detailed study of Beld & Ariëns (1974), using a membrane fraction of smooth muscle and brain tissue, did not take into account a possible stereoselectivity of unspecific binding sites. A useful chemical procedure was evaluated by Blaschke (1972); by using polyacrylamides substituted by either left or right rotating substances, racemic compounds could be separated by this mode of column chromatography.

A further conclusion may be drawn from the results shown in Figure 8. As has already been stated, the binding curve for levetimide exhibits a negative slope at a concentration range corresponding to the filling of only a saturable unspecific compartment. It is therefore possible that the corresponding negative slope for dexetimide is due to the filling of not only a specific but also an unspecific compartment. In addition, from the negative diverging slopes it is not possible to determine the degree of stereoselectivity shown towards the two isomers. Higher affinity to the tissue binding sites shown at the lower concentration range may parallel an increasing ability to discriminate between the two isomers, which cannot be quantitatively determined. Thus a reliable estimation of the size of the receptor compartment in the case of dexetimide is not possible.

The decline curves depicted in Figure 5 reveal that an initial 'plateau' phase is present at higher concentrations of both isomers. This may be interpreted as reflecting a high initial release of the drug predominantly from the unspecific binding sites. Thus a relatively high antagonist concentration is kept in the biophase, thereby delaying the release of the drug from the receptor during this initial phase. Thereafter, when the concentration of antagonist in the biophase falls to a critical level, the release from the receptor obeys a monoexponential function. The 'plateau' may thus be explained in terms of an overwhelming initial contribution to the diffusion process by the unspecific binding sites. From this phenomenon it is clear that unspecific binding sites may be closely involved in the kinetic behaviour of benzetimide.

The quantitative nature of this phenomenon is at present under investigation together with the mechanism underlying the unusually high tissue accumulation of benzetimide.

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