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Development of a sensitive radioreceptor assay for oxyphenonium in plasma and urine

K. ENSING*, F. KLUIVINGH, T. K. GERDING AND R. A. DE ZEEUW
Department of Toxicology, State University A. Deusinglean 2, 9713 AW Groningen, The Netherlands

A radioreceptor assay (RRA) for oxyphenonium has been developed. It is based on competition between [^{3}H]dexetimide and oxyphenonium for binding to muscarinic receptors from calf striata. The RRA is optimized towards incubation medium and to extraction by ion pair formation with sodium picrate. At least 4×10^{-10} M of oxyphenonium is necessary to permit a reliable assay. This corresponds to a detection limit of drug of 2 ng ml^{-1} urine. After extraction, drug at 100 pg ml^{-1} of plasma can be estimated using 4 ml samples. The method is applicable to monitoring the drug and to the determination of its pharmacokinetics after therapeutic dosing. Urine levels can also be monitored.

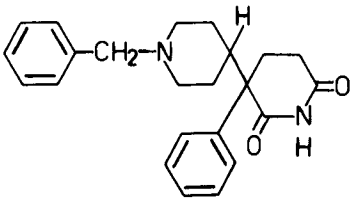
Oxyphenonium bromide (Antrenyl, Ciba-Geigy) is a potent antimuscarinic drug used in the treatment of chronic aspecific respiratory conditions to obtain bronchodilation (Greving et al 1978).

The drug antagonizes the bronchus-obstructing effect of acetylcholine (van Bork et al 1979) and may also be useful as a protective against certain irritants (Koëter 1983, unpublished results). For a proper understanding of the pharmacological action of the drug and to obtain optimum therapeutic efficacy, it is necessary to determine its pharmacokinetic profiles after intramuscular and oral administration as well as after inhalation, and to correlate these findings with the observed lung function data.

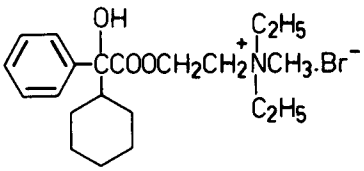
Because of its potency, the drug concentrations in plasma are usually in the lower ng ml^{-1} range after intramuscular and/or oral administration (Greving et al 1978). To determine these low levels, we decided to investigate the potential of radioreceptor assays (RRA) based on competition between a radiolabelled ligand ([^{3}H]dexetimide), and oxyphenonium for binding to muscarinic receptors obtained from a suitable animal. We now describe the development of the assay for oxyphenonium in plasma and urine as sensitive as the high femtomole range. Direct assays in the biofluids could not be carried out because of interference of the endogenous matrix, so the possibilities of an ion pair extraction and concentration of the drug were investigated and optimized towards reproducibility and lowest detection limit.

MATERIALS AND METHODS

Chemicals. [^{3}H]Dexetimide (15 Ci mmol^{-1}) was supplied by IRE (Utrecht, The Netherlands). Unlabelled dexetimide was kindly donated by Janssen Pharmaceutica (Beerse, Belgium). Oxyphenonium bromide was a gift from Ciba-Geigy (Basel, Switzerland). Tetrapentylammonium was obtained as its iodide from Eastman Kodak (Rochester, NY, USA). All other chemicals and solvents of analytical grade were obtained from Merck (Amsterdam, The Netherlands). Polyethylene tubes were obtained from Greiner (Alphen a.d. Rijn, The Netherlands).



Dexetimide



Oxyphenonium bromide

Sovirel tubes were obtained from Quickfit S.A. (Epernon, France). GF/B filters were from Whatman (Maidstone, UK). The 50 mM sodium potassium phosphate buffer (pH = 7.4) was composed from 4 volumes 50 mM potassium dihydrogen phosphate and 1 volume 50 mM disodium hydrogen phosphate. Plasmasol was used as scintillation liquid and obtained from Packard Instruments (Groningen, The Netherlands).

* Correspondence.

Preparation of receptor material. Freshly prepared striata from calf brains were homogenized in 12 volumes of icecold buffer using a Teflon-glass Potter-Elvejehem homogenizer at 1200 rev min^{-1} (R.W. 18, Janke & Kunkel., Staufen i. Breisgau, FRG).

Initially the homogenate was either used directly after preparation or stored at −80 °C when there was no significant loss in affinity or receptor concentration. Later the tissue was homogenized in 12 volumes 0·32 M sucrose and centrifuged, 10 min at 1000 *g*. The pellet was discarded and the supernatant centrifuged, 60 min at 100 000 *g*. The latter pellet (P_2) was resuspended in buffer and used, or stored at −80 °C. The receptor concentration of the prepared receptor suspension was determined as follows: The receptor suspension was incubated with increasing concentrations of [^{3}H]dexetimide over the range 10^{-10}–10^{-8} M, with or without 2×10^{-6} M unlabelled dexetimide. The difference in binding is receptor-bound radiolabelled ligand, and by means of Scatchard analysis (Scatchard 1949) the receptor concentration can be calculated. Then, the receptor suspension is diluted with buffer to a final concentration of $1{\cdot}15 \times 10^{-8}$ M.

Radioreceptor assay (initial procedure). To triplicate polyethylene tubes were added solutions of oxyphenonium in buffer, a biofluid or a suitably prepared aqueous extraction solution in concentrations varying from 10^{-10} to 10^{-7} M. In other assays the drug was replaced by 2×10^{-6} M unlabelled dexetimide. Then 50 µl of radiolabelled dexetimide (concn $4{\cdot}6 \times 10^{-8}$ M) and buffer was added to 1100 µl. After mixing, 50 µl of freshly prepared or freshly thawed receptor suspension was added, giving a final concentration of 5×10^{-10} M. Polyethylene tubing was used because of its low aspecific binding of the radiolabelled ligand. After mixing, the tubes were incubated for 10 min at 37 °C. Then, they were mixed again with addition of 4 ml icecold phosphate buffer. The samples were immediately filtered through Whatman GF/B glass fibre filters under vacuum, using a filtration apparatus (Multividor 40 S, Janssen Scientific Instruments, Beerse, Belgium). The tubes were rinsed with 4 ml icecold buffer, which was also filtered. The filters were washed with 4 ml icecold buffer and dispersed in 6 ml Plasmasol by shaking for 60 min. The total filtration, rinsing and washing process taking place in approximately 15 s, was carried out on each tube in turn. The vials were counted for 10 min or 40 000 counts in a liquid scintillation counter (Isocap 300, Nuclear Chicago Division (Searle), Chicago IL, U.S.A.).

Extraction and re-extraction of oxyphenonium. To 10 ml-Sovirel tube 1 ml plasma or water containing 10^{-7} to 10^{-10} M drug and 100 µl 10^{-3} M sodium picrate in 50 mM sodium phosphate buffer was added, to give a final picrate concentration of 10^{-4} M. Five ml DCE was added, the tubes were vortexed during 30 s and centrifuged, 10 min 5000*g*. The water phase was discarded, 4·5 ml of the DCE was transferred to an other Sovirel tube and 2·0 ml 10^{-6} M tetrapentylammonium iodide (TPA) in buffer was added. The mixture was vortexed for 30 s and centrifuged, 10 min 5000*g*. Three 0·5 ml aliquots of the aqueous TPA-extract were transferred to 10 ml polyethylene tubes and the RRA was carried out.

Radioreceptor assay (final procedure). For the assay of oxyphenonium aliquots of 1·0 to 5·0 ml plasma or water, 0·1 to 0·5 ml 10^{-3} M sodium picrate in 50 mM sodium phosphate buffer and 5 ml dichloroethane (DCE) were added to 10 ml Sovirel tubes and extracted and re-extracted as described above. Three aliquots of 0·5 ml aqueous TPA-extract were transferred to polyethylene tubes and lyophylized over 48 h. The lyophylized samples were redissolved in 0·5 ml water, 25 µl $4{\cdot}6 \times 10^{-8}$ M [^{3}H]dexetimide and 25 µl resuspended P_2-pellet was added to give a final concentration of 2×10^{-9} M [^{3}H]dexetimide and a receptor concentration of 5×10^{-10} M, respectively. The tubes were vortexed for 5 s and incubated at 37 °C for 10 min. The incubation was stopped by the addition of 4 ml icecold buffer, filtration and counting was done as in the initial procedure. Calibration graphs were constructed by carrying out the incubation in the presence of increasing quantities of drug (10^{-7} to 10^{-10} M) which were added to blank plasma or water and handled in the same way as the samples to be analysed. All binding values were expressed as specific binding, which is total binding minus aspecific binding. The counts s^{-1}-values were converted to d s^{-1}, the counting efficiency of these samples was always between 47 and 50%. The amount of drug in the standards was plotted against the d s^{-1} values. The recovery of the method was determined by comparing the values of the standard curve with aqueous TPA solutions containing fixed amounts of oxyphenonium. The reproducibility of the method was determined by trifold determination of a standard curve and a select determination of some spiked samples in random order.

RESULTS AND DISCUSSION

When inhibition experiments were carried out in buffer, the plotted data allow calculation of the minimum amount of drug required to give 10% inhibition of specific [^{3}H]dexetimide binding. This is in the order of 4×10^{-10} M and can be considered as the determination limit of the method.

When blank plasma was used in the RRA, the [^{3}H]dexetimide binding became inhibited depending on the volume of the plasma. Addition of 100 µl plasma already caused an inhibition of about 20%. This indicates the occurrence of non-competitive binding of dexetimide to plasma constituents (proteins) which has a detrimental effect on the sensitivity of the assay. In view of the detection limit required (<1 ng ml^{-1} plasma) this non-specific binding to plasma constituents made it impossible to apply the RRA directly to plasma.

Therefore it was decided to try and isolate the drug from plasma by means of an extraction that, at the same time, would also serve as a concentration step. For this purpose ion pair extraction seemed logical. Greving (1981) determined the extraction constants of oxyphenonium with iodide, perchlorate and picrate from water to 1,2-dichloroethane. Picrate offered the best opportunities for quantitative extractions at lower concentrations. Experiments in buffer with oxyphenonium concentrations ranging from 5×10^{-4} to 5×10^{-6} M were successful, but when plasma was used recovery losses were observed in attempts to redissolve the residue obtained after evaporating the DCE-extract. These losses may be ascribed to fatty endogenous compounds from the plasma. We then investigated the potentials of a liquid-solid extraction using Amberlite XAD-2 columns. Again losses were observed when we were trying to redissolve the residue of the organic extract.

Because of these problems, the possibilities of a re-extraction with tetrapentylammonium (TPA) after ion pair extraction with picrate were investigated. TPA has a more than 1000-fold higher extraction constant with picrate than oxyphenonium, log K_e being 8·4 for the former (Gustavii & Schill 1966). So, when the amount of TPA is larger than the total amount of oxyphenonium in the organic layer a nearly 100% re-extraction of drug in the aqueous layer can be expected. This type of re-extraction may also be called a displacement extraction as TPA displaces oxyphenonium in the picrate ion pair. Unfortunately, the excess of TPA, necessary for the extraction, affected the binding properties of the muscarinic receptors. When TPA was added to the radioreceptor assay instead of oxyphenonium, the [^{3}H]dexetimide binding became substantially inhibited at concentrations above 5×10^{-6} M (Fig. 1). The assays were carried out with either 0·5 or 1·0 ml aqueous TPA-extract or phosphate buffer, a receptor concentration of 5×10^{-10} M and 2×10^{-9} M radiolabelled dexetimide.

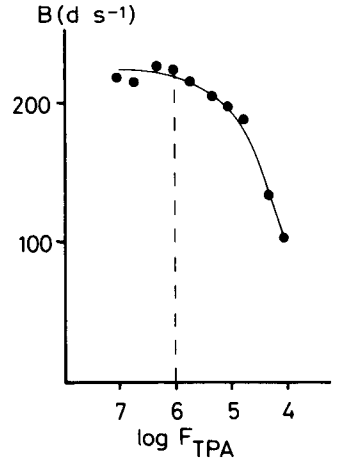


FIG. 1. Inhibition of [^{3}H]dexetimide binding by TPA; F_{TPA} is concentration free TPA; B is bound radiolabelled ligand.

When inhibition curves with comparable oxyphenonium concentrations in buffer and in TPA-extracts are determined, a remarkable difference in total binding between both curves is seen. The loss of 20 to 50% specific binding in the TPA-extracts in comparison with the assay in buffer cannot be due to the TPA present as can be seen from Fig. 1. We found that the interference was caused by trace amounts of dichloroethane which remained in the aqueous phase after extraction. Despite the low solubility of dichloroethane in water, 0·8% at 25 °C, it destroyed specific binding in a non-competitive manner. Organic solvents like *o*-dichlorobenzene and chlorobutane were less destructive but unsuitable for the ion pair extraction. The only method for a complete removal of the DCE which would not influence the reproducibility of the RRA seemed to be lyophylization of the TPA-extracts. Though it introduces an extra step in the overall procedure, lyophylization offers extra advantages in that a concentration can be obtained at the same time, thus lowering the detection limit. In addition, it provides a safe way to preserve and store plasma extracts. After lyophylization for 48 h no disturbances of specific binding were noticed.

In an attempt to further optimize the RRA, the concentration of the radiolabelled ligand was varied.

When the concentration of the free radiolabelled ligand (F_D) is equal to or near its K_D-value, K_D being the equilibrium dissociation constant, it can be assumed on theoretical grounds that the most ideal system is obtained. The IC50, being the concentration of competitive ligand that causes 50% inhibition of receptor-bound radiolabelled ligand (B_D), becomes equal to its K_i, the equilibrium dissociation constant of the competitive ligands, if F_D gets near to zero. When the F_D is equal to K_D the IC50 is $2 \times K_i$. Specific binding of [^{3}H]dexetimide is then 50% of the maximal specific binding and the detection limit, which we define as the concentration of drug giving 10% inhibition of specific [^{3}H]dexetimide binding, is $0{\cdot}2 \times K_i$. Decreasing the concentration of radiolabelled ligand may decrease the detection limit theoretically by a factor 2 but this is counteracted by the fact that B_D will decrease by a factor larger than 2. On the other hand, an increase in the concentration of radiolabelled ligand will be accompanied by an increase in B_D, but also to an increase in aspecific binding and in the detection limit, as is depicted in Fig. 2.

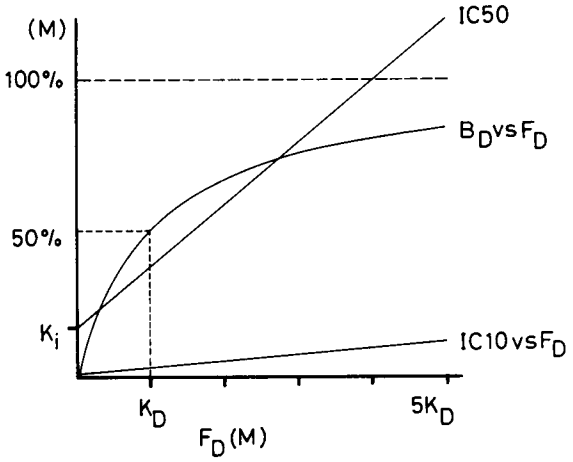


FIG. 2. Optimization of the RRA, relations between the concentration free radiolabelled ligand (F_D) and the concentration bound radiolabelled ligand (B_D), the IC50 and IC10 respectively. The IC50 and IC10 are the concentrations of competitive ligand that cause 50 and 10% inhibition of receptor-bound ligand, respectively. The IC10 is considered the detection limit of the RRA for the competitive ligand. K_D and K_i are the equilibrium dissociation constants of radiolabelled and competitive ligand, respectively.

After having established the optimum radiolabelled ligand concentration at 2×10^{-9} M, giving a free [^{3}H]dexetimide concentration slightly above its K_D, we investigated the influence of incubation time after the addition of [^{3}H]dexetimide. All constituents of the RRA, except the radiolabelled ligand, were mixed and incubated at 37 °C for 10 min before the radiolabelled ligand was added. Incubation was then repeated for 4, 7·5, 10 and 60 min at 37 °C. The obtained inhibition curves are shown in Fig. 3 from which it can be concluded that a small shift of the curves to the right with increasing incubation time occurs but that there is no significant shift of the desired concentration that causes 10% inhibition of specific [^{3}H]dexetimide binding.

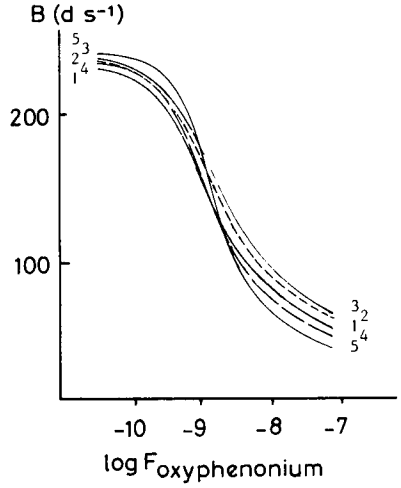


FIG. 3. Inhibition curve after 10 min incubation with oxyphenonium and [^{3}H]dexetimide without preincubation (1), inhibition curves, 10 min preincubated with drug and 4 (2), 7·5 (3), 10 (4), 60 (5) min incubated after addition of [^{3}H]dexitimide; F_{OX} is concentration free drug; B is bound radiolabelled ligand.

For practical reasons, a total homogenate of striatal tissue was used during the development of the RRA for oxyphenonium. Yet, a better defined and more homogenous receptor suspension is preferable for various reasons. Therefore the striata were homogenized in 0·32 M sucrose and fractionated as described by Yamamura & Snyder (1974). The obtained P_2-pellet is resuspended in buffer to give a receptor concentration of $1{\cdot}15 \times 10^{-8}$ M. 25 µl of this suspension is added to 0·5 ml aqueous TPA-extract for the RRA. The receptor concentration was chosen at 5×10^{-10} M, so that with a ligand concentration of 2×10^{-9} M, we were able to count the samples within 10 min and 0·5% relative standard deviation.

When blank urine was used in the RRA, the [^{3}H]dexetimide binding was inhibited similarly to the observations in plasma. Addition of 10% urine

caused about 10% inhibition of specific [^{3}H]dexetimide binding. Yet, because of the higher concentrations of drug (>1 ng ml^{-1}) in urine this allows to determine oxyphenonium directly in urine diluted with 9 volumes of buffer. A standard curve with concentrations of drug in urine varying from 10^{-9} to 10^{-6} M was determined. Thus determination of fmol quantitites of oxyphenonium in plasma is possible with the radioreceptor assay as the drug can be determined at a final concentration of at least 4×10^{-10} M. This corresponds to a detection limit of 100 pg. Using 4 ml plasma samples, a detection limit of 100 pg ml^{-1} can be obtained. If the drug is estimated in 10% urine, the detection limit is 2 ng ml^{-1} urine. Lower detection limits can be reached, but at the expense of precision.

In comparison with the chemical method of Greving et al (1978), the RRA has a 10 fold higher sensitivity. This gain offers possibilities for the determination of plasma concentrations after inhalation of oxyphenonium and an opportunity for the determination of the active enantiomer. As the metabolism of oxyphenonium has been found to be neligible (Levine 1957), we have considered the possibility of cross reactivity with metabolites to be irrelevant. The assay is sufficiently reproducible when carried out with a resuspended P_2-pellet and a concentration of radiolabelled ligand close to its K_D-value. The following reproducibilities were obtained: 100 pg ± 8%; 200 pg ± 8%; 500 pg ± 10%; 1000 pg ± 14% (n = 9, % = coefficient of variation). The effective linear range of the method lies between 100 and 5000 pg. For higher concentrations, a dilution of the sample is necessary. The overall recovery was 95–98% over the entire concentration range. The extraction and re-extraction of drug from plasma and lyophylization of the re-extract is time-consuming when only a few samples are handled. The total procedure can be carried out within 3 days but we are able to analyse 40 samples a day.

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