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Application of radioreceptor assays for systematic toxicological analysis — 1. Procedures for radioreceptor assays for antihistaminics, anticholinergics and benzodiazepines

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Abstract: Radioreceptor assays can be a useful tool for systematic toxicological analysis in that they can be applied for the detection of an entire pharmacological class of drugs. In the present paper procedures for radioreceptor assays for benzodiazepines, anticholinergics and antihistaminics have been described in detail. The development of the assay for antihistaminics in urine is given in order to illustrate the prerequisites for these types of assays with regard to the incubation conditions. In part 2 the applicability of the three assays for systematic toxicological analysis will be evaluated on the basis of testing a large number of urine samples after administration of a selected number of drugs to healthy volunteers and patients.

Keywords: *Radioreceptor assays; systematic toxicological analysis; screening; benzodiazepine; anticholinergic; antihistaminic; flunitrazapem;* N*-methylscopolamine; mepyramine; therapeutic drug monitoring.*

Introduction

Quantitative radioreceptor assays (RRA) have been developed and used for the determination of various drugs in biofluids [1–7]. RRA can be advantageous over chemical and physical as well as over immunoassays, in that they pair a sufficiently high sensitivity (directly related to the potency and affinity of these drugs, mainly antagonists) with a selective determination of biologically active compounds such as the eutomer of a racemic drug and/or active metabolites that contribute to the desired (and undesired) actions of the parent compound [1, 4, 8–11].

Systematic toxicological analysis (STA) comprises the logical chemical analytical search, detection and identification, for potentially harmful substances (a.o. drugs or metabolites present in biological matrices), whose presence is unsuspected and whose identity is unknown. STA can be applied in different areas of analytical toxicology which mainly differ in the interpretation of the analytical results. Forensic toxicology comprises the analysis of hazardous substances in body fluids or tissues and the evaluation of their rôle in the cause and manner of death or other alledged cases under investigation of criminal or civil justice. In clinical toxicology the analysis of hazardous substances in body fluids and the evaluation of their rôle in the cause and manner of clinical intoxications is studied. In drug abuse testing the intake of hazardous substances for purposes other than intended, to the extent that they may be detrimental to the user, to others in the environment or to society, is monitored. It should be noted that the speed and sensitivity of analytical methods are strongly dependent on the application areas. An intoxication should be identified before the patient dies, whereas drugs of abuse should be detected with the most sensitive method.

In general, a screening procedure is combined with a more selective analytical confirmation method. So far chromatographic systems like thin layer chromatography and gas

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chromatography have been successfully applied, whereas liquid chromatographic approaches are currently evaluated.

Immunochemical techniques are often applied for screening for reasons of time and money, though these techniques cannot be considered for a systematic approach. Immunoassays can either identify a single substance or a group of structurally related substances with the limitation that the sensitivity for each individual compound can differ substantially and is completely independent of therapeutically or toxicologically relevant concentrations. Moreover, the selectivity or cross-reactivity can only be determined adequately by testing all other compounds that might appear in the biofluid. In screening assays you have to deal with false positive and false negative outcomes. The first category should be limited for economical reasons though these samples can be identified by a negative outcome of the confirmation test. The latter category should preferably not exist since no further tests will be conducted that can correct for this misclassification. While many drugs exert their pharmacological and toxicological effect via a receptor and the occupancy of the receptor is most often related to the extent of this effect, receptor binding properties can be employed for the detection of these drugs. RRA can therefore principally contribute to STA, though this approach has not yet been evaluated in detail [8, 12].

RRA are based on the competition between a radiolabelled ligand and a drug (unlabelled ligand) for binding to a certain receptor type [9, 11]. When a drug is added to a mixture containing fixed concentrations of receptors and labelled ligand, the competitive drug will displace a certain amount of labelled ligand depending on its equilibrium constant, K_d, and the added concentration of competitive drug. The remaining bound fraction of labelled ligand is inversely related to the concentration of the competitive drug. The labelled bound fraction is collected by filtration and determined by liquid scintillation counting. By means of centrifugation bound and free fractions can be separated as well, allowing measurement of the free fraction of the radiolabelled ligand [13]. A representative calibration curve for receptor assays is given in Fig. 1. In this paper detailed experimental procedures for the RRA for antihistaminics, anticholinergics and benzodiazepines are presented. In order to obtain insight in the experimental conditions required for this type of assays, the optimization of the RRA for antihistaminics is described in more detail.

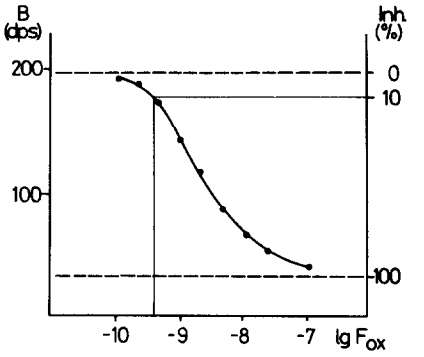

Figure 1
Calibration curve of a radioreceptor assay. B is bound radiolabelled ligand; F_{ox} is the concentration of competitive drug. Inh. is the inhibition of receptor-bound radiolabelled ligand, 100% inhibition is considered the amount of non-specific bound radiolabelled ligand. Ten per cent inhibition is considered the detection limit of the RRA.

In part 2 the potential of RRA as a screening method in STA will be addressed, based on literature data [14]. Identification and confirmation of substances requires additional techniques. Furthermore, the three RRA will be applied to urine samples of patients and volunteers who obtained multiple or single therapeutic doses of various drugs, respectively. The majority of patients also received more than one drug during these experiments.

Materials and Methods

Chemicals

^{3}H-mepyramine (29 Ci mmol^{-1}; ^{3}H-MEP), ^{3}H-N-methylscopolamine chloride (87.5 Ci mmol^{-1}; ^{3}N-NMS), and ^{3}H-flunitrazepam (81.8 Ci mmol^{-1}; ^{3}H-FLU) were obtained from NEN (Boston, USA). Chemical structures of these compounds are depicted in Fig. 2. Mepyramine, promethazine, scopolamine and lorazepam were of pharmacopoeial quality. Tris(hydroxymethyl)-aminomethane, disodium hydrogen phosphate dihydrate and sucrose were from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate dihydrate and hydrochloric acid 36% were

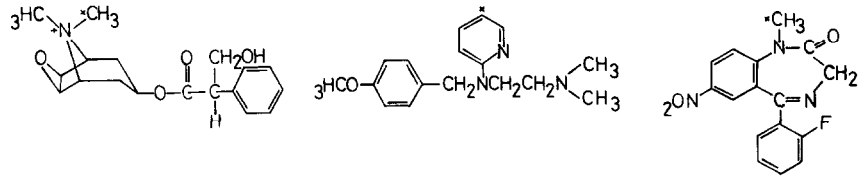

Figure 2
Chemical structures of ^{3}H-N-methylscopolamine, ^{3}H-mepyramine and ^{3}H-flunitrazepam.

from Brocacef (Maarssen, The Netherlands). Polyethylene tubes (10 ml) were obtained from Greiner (Alphen a/d Rijn, The Netherlands). The GF/B glassfibre filters were from Whatman (Maidstone, UK). Aqualuma Plus was used as scintillation liquid, obtained from Lumac (Oud Beijerland, The Netherlands), in combination with mini-scintillation counting vials from Packard (Groningen, The Netherlands).

Preparation of solutions

The 50 mM phosphate buffer (pH 7.45) was prepared by dissolving 1.56 g of $NaH_2PO_4 \cdot 2H_2O$ and 7.12 g of $Na_2HPO_4 \cdot 2H_2O$ in 1 l of distilled water. The 50 mM Tris–HCl buffer was prepared by dissolving 6.06 g of tris(hydroxymethyl)-aminomethane in demineralized water, and the pH was adjusted to 7.4 with concentrated HCl. Stock solutions (10^{-3} M) of mepyramine, promethazine and scopolamine were prepared in phosphate buffer, lorazepam was dissolved in ethanol and diluted with 9 vol Tris–HCl buffer. Stock solutions of the labelled ligands were prepared in ethanol, working solutions were prepared by diluting with assay buffer so that <1% of organic solvent was present. The following working concentrations were obtained, ^{3}H-MEP 2×10^{-8} M; ^{3}H-NMS 5.5×10^{-9} M; ^{3}H-FLU 6×10^{-9} M.

Preparation of receptor material

Freshly prepared calf brains without cerebellum were homogenized in 10 vol (w/v) ice-cold 0.32 M sucrose using a Teflon-glass Potter Elvejehem homogenizer (R.W. 18, Janke & Kunkel, Staufen i. Breisgau, Germany) at 1200 rpm. The homogenate was centrifuged, 10 min at 1000*g*. The pellet was discarded and the supernatant centrifuged, 60 min at 100,000*g*.

The latter pellet was resuspended in phosphate buffer, recentrifuged for 20 min at 70,000*g*, and this procedure was repeated once. The washed pellet was resuspended in 5 vol (w/v) phosphate buffer. The entire procedure was completed at 4°C. The obtained homogenate was frozen with liquid nitrogen and lyophilized in a Hetosicc CD-52 lyophilizer (Heto, Birkerup, Denmark). The lyophilized preparation was stored in the refrigerator at −20°C [15].

For the RRA for antihistaminics 10 mg of lyophilized material was dissolved in 1 ml of phosphate buffer. For the RRA for anticholinergics 2.5 mg of lyophilized material was dissolved in 1 ml of phosphate buffer. For the RRA for benzodiazepines 2.5 mg of lyophilized material was dissolved in 1 ml of Tris–HCl buffer.

Procedures for radioreceptor assays for antihistaminics, anticholinergics and benzodiazepines

Inhibition experiment. To duplicate polyethylene tubes 50-µl aliquots of solutions of mepyramine–promethazine were added, giving final concentrations ranging from 1×10^{-10}–1×10^{-6} M (for anticholinergics, scopolamine 1×10^{-10}–5×10^{-7} M; for benzodiazepines, lorazepam 5×10^{-10}–2×10^{-6} M). Then 50 µl of an aqueous 2×10^{-8} M ^{3}H-MEP solution was added giving a final concentration of 2×10^{-9} M (for anticholinergics, 5.5×10^{-9} M ^{3}H-NMS, assay concentration 5.5×10^{-10} M; for benzodiazepines, 6×10^{-9} M ^{3}H-FLU, assay concentration 6×10^{-10} M). Finally, 400 µl of receptor preparation was added to each tube, to give a total volume of 500 µl. After mixing for 5 s, the tubes were incubated 90 min 20°C (for anticholinergics, 30 min 37°C; for benzodiazepines, 45 min 0°C). Then they were mixed again under the addition of 4 ml of ice-cold phosphate buffer. The samples were immediately filtered through Whatman GF/B glass-

fibre filters under vacuum using a custom made filtration apparatus (48S, University Centre for Pharmacy, Groningen, The Netherlands). The tubes were rinsed twice with 4 ml of ice-cold buffer which was also filtered. The total filtration and rinsing process, taking place in approximately 15 s, was carried out on each tube in turn (for anticholinergics, using the same phosphate buffer; for benzodiazepines, using Tris–HCl buffer). The filters were transferred to mini-scintillation vials and dispersed in a 3.5 ml scintillation cocktail by shaking for 120 min. The vials were counted for 40,000 counts or 5 min in a liquid scintillation counter, whatever came first (Minaxi, Packard, Groningen, The Netherlands). Fifty microlitres of the ^{3}H-MEP (^{3}H-NMS, ^{3}H-FLU) solution was added to two mini-scintillation vials and measured.

Saturation experiments. For the determination of the affinity of ^{3}H-MEP and the receptor density of the lyophilized preparation 400 µl of the receptor suspension was incubated with 50-µl aliquots of solutions of ^{3}H-MEP over the range 2.5×10^{-9}–1×10^{-7} M (final concentrations ranging from 2.5×10^{-10}–1×10^{-8} M) with or without 50 µl 2×10^{-5} M promethazine. Non-specific and total binding values were thus obtained, respectively. The specific binding (= receptor bound radiolabelled ligand) has been defined as the difference between total and non-specific binding. Receptor concentrations and equilibrium dissociation constants were calculated with the ligand curve fitting program [16].

Results and Discussion

Assessment of the optimal conditions for the radioreceptor assay to detect antihistaminics

Previous binding studies with ^{3}H-mepyramine and guinea-pig brains revealed that histamine H1 receptors are present in all brain regions while receptor densities in the cerebellum are about twice those of the cortex [17]. The distribution of histamine H1 receptors in the rat differs from that in the guinea-pig in that the receptor density in the cortex is about twice the density in the cerebellum, although the overall receptor density is higher in the rat [18]. The amount of receptor material required for large numbers of quantitative radioreceptor assays would result in an unacceptable consumption of laboratory animals. Calf brains, available in large quantities at low costs in the local slaughterhouse, form an excellent alternative as a source for muscarinic and benzodiazepine receptors. For reasons of stability and convenience receptor preparations were lyophilized. For the RRA for anticholinergics and benzodiazepines lyophilized preparations were previously evaluated and proved to have the same specifications as freshly prepared receptor materials [7, 10]. The lyophilized calf brain membrane preparation proved also to be a good source for histamine H1 receptors.

Densities of the histamine H1 receptor were expected to be much lower than the densities of muscarinic and benzodiazepine receptors, which forced us to increase the amount of lyophilized material per assay in order to allow more accurate quantitation of bound radiolabelled mepyramine. The amount of receptor material is limited to 6 mg by the filtration capacity of the glassfibre filters but should also not exceed amounts that no longer give a proportional increase in receptor-bound mepyramine. Specific binding of ^{3}H-mepyramine was linear up to 5 mg lyophilized receptor material per tube as shown in Fig. 3. In further experiments 4 mg of receptor material was used.

In Fig. 4 a representative saturation curve is presented for the histamine H1 receptors labelled with ^{3}H-mepyramine. The promethazine insensitive binding (non-specific binding) increased linearly with the concentration of ^{3}H-mepyramine, while the promethazine sensitive binding, taken to represent receptor specific binding was saturated. The ratios of specific/non-specific binding varied between 0.8 and 2.5. The calculated receptor density was 13.9 ± 1.1 pmol g^{-1} lyophilized receptor preparation and the dissociation constant K_d was 1.8 ± 0.1 nM.

Assay concentrations of radiolabelled mepyramine below 3×10^{-9} M offered the best ratio of specific/non-specific binding. For inhibition experiments the assay concentration of ^{3}H-mepyramine was set at 2×10^{-9} M in combination with 4 mg lyophilized receptor material per assay.

Comparison of the binding characteristics of the calf brain receptor preparation with those of rat brain and guinea-pig brain revealed some remarkable differences between the species. The K_d of ^{3}H-mepyramine for the calf brains histamine receptor is in good agreement with the K_d for guinea-pig brains, 1.6 ± 0.2 nM

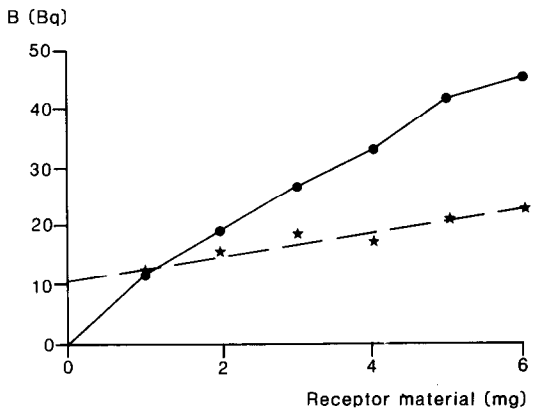

Figure 3
Determination of the optimum amount of lyophilized material for the RRA for antihistaminics; specific (●) and non-specific (★) binding of ^{3}H-mepyramine.

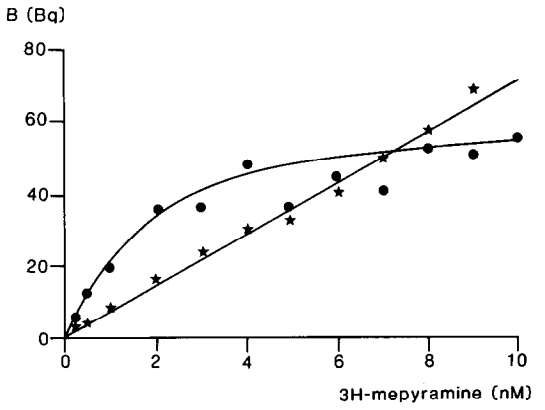

Figure 4
Determination of the optimum concentration of radiolabelled ligand for the RRA for antihistaminics; specific (●) and non-specific (★) binding of ^{3}H-mepyramine.

[17], but roughly 10 times smaller than the K_d for rat brains, 10–30 nM [18]. Surprisingly, the extent of non-specific binding relative to total ^{3}H-mepyramine binding, is more favourable for calf brains (<30%) in comparison with guinea-pig brains (60%), in case of a concentration of radiolabelled ligand (2 nM) slightly above the K_d of ^{3}H-mepyramine [13].

The impact of the pH on specific and non-specific binding was evaluated. The optimum incubation pH turned out to be between 7.4 and 7.8. Higher or lower pH values were accompanied by increases in non-specific binding resulting in a lower ratio of specific/non-specific binding.

The incubation time and temperature were then varied and the effects on specific and non-specific binding were determined. The highest ratios of specific/non-specific binding were found with an incubation time of 60 min at 25°C and 90 min at 20°C. The difference between the ratios was very small. Incubation at 20°C during 90 min was selected for further experiments, due to the fact that the absolute value of the specific binding was higher, allowing better counting precision. The inhibition of binding of 2×10^{-9} M ^{3}H-mepyramine by non-radioactive mepyramine is shown in Fig. 5. The extent of non-specific binding is approximately 40% of the total binding of ^{3}H-mepyramine.

A critical factor in the development of a bioassay is the interference caused by the biological matrix in which the drug of interest is presented. The addition of 50 µl of urine caused <10% inhibition of the specific binding of radiolabelled mepyramine. The addition of 100–200 µl of urine caused 40–60% inhibition of the specific binding. Therefore a direct assay, without sample pretreatment may be possible using small volumes of urine. It is anticipated that the addition of 25 µl of urine to the RRA for antihistaminics will not seriously affect the outcome of the assay.

In comparison with the receptor assays for anticholinergics and benzodiazepine, the receptor assay for antihistaminics is more sensitive to changes in pH and incubation temperature [7, 11]. The relatively low density of histamine H1 receptors reduces the ratio of specific/non-specific binding and will have a negative effect on the obtainable accuracy for quantitative purposes. However, for screening purposes a cut-off value can be chosen to limit the number of false positive conclusions.

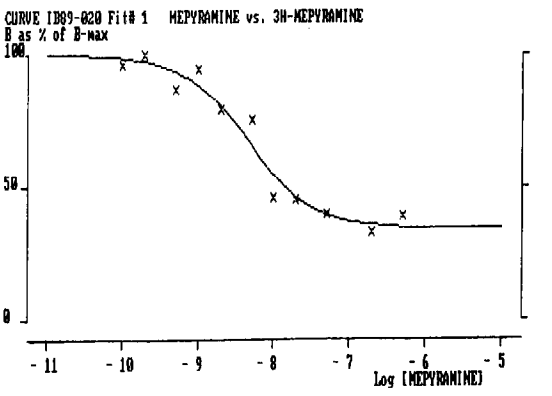

Figure 5
Representative inhibition curve after 90 min incubation at 20°C with mepyramine, 4 mg lyophilized receptor material and 2 mM ^{3}H-mepyramine; B is the total amount of bound radiolabelled drug; [mepyramine] in mol l^{-1}.

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