Pergamon

Journal of Pharmaceutical & Biomedical Analysis. Vol. 12, No. 6, pp. 723–745, 1994

0731–7085/94 $7.00 + 0.00

Review

Methodological aspects of quantitative receptor assays

J. ŠMISTEROVÁ,*†‡ K. ENSING† and R.A. DE ZEEUW†

† *University Centre for Pharmacy, A. Deusinglaan 2, 9713 AW Groningen, The Netherlands*
‡ *Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dubravska cesta 9, 842 16 Bratislava, Slovak Republic*

Abstract: Receptor assays occupy a particular position in the methods used in bioanalysis, as they do not exploit the physico-chemical properties of the analyte. These assays make use of the property of the analyte to bind to the specific binding site (receptor) and to competitively replace a labelled ligand from the same binding site. The amount of labelled ligand replaced is a measure of the amount as well as the affinity of the analyte. Thus, receptor assays offer additional information about the biological (pharmacological) activity of the analyte by distinguishing the compounds on the basis of their specific binding rather than specific molecular structure (chromatographic and non-chromatographic methods). This paper, starting with the general principles of receptor–ligand interaction, focuses on the application of ligand-binding techniques to the quantitative analysis. The factors which influence the sensitivity and the specificity of quantitative receptor assays, as well as the main directions in the improvement of the receptor preparation by using the solubilized and purified receptor are discussed. In order to enhance the use of these assays in routine practice, the development of solid-phase receptor assays is considered.

Keywords: *Quantitative receptor assay; solubilized receptor; immobilized receptor; solid-phase receptor assay.*

Introduction

The major aims of biopharmaceutic analysis is to detect (qualitative analysis) and/or to measure (quantitative analysis) a particular (usually organic) analyte, in order to answer the questions of pharmaceutical, pharmacological or toxicological relevance. These analytes can be subdivided into parent compounds and metabolites. For chiral analytes one may wish to distinguish between the individual enantiomers or other stereoisomers.

It is often important to measure one or more compounds selectively with adequate sensitivity in the presence of a structurally related compound (e.g. the parent drug in the presence of its metabolite). When the analyst wants to meet this demand, the following situations will govern the choice of an appropriate analytical method:

(A) The properties of the analyte are so unique that it can be measured directly in a sample.

(B) The properties of the analyte are similar to those of structurally related or other interfering compounds present in the sample, which necessitates the use of a separation procedure (sample pretreatment–extraction, chromatographic separation step).

(C) The properties of the analyte do not allow adequate detection which implies that another compound should be measured, the concentration of which forms a reflection of the concentration of the analyte (e.g. chemical derivatization of the analyte makes it compatible to quantitation by a method under A or B).

Thus, the choice of the method selected is governed either by the intrinsic physico-chemical and pharmacological properties of the analyte or its amenability to chemical derivatization and by the purpose for which the analysis has to be performed. The method of choice then determines the degree of sample preparation and clean-up.

Generally, the methods used in bioanalysis can be subdivided as follows:

(1) Non-separation (direct) methods: spectrophotometry; luminescence spectrophotometry; and differential pulse polarography.

* Author to whom correspondence should be addressed.

(2) Separation methods: thin layer chromatography (TLC); high-performance liquid chromatography (HPLC); gas chromatography (GLC); and electrophoresis.

(3) Ligand binding assays: immunoassays (IA); receptor assays (RA); and protein binding assays (PBA).

The non-separation direct methods and the separation methods are applicable to all situations under A, B and C. The ligand binding assays have a particular position in that they do not exploit the physico-chemical properties of the analyte and that they are exclusively categorized under (C) since the measurements are indirect. These assays are based on the phenomenon that an analyte (ligand) may bind to a binding site present on an immunoglobulin molecule (IA), on a receptor protein imbedded in a cell membrane (RA) or on a plasma protein (PBA). In doing so, the analyte competitively replaces a labelled ligand from the binding site and the amount of labelled ligand replaced is a measure of the amount of analyte that was present.

This paper will focus on ligand binding assays, especially on receptor assays, the methodological aspects of which are being reviewed.

Ligand Binding Assays

These assays exploit the property of a ligand to bind to the specific binding site on an immunoglobulin molecule (immunoassays — IA), on a receptor protein (receptor assays — RA) or on plasma protein (protein binding assays — PBA).

Immunoassays

Immunoassays are based on the ability of a given analyte (drug, hormone, etc.) to inhibit the reaction between the antibodies raised against the analyte (or structurally related substance) and labelled ligand or its derivative. The labelled ligand should closely resemble the analyte structurally and should participate in the immunochemical reaction with the antibody (Ab) as if it were the analyte.

In qualitative analysis, immunoassays are excellent screening methods (where there is a high number of samples, less emphasis on specificity and more emphasis on simplicity and sensitivity). In quantitative analysis immunoassays are especially useful in monitoring drugs in pediatric therapy where small volumes of sample with an analyte are sufficient. Sample pretreatment is usually not necessary.

Sensitivity is a chief advantage of immunoassays. Generally, analyte concentrations in the picogram to low nanogram range can be determined. However, specificity is not as good as for chromatographic methods (HPLC, GLC, TLC). There are two reasons for the lower specificity: (1) cross-reactivity and (2) heterogeneity of antibodies.

The binding between antigen and antibody is realized by particular sites on the surface of their molecules. A particular functional group on an antigen molecule, called the antigen determinant, is reorganized by a specific binding site on the antibody molecule (immunoglobulin). There may be various determinant sites on an antigenic substance towards which antibodies are formed. If one or more determinant groups are inaccessible during the raising of the antibodies, a low analytic specificity of antibodies may result since the homologous and heterologous compounds of similar basic structures may cross-react in relatively low concentrations on a more or less equal basis.

The immune response (the raising of antibodies) is evoked solely by the antigen, endogenous compounds of high molecular weight of another animal species (e.g. protein, lipoprotein, glycoprotein, nucleoprotein, etc.). A compound of low molecular weight cannot cause the synthesis of antibodies. In order to raise antibodies, such a small compound, the hapten, must be conjugated with a suitable carrier (of high molecular weight), e.g. serum albumin. Antibodies which recognize the hapten are prepared by inoculation of an animal with this synthesized antigen. This procedure must be repeated several times. Antiserum obtained from an animal after first inoculation may be different from that obtained after second inoculation. Thus, antibodies vary in their ability to differentiate between analyte, cross-reacting compounds, and/or metabolites.

In summary, the development of IA requires the chemical synthesis of analyte–protein conjugate, inoculation of a suitable animal species (e.g. rabbit) over a period of at least 4–6 weeks to produce the antiserum, chemical synthesis of labelled ligand, the testing of the entire system for specificity, sensitivity and cross-reactivity due to metabolites or other drugs.

Receptor assays

The general principles first developed for immunoassays have been exploited for receptor assays. The receptor assays are based on the ability of an analyte (drug, hormone, neurotransmitter, etc.) to compete with a labelled ligand for a specific receptor binding site. Thus, analyte (L), that exerts its pharmacological action through interaction with a given receptor (e.g. beta-adrenergic drug with beta-adrenergic receptor, anticholinergic drug with muscarinic receptor) can be analysed qualitatively as well as quantitatively when an aliquot of the sample is added to a solution that contains a fixed amount of receptor (R) and a fixed amount of labelled ligand (L*). This will result in the following equilibrium:

$$R + L + L^* \Leftrightarrow RL^* + RL. \qquad (1)$$

The unknown quantity of analyte can be calculated by determining the percentage inhibition of labelled ligand binding and comparing this to the inhibition produced by known quantities of analyte in calibration samples. In contrast to immunoassays, in which the specificity is determined by sites on the analyte molecule that are recognized by antibodies prepared from immunized animals, specificity in receptor assays is determined by binding to the biological receptor that mediates the action of the analyte. Receptors are protein (glycoprotein) macromolecules which naturally occur on cell membranes of different tissues and organs. Hormones, drugs and neurotransmitters bind to the receptors in order to evoke a corresponding response. Thus, RRA utilizes the first step (binding) in a biological cascade of physiological reaction (Fig. 1). This binding is reversible, specific, saturable and of high affinity. Such tight binding is necessary because the ligand levels in the living organism are low.

Unlike immunoassays (antibodies against the analyte have to be generated before the assay by immunization of animal), the receptor material for receptor assays can be easily prepared from certain animal organ or tissue. In principle, homogenized suspensions of freshly isolated tissue may be used, but in practice crude membrane fractions or more purified receptor preparations are preferred, because of their enhanced specificity. From the chosen tissue, the receptor preparation used in an assay is prepared by homogenization and

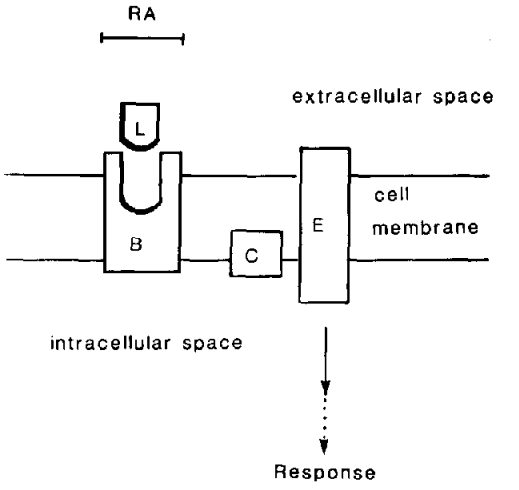

Figure 1
Schematic representation of the cascade of physiological events in a cell that take place when a bioactive compound binds to the binding site of a receptor. Receptor assays utilize only the first step of this cascade-binding. The binding site of receptor is also the place of cooperativity and regulation. L = bioactive compound (ligand); B = binding component (receptor); C = coupling component (e.g. G-protein); E = effector (e.g. adenylate cyclase, activation of which leads to the production of cAMP, a second messenger).

centrifugation. The particular conditions depend on the nature of the tissue and on the localization of the receptors in the target cells.

From the above principles it follows that the sensitivity of receptor assays may be very high (picograms to nanograms of analyte can be measured) if the analyte has a high affinity to the receptor. On the other hand, an analyte with a lower affinity is usually not excluded from being measured by RRA since it requires higher doses to be pharmacologically active, which in turn, will result in higher concentrations in the respective body fluid. A key element in the receptor assay is specificity. Receptors are defined on the basis of binding specificity for a particular class of compounds (analyte). Thus, by the receptor assay only the active forms of ligand, including its metabolites can be specifically and rapidly detected. This means that the specificity of receptor assays is different from that of immunoassays. While in immunoassays, analyte related compounds can cross-react whether they are biologically (pharmacologically) active or not, in receptor assays the cross-reactivity is caused only by pharmacologically active compounds present in the sample. Also receptor assays cannot distinguish between parent drug and its active metabolite if they both bind to the same

binding site, although their physico-chemical properties are different.

On the other hand, the specificity of the two assays (IA and RA) can be enhanced, if they are used in combination with a chromatographic method such as HPLC which by recognizing physico-chemical differences, may be able to distinguish between parent compound(s) and metabolite(s).

The above mentioned properties of receptor assays make them preferable over immunoassays and physico-chemical methods in the following situations. RA provides indices of biological activity which is particularly meaningful when racemic drugs are to be determined. In general the pharmacological effects of enantiomers are different, not only in potency (receptor affinity) but sometimes the individual enantiomers interact with different types of receptors, mediating different effects. Also RA can distinguish enantiomers (receptor sites usually bind only one of a pair of enantiomers) or in distinguishing pharmacologically active parent drugs and metabolites from inactive metabolites. The results of receptor assays (depending on the class of drug) can better reflect the overall effect of a drug — e.g. for beta-blockers, a good correlation between plasma levels measured by receptor assay and effect was found [1].

Secondly, RA is useful in measuring active substances for which antibodies are not readily available (e.g. dexamethasone and prednisone receptor assay [2]).

Thirdly, receptor assays provide major insights into the pathogenesis of endocrine disorders that involve auto-antibodies to hormone receptors [3].

The areas of application of receptor assays. With the appearance of a large number of labelled ligands for different types of receptor, RAs have first been applied on qualitative analysis — in basic studies on receptors, endogenous ligands for the receptors and discovery and identification of biologically active chemical entities (pharmacological screening).

Later on, receptor–ligand interaction has been found to be a versatile tool for the measurement of the concentrations of hormones and neurotransmitters. The first example of quantitative receptor assay was described by Lefkowitz *et al.* [4] who measured ACTH by competition with ^{125}I-ACTH for specific sites in homogenates of adrenal cortical tissue. There followed other similar assays for hormones and neurotransmitters [5, 6], neuroleptics [7], beta-adrenergic antagonists [8], tricyclic anti-depressants [9], bradykinin [10], dihydropyridines [11], endogenous neuroactive compounds [12, 13], opiates [14], etc. In addition to this, quantitative receptor assays have been applied to the determination of receptor- and depolarization-stimulated production of second messengers in the tissues [15, 16], receptor auto-antibodies [3] and receptors during purification procedures.

Though the principle of receptor–ligand interaction which is valid for qualitative assays can be applied for quantitative analysis, the use of RA for quantitative assays which combine high sensitivity with high reproducibility requires a different approach.

In this paper we will discuss the basic factors which are to be considered in quantitative RA and the methodological aspects of assays with special interest in new directions in the improvement of the receptor material and the performance of assays.

Receptor–ligand binding theory. The interaction of a ligand and a labelled ligand with one class of receptor sites is expressed by the following equations:

$$L^* + R \overset{K_d^*}{\leftrightarrow} B_s^* \qquad (2)$$

$$L^* + R \overset{K_d}{\leftrightarrow} B_s \qquad (3)$$

where K_d^* and K_d are the dissociation constants of equations (2) and (3), respectively.

The concentration of bound labelled ligand $[B^*]$ in the presence of unlabelled ligand can be expressed:

$$[B^*] = [B_s^*] + [B_n^*] = \frac{[R]_o\,[L^*]}{K_d^*\,(1 + [L]/K_d) + [L^*]} + K_n^*\,[L^*], \qquad (4)$$

where L = free unlabelled ligand, L^* = free labelled ligand, B^* = bound labelled ligand, B_s^* = specifically bound labelled ligand, B_n^* = nonspecifically bound labelled ligand, R = receptor, K_n^* = constant characterizing nonspecific binding of labelled ligand and $[R]_o$ = total receptor concentration.

If unlabelled ligand is introduced into the

assay, it will compete for the binding site, displacing the labelled ligand. At equilibrium, the remaining fractions of bound and free labelled ligand are a function of the amount and affinity of unlabelled ligand present. It should be mentioned that — at least up until now — radioactivity is almost exclusively used as the label.

The standard curve. The amount of receptor-bound labelled ligand is related to its free concentration in the incubation medium. The free concentration of labelled ligand increases when unlabelled ligand (analyte) is added, because receptor-bound labelled ligand is released. On the other hand, unlabelled ligand itself is bound to the receptor, which results in a decrease in the free concentration.

Depending on the method of separation used, the inhibition of receptor-bound labelled ligand by an increasing concentration of free unlabelled ligand can be measured as a decrease in the amount of receptor-bound labelled ligand (B*) or an increase in the concentration of free labelled ligand (F*). This is shown in Fig. 2.

Most of the time we are interested in the amount of labelled ligand bound to the receptor, which is inversely related to the amount of unlabelled ligand present. In this case the competition isotherms with unlabelled ligand (see Fig. 2(a)) are used as calibration curves and equation (4) can be simplified to

$$[B^*] = \frac{[B^*]_o}{(1 + [L]/IC_{50})} + K_n^*, \qquad (5)$$

where $[B^*]_o$ is the amount of bound labelled ligand in the absence of unlabelled ligand and IC_{50} is the concentration of the analyte which causes the decrease in the amount of bound labelled ligand by 50%. Unknown concentrations of the analyte can be read or computed from such calibration curves.

The separation of bound and free labelled ligand. In receptor assays, labelled and unlabelled ligand (analyte) are incubated with a receptor preparation to achieve equilibrium. The incubation time depends on the association and dissociation rates of both ligands. The technique to be used for the separation of the bound and free fractions is mainly dependent on the dissociation rate of the labelled ligand. Accordingly the separation can be carried out under equilibrium or non-equilibrium conditions. The separation method and the additional conditions (temperature) have to be chosen to prevent significant losses of bound labelled ligand.

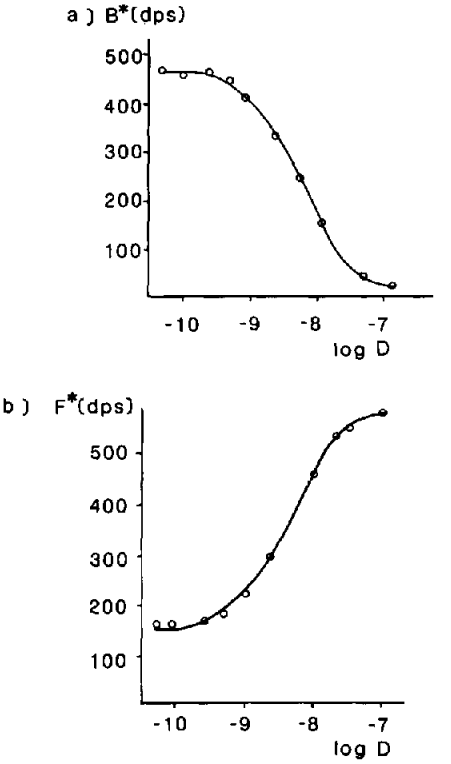

Figure 2
Experimental inhibition curves for oxyphenonium obtained when bound labelled ligand was measured (filtration method) (a) and when free labelled ligand was measured (centrifugation method (b) (from [17]). F* and B* indicate the concentration of free and bound labelled ligand, respectively; D indicates the concentration of added drug [in mol l^{-1}].

The separation methods can be subdivided according to the receptor material used, into those for membrane-bound and for solubilized receptor. The criteria for solubilized receptor preparation are (1) disappearance of lamellar structure observed by electron microscopy, (2) the passing through filters with particle retention higher than 0.22 μm and (3) no sedimentation when centrifuged at 100,000*g* for 60 min.

Separation methods for membrane bound receptor can be based on centrifugation or on filtration through glass fibre filters. Separation methods for solubilized receptor can be based on dialysis, gel-filtration (Sephadex G-50), precipitation of receptor–ligand complex followed by centrifugation or filtration (PEG 6000–8000, ammonium sulphate), adsorption

of free ligand followed by centrifugation (activated charcoal), adsorption of the receptor–ligand complex (ion-exchange filter-DEAE 81, PEI-treated glass fibre filters).

In case of centrifugation or filtration, free and bound fractions can be measured separately. In centrifugation the ligand–receptor complex is pelleted and separated from the free ligand. Aliquots of supernatant are pipetted into the counting vials and are measured after the addition of scintillation cocktail. Alternatively, the pellet can be measured as well.

In filtration, various types of glass microfibre filters are commonly used with thicknesses varying from 0.26 to 0.68 mm, which retain particles with sizes larger than 0.7–2.7 μm.

Filtration, gel filtration or ligand adsorption assays are performed under non-equilibrium conditions. During the assay the concentration of free ligand in the vicinity of the receptor binding site is being reduced which initiates ligand dissociation. Thus, these methods may be unfavourable for the measurement of a rapidly dissociating labelled ligand.

Centrifugation and filtration techniques were compared with the quantitative receptor assay of anticholinergics by Ensing *et al.* [17]. In centrifugation assays the pipetting of the supernatant may have considerable impact on the precision (the error in free labelled ligand increases with increasing percentages of inhibition). The precision of the centrifugation method was better than for the filtration technique for low concentrations of analyte. Equilibrium dialysis and gel filtration are not practical for multiple assays since both methods are rather time consuming. However, a comparison of dialysis (performed at equilibrium conditions) with filtration or centrifugation (non-equilibrium conditions) can be advisable when suspicion arises about inadequate separation of bound and free labelled ligand with the latter methods.

The major drawback of precipitation methods is the high nonspecific binding (during precipitation, the free labelled ligand can become attached to the receptor–ligand complex). Incubation with the precipitation agent prior to filtration or centrifugation can lead to disturbances of the equilibrium between bound and free fractions.

When adsorption of free labelled ligand is applied as the separation method, an equal volume of a charcoal/BSA solution is added, and after the centrifugation (microcentrifuge, 13,000*g*) the supernatant with bound labelled ligand is pipetted into scintillation vials. The possible drawback of this procedure lies in the pipetting error.

Analytical characteristics of receptor assays. Sensitivity. Since receptor assays employ the specific binding site of a ligand for its detection, the sensitivity is related to the ratio of concentration/K_d of a ligand being assayed. In other words, low concentrations of a ligand with a low K_d-value (high affinity) can be measured and vice versa.

Limit of detection (LOD) can be defined as the concentration of analyte at which the fraction bound label is statistically significantly smaller than the fraction bound label in the absence of analyte. A statistically significant difference means, that if the errors of the measurements of $[B^*]_o$ and $[B^*]_1$ are normally distributed and the standard deviations S_1 or the mean values $[B^*]_o$ and $[B^*]_1$ are the same, then these values differ according to the criteria of the Student's *t*-test [18]:

$$t = \frac{[B^*]_o - [B^*]_1}{S_1 \sqrt{(1/n_o + 1/n_1)}} \geq t_\alpha, \qquad (6)$$

where n_o and n_1 are the numbers of the measurements of $[B^*]_o$ and $[B^*]_1$, respectively and t_α is the student t statistic with degrees of freedom $n_o + n_1 - 2$ at a significance level α.

Hence, the smallest difference between $[B^*]_o$ and $[B^*]_1$ will be at $t = t_\alpha$. In this case:

$$\Delta[B^*]_o = [B^*]_o - [B^*]_1 = t_\alpha S_1 \sqrt{(1/n_o + 1/n_1)}. \qquad (7)$$

Providing that ϵ, the relative error of the measurement of $[B^*]$, is constant [19] then $S_1 = \epsilon[B^*]_o$ and

$$\Delta[B^*]_o = t_\alpha \epsilon \sqrt{(1/n_o + 1/n_1)}\,[B^*]_o = \gamma_1[B^*]_o \qquad (8)$$

where $\gamma_1 = t_\alpha \epsilon \sqrt{(1/n_o + 1/n_1)}$.

Then LOD can be expressed by the following equation:

$$\mathrm{LOD} = \frac{\Delta\,[B^*]_o}{|d[B^*]/d[L]|_o} = \qquad (9)$$

$$\frac{\gamma_1[B^*]_o}{|d[B^*]/d[L]|_o},$$

where $|d[B^*]/d[L]|_o$ is the absolute value of $[B^*]$ dependence on $[L]$ at $[L] = 0$.

After differentiation of equation (4) according to $[L]$, substitution in equation (9) and taking into account that $[L] = 0$, when determined $[B^*]_o$ and $|d[B^*]/d[L]|_o$, the final form of equation for LOD will be:

$$LOD = \gamma_1 \left[1 + \frac{K_n^* K_d^*}{[R]_o}\left(1 + \frac{[L^*]}{K_d^*}\right)\right]$$

$$\left(1 + \frac{[L^*]}{K_d^*}\right) K_d, \qquad (10)$$

where γ_1 is the parameter from Student t-test and characterizes the error of the determination of $[B^*]_o$ (the concentration of bound radioligand in the absence of unlabelled ligand).

The determination of the sensitivity of a receptor assay together with other analytical characteristics is shown in Fig.3. LOD can be determined experimentally by performing the replicate measurements of the zero standard (amount of bound labelled ligand in absence of analyte). The experimental data are then treated statistically to obtain the standard deviation (SD) of these measurements and to determine where -2 SD intersects on a composite standard curve.

From the above equations it can be seen that sensitivity is a function of several factors, including the affinity of labelled ligand towards receptors, the affinity of analyte towards receptors, the concentration of labelled ligand,

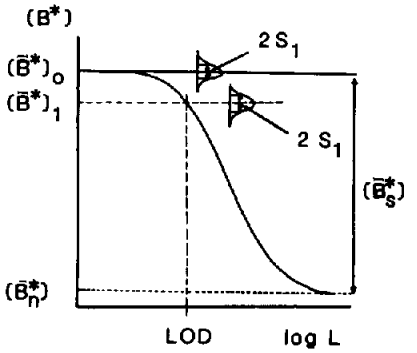

Figure 3
Assessment of the limit of detection (LOD) from the standard curve in receptor assay. $[B^*]_o$ is the mean value of the amount of bound labelled ligand in the absence of analyte; $[B^*]_1$ is the mean value of the amount of bound labelled ligand in the presence of analyte; $[B_s^*]_o$ is the mean value of the amount of specifically bound labelled ligand in the absence of analyte; $[B_n^*]$ is the mean value of the amount of nonspecifically bound labelled ligand.

receptor concentration, as well as the specific activity of labelled ligand and the assay volume. It can be concluded that:

(1) When labelled ligand has a high specific activity, a lower concentration of labelled ligand can be used and the sensitivity can be increased;

(2) The higher the error of determination of $[B^*]_o$ the higher LOD;

(3) LOD is directly proportional to K_d of the ligand being measured;

(4) The lower the nonspecific binding and the concentration of labelled ligand (the lower value $K_n^* K_d^*/[R]_o$) the higher the sensitivity;

(5) LOD depends on the concentration of labelled ligand. A decrease in this concentration is accompanied by a decrease in detection limit. Therefore a minimum concentration of labelled ligand should be used. Free concentration of labelled ligand around its dissociation constant ($F^* = K_d^*$) is considered to be an acceptable compromise.

One of the factors which limits sensitivity is the precision of the measurements. From Fig. 6 it can be seen that at the stated acceptable precision lower than 15%, the lowest concentration of analyte (PAF) which could be quantitated was 50 ng tube^{-1} and the highest 6000 ng tube^{-1}. The highest and lowest concentrations of analyte, which can be measured with stated acceptable precision, determine the working range in receptor assays.

The nonspecific binding of (labelled) ligand can impair the precision as well as accuracy of the assay. The aggregation, sedimentation and other factors which influence the uniform distribution of receptor material in the assay can also affect the precision.

(A) The improvement of sensitivity: Receptor assays can be used only for the determination of ligands of which the pharmacological effect is mediated by a ligand–receptor interaction. Depending on the class of drug, the concentrations measured by receptor assays reflect the therapeutic effect of a drug. This property makes receptor assays useful especially in therapeutic drug monitoring. Therefore, the assay conditions and the composition of the incubation medium have to be comparable with the physiological medium in man. Otherwise, no meaningful quantitation of the total biological activity can be obtained [20].

On the other hand, the limit of detection in receptor assays depends on the affinity of the

ligand being measured towards the receptor preparation used in the assay. Thus, in order to increase the sensitivity, assay conditions at which the affinity of ligand is maximum, have to be established.

The approach to meet these demands depends on the purpose the receptor assay is performed for, as well as on the presence or absence of active metabolites of the ligand being measured.

When the action of a ligand is mediated by only one active species, any adaptation of incubation conditions (time, temperature, pH) that improves the assay performance should be implemented. In this approach lies the potential of receptor assays to measure very low therapeutic plasma levels of pharmacologically highly potent drugs.

When the actions of a ligand are mediated by more than one species, two situations have to be distinguished: (1) a change in the incubation conditions affects the affinities of all active species to the same extent, (2) a change in the incubation conditions affects the affinities to a different extent.

In the first case, the relationship between concentrations and effect will be unaffected. Here, again, any adaptation that improves the assay sensitivity should be implemented. The latter case can lead to wrong information about the relative potencies of individual active species present in the sample. However, when the differences between the affinities of active species are high enough (more than two decades), such an approach can be useful for the selective determination of one of the species present (with the highest affinity), (see paragraph about specificity of receptor assay).

In receptor assays the labelled ligand, analyte and receptor preparation are usually incubated under certain conditions (time, pH and temperature) which are chosen to allow equilibration between bound and free fractions of labelled ligand and analyte. These conditions have to be optimized in order to improve the sensitivity of assay.

(B) The influence of temperature and pre-incubation: The limit of detection in receptor assays is dependent on the affinity (dissociation constant) of analyte being assayed. The affinity of ligand binding to the receptor depends on temperature. It has been observed that the dissociation constant decreases with temperature. Some authors, therefore, suggested that pre-incubation of receptors with an analyte to be assayed at 0°C before the addition of labelled ligand and further incubation at 0°C may decrease the detection limit. This approach was applied for instance to anticholinergics [21] and vitamin D [22]. Thus, analyte bound to the receptor during pre-incubation at 0°C is more difficult to replace by the labelled ligand than at 37°C under equilibrium conditions. Such non-equilibrium conditions may bring a considerable gain in sensitivity as can be seen in Fig. 4 for the anticholinergic scopolamine. With dexetimide as labelled ligand, a gain in sensitivity of factor 6.7 could be obtained by applying pre-incubation at 0°C [21]. This approach can be utilized to reduce plasma sample volumes, provided that the incubation conditions can be controlled satisfactorily in order to obtain reproducible results.

Another mechanism (during pre-incubation), that influences the sensitivity of receptor assays, can take place when the whole cells are used as the source of receptors. This mechanism, called down regulation, results in the loss of cell-surface receptors due to internalization of the receptors during the pre-incubation with analyte and these receptors are then no longer available for the competition process. For instance, very low concentrations of human growth hormone (10^{-10} M) will lead to a loss of cell-surface binding of labelled hGH in cultured human lymphocytes [23]. Such an approach has also been applied to hGH receptor assays, where IM-9 cultured human lymphocytes, used as receptor preparation, were pre-incubated with the analyte (hGH) for a short period, which led to a decrease in the number of receptor sites. After

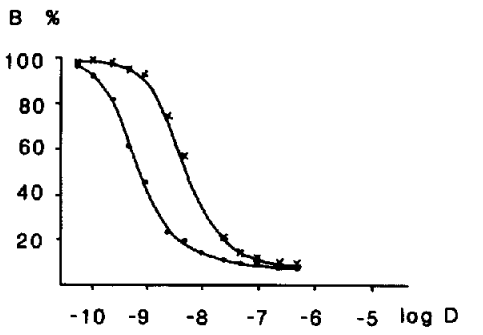

Figure 4
Inhibition of ^{3}H-dexetimide binding by scopolamine under equilibrium (x) and non-equilibrium (●) conditions (from [21]). B indicates bound labelled ligand, 100% represents the total binding in the absence of scopolamine; D is the concentration of scopolamine (mol l^{-1}).

addition of labelled ligand its bound fraction was lower than without pre-incubation; at the same concentration of analyte [24].

Low-temperature conditions are necessary when receptors used in assays undergo degradation by proteases. This can be the case when solubilized or purified receptors are removed from their natural surroundings, resulting in lower stability [25].

For some receptor assays, factors other than affinity of the ligand being assayed affect the binding and, consequently, the sensitivity of assay. For example, the dissociation rate of insulin from its receptor is a function of receptor occupancy. This phenomenon is referred to as negative cooperativity, which means accelerated dissociation of bound ligand (labelled ligand) as a result of an increasing receptor occupancy by unlabelled ligand (analyte). Thus, the drop in the amount of bound labelled insulin will be a function not only of an increasing amount of unlabelled insulin bound to its receptor (competition), but also a function of accelerated dissociation [26].

(C) The influence of pH: Ligand binding can be pH-dependent. For example, the binding of insulin to membrane receptors is highly pH-dependent with maximum binding between pH 7.8 and 8.0 [2]. The affinity of dexetimide (anticholinergic agent) is optimal and constant around the physiological pH, but the affinity of another anticholinergic, pirenzepine, increases with decreasing pH values, as can be seen in Fig. 5 [27]. It has to be noted, however, that the optimal pH for the analyte may not necessarily coincide with the optimum value for the labelled ligand. A good set of examples are dexetimide and pirenzepine when they are used as labelled ligand and analyte in the same

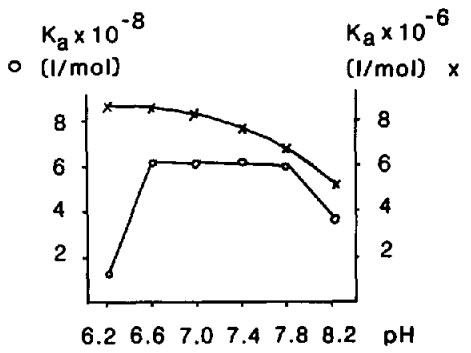

Figure 5
Equilibrium association constants $K_a = 1/K_d$ as a function of pH for ^{3}H-pirenzepine (x) and ^{3}H-dexetimide (O) (from [27]).

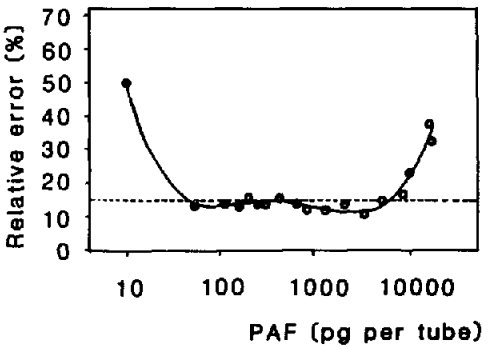

Figure 6
Precision profile of the radioreceptor assay of platelet activating factor (PAF). The working range of the assay, giving a precision error ≤ 15%, was 50–6000 pg tube^{-1} (from [55]).

assay. In this situation a good compromise would be pH 6.6 as indicated by Fig. 5.

Other factors that may affect the sensitivity of the assay are ionic strength, the presence of enzymes which degrade receptor or ligand, the presence of certain ions which influence the ligand–receptor binding. For instance sodium can selectively increase the sulpiride (dopaminergic agent) affinity in displacing ^{3}H-spiperone binding in striatum and anterior pituitary membranes [25].

Accuracy and precision. Dependent on the application of receptor assay, accuracy and precision should be within preset values. Accuracy problems due to cross-reacting metabolites or endogenous substances should be eliminated by adequate sample pretreatment procedures. However, accuracy and precision of the receptor assay itself is strongly dependent on the choice of receptor material and labelled ligand. The combination determines the extent of specific and non-specific binding. While for the assay only specific binding is required, non-specific binding will cause variations in the free and specifically bound fractions of both the labelled and unlabelled ligand. Due to the fact that determination of the non-specific binding can be affected by the separation method, e.g. adsorption to filters or removal of non-specific binding by the washing procedures, correction for non-specific binding is never perfect. While curve-fitting procedures are often based on a mathematical description of the receptor binding process, accuracy problems can also occur.

The nonspecific binding results from the combination — the tendency of labelled ligand

(as well as analyte) to bind nonspecifically (e.g. hydrophilic versus lipophilic labelled ligand) and the character of receptor preparation (cell culture, crude membrane homogenate, purified receptor, etc.). The nonspecific binding is due to the attachment of the labelled ligand to membrane components which are not receptors and/or the adsorption of ligands to the filters, tubes, etc.

In case that nonspecific binding is caused by labelled ligand binding to membrane proteins, this can be reduced by using enriched receptor preparations (the sensitivity is dependent on the concentration of receptors in assay — $[R]_o$, as follows from equation (4)). In case that the major part of nonspecific binding is due to labelled ligand binding to filters, tubes, etc. (opioid peptides), it is important to coat these materials with polyamines (polyethyleneimine, polybrene, protamine), serum albumin, etc. [28]. The advantage of coating filters with polyamines lies in preventing the binding of positively charged ligands by blocking the centres of nonspecific binding. For instance, nonspecific binding of beta-endorphins can be prevented by treating filters with bovine serum albumin or myelin protein. Coating with polylysine, bacitracin or silicone reduces the opioid peptides, substance P or calcitonin binding to the assay tubes. The appropriate choice of material of assay tubes is also important, for instance the lowest binding of ^{3}H-dexetimide was observed for polypropylene tubes when compared to the glass and polystyrene tubes [20]. On the other hand, the centrifugation method should be considered if filter-binding remains important after coating.

Specificity. Receptor assays discriminate between pharmacologically active compounds (bound with high specificity and affinity to the receptor material used in assay) and inactive compounds. Receptor assays do not discriminate between compounds of the same pharmacological class.

Since a single receptor may bind more than one ligand and the same ligand may bind to more than one receptor, the specificity of a receptor assay is determined by the receptor preparation, the labelled ligand used in assay and the binding properties of ligand being assayed.

The following situations and their combination will modulate the specificity of receptor assay: (1) the receptor preparation used contains a single or a heterogenous population of binding sites; (2) the labelled ligand used binds to a single or a heterogenous population of binding sites; and (3) the analyte under assay binds to a single or a heterogenous population of binding sites.

The use of a selective receptor preparation (with a single population of binding sites) excludes the necessity to use a selective labelled ligand. On the other hand, provided that the labelled ligand as well as the analyte bind with a high affinity to a single (and the same) population of binding sites, a receptor preparation containing a heterogenous population of binding sites is appropriate to warrant the specificity of receptor assay. If the labelled ligand and the ligand being assayed tend to bind to more than one class of receptors, the heterogeneity of the receptor preparation used may result in depletion of both ligands by irrelevant binding sites. This subsequently results in the decrease of free ligand concentration and the affinity of ligand (analyte) will be wrongly determined from the competition curve. For instance, ^{3}H-spiperone labels D_2-receptors but also serotonergic receptors in forebrain regions [29]. Thus, it would be inappropriate to use whole brain as a source of receptors to assay drugs known to interact with dopaminergic receptors. Preferably corpus striatum should be used in which the dopaminergic receptors prevail. The better the choice of receptor source, or, alternatively the purification of the receptor preparation the more specific the assay will be. Another possibility to obtain a receptor assay specific for dopaminergic agents is using a D_2-selective labelled ligand (^{3}H-sulpiride), which specifically labels D_2-receptor sites, as compared to ^{3}H-spiperone which labels D_2 as well as serotonergic receptors, or ^{3}H-domperidone which labels D_2- and alpha$_1$-receptors [25].

When the same receptor interacts with more than one class of ligand, the situation is more complex. For example, prolactin receptor of rat liver binds human prolactin and human growth hormone with equal affinity. On the other hand, cultured human lymphocytes contain growth hormone receptor which does not react with prolactin or with nonhuman growth hormone. Thus, the latter receptor source provides a more specific assay for human growth hormone [2].

In some cases the receptor containing tissue contains endogenous compounds which can

bind to this particular receptor. For instance, the rat liver contains receptors for insulin, insulin-like growth factor (IGF-II) and multiplication stimulating activity (MSA). When IGF receptor assay was developed using liver membrane preparations, the removal of insulin, pro-insulin and other interfering factors prior to assay was necessary [2]. There are some special situations in which the specificity of different receptor sources may vary, depending on the conditions of isolation of the membranes and the physical conditions of the incubation. The TSH receptor offers a good example of such a situation. TSH binding sites are present in thyroid, testes, fat cells, plasma membranes, and lymphocytes. The specificity for these receptor preparations should be relatively similar. However, under conditions of low temperature, pH and ionic strength, there is predominance of the sites with low affinity and specificity but high capacity. At physiological temperature and pH, and at salt concentration of 50 mM NaCl or higher, there is a predominance of sites with high affinity, high specificity and low capacity. Pretreatment of thyroid membrane with higher salt concentration and performing the TSH receptor assay at physiologic salt concentrations gave a specific assay without cross-reactivity of normal immunoglobulin, thyroglobulin, cholera toxin or gangliosides which normally cross-react with the assay [30].

In summary, the specificity in receptor assays may be improved by (1) exploring the different receptor sources and possibly finding a tissue with a single population of binding sites, (2) using labelled ligand which binds to the single population of binding sites, or (3) establishing appropriate incubation conditions for a receptor that enhance its specificity.

Stereoselectivity. A stereoselectivity assay is generally based on the difference in interaction between the chiral part of the analyte molecules and the chiral part of the interacting agent. For RA the interacting agent is a receptor molecule, with a binding site in a chiral environment. Stereoselectivity in receptor binding will only be observed where the chiral parts of the analyte molecule are involved in the interaction with the receptor binding site. For instance, the binding of catecholamines to the specific alpha- or beta-adrenergic receptors is stereoselective in contrast to polypeptide hormones which do not bind in a stereospecific fashion.

In the former case the receptor assay rather selectively measures the enantiomer with the higher affinity. When the differences between affinities of individual enantiomers are high enough, eudismic ratio >100, the concentration of the eutomer, the enantiomer with the highest affinity, can be read from a standard curve prepared with its racemate. For instance, a good agreement has been obtained between the determination of the (−)-enantiomer of propranolol by stereospecific HPLC and the receptor assay using a standard curve prepared with racemic propranolol [1]. Though in cases the concentration of the distomer is high relative to the eutomer, the contribution of the distomer is no longer negligible and the read-out should be expressed as eutomer equivalents instead of a true concentration of the eutomer.

Methodology of receptor assays.

The receptor preparation. The receptor material for receptor assays can usually be obtained conveniently from animal tissues and organs. The choice of appropriate tissue or organ is governed by the ligand being assayed (its pharmacological properties), by availability of tissue and by the concentration of receptors of interest in this tissue. In most receptor assays the tissue used as the source of receptors represents the pharmacological target tissue for the ligand being measured. The membranes of cells of such a tissue usually also contain other receptors as well as non-receptor proteins (from the point of view of receptor assays causing the irrelevant non-specific binding). The concentration of receptor binding sites among all these proteins is in the range of fmol–pmol/mg^{-1} of proteins and this fact prompts a careful choice of the receptor source. From the feasible tissues the one with the highest receptor concentration has to be selected.

The most common way of preparing membrane homogenates for the receptor assays includes the homogenization of tissue or organ from killed or anaesthetized animals in 0.32 M sucrose. In some cases, e.g. for tissues containing fibrous components (blood vessels), filtration of this homogenate may be necessary. From the first homogenate the individual subcellular fractions, such as the crude nuclear pellet, crude mitochondrial pellet or synapto-

somal fraction, microsomal and ribosomal fractions can be prepared step by step by differential centrifugation. The choice of the appropriate fraction depends on the content of a certain receptor. For instance, the crude nuclear pellet from rat brain homogenate was used as the source of beta-adrenergic receptors, while the crude mitochondrial pellet from the same homogenate was suitable for the receptor assay of ligand acting via alpha-adrenergic receptors [31].

In addition to the target tissues, nontarget tissues may possess specific binding sites for ligands and can be used in receptor assays, e.g. cultured human lymphocytes possess specific binding sites for insulin, human growth hormone and calcitonin. This receptor material is convenient as it does not require enzymatic treatment to isolate the cells, which is necessary in other receptor assays using whole cells from target tissue (liver or fat cells as the source of insulin receptors, adrenal cells for ACTH, etc.) [2]. Usually, receptor preparations in RA contain heterogenous populations of binding sites. In order to ensure the specificity of an assay with respect to one pharmacological class of binding sites, the approach previously described can be considered. In addition to this, the use of purified receptor preparations will be discussed later.

In only a few cases can a receptor preparation with a single class of receptors be obtained. In this regard, the availability of recombinant receptor may be an important step forward. For instance, receptor material for a hGH receptor assay was prepared by the expression of cloned cDNA for the binding domain of the receptor in *E. coli*, which resulted in the secretion of this specific binding protein in the culture medium. The binding properties of this protein were the same as for the full-length receptor and enabled a sensitive and specific receptor assay of hGH in the presence of plasma [24]. Another example can be the receptor assay for thyrotropin receptor autoantibodies, using the recombinant receptor protein [32]. High levels of the human TSH receptor were expressed by a transfected Chinese hamster ovary cell line. The advantage of using such a receptor preparation is that the cell line which produces the receptor protein can be adapted to growth in suspension cultures, which permits the large scale production. However, the application of recombinant receptors in quantitative receptor assays is not yet common practice, despite the fact that such an assay is much easier to standardize than the assay which uses the whole cells or crude membrane homogenate, because of the elimination of batch-to-batch variations of receptor material.

The choice of labelled ligand. In this paper, labelled ligand refers to a radiolabelled ligand. However, it should be mentioned that the introduction of fluorescence-labelled ligands represents a new direction in the development of receptor assays, which eliminates problems associated with the handling of radioactive material.

There are some general criteria for the labelled ligand used in a binding assay which are independent of the nature of labelling. The most important of them are the following:

(1) The labelled ligand should bind to the same receptor as the unlabelled ligand, with high affinity.

(2) The nonspecific binding of the labelled ligand (to the non-receptor binding sites and utensils) should be minimal.

(3) The labelled ligand should be chemically stable and resistant to enzymatic degradation. This criterion is met by most labelled ligands, with the exception of labelled peptides used in neuropeptides receptor assays [33].

(4) The labelled ligand should preferably be the eutomer, the pharmacologically most potent enantiomer.

When the labelled ligand is a radioligand, additional criteria have to be fulfilled:

(5) The ligand must be of high specific activity. This demand is governed by the fact that the density of binding sites in tissue is very low. From this point of view, ^{125}I-labelled ligands with specific activities up to 2000 Ci mmol^{-1} are more suitable than tritium labelled ligands. On the other hand, the half-life of ^{3}H is much longer than for ^{125}I, which may make the use of tritium-labelled compounds preferable.

(6) The radiochemical purity of the labelled ligand has to be ensured. Labelled impurities can cause artifacts (curvilinear Scatchard plot, Hill coefficient smaller than unity, poor specific to nonspecific binding ratio). Thin layer chromatography or HPLC are the methods of choice for the checking of the radiochemical and chemical purity of labelled ligands [34].

The criteria for the binding characteristics of

labelled ligands, such as the affinity towards the receptor and selectivity to a certain class of binding sites, were discussed in the paragraphs about the specificity and accuracy of receptor assay. However, some additional notes have to be made about the selectivity of labelled ligand.

(A) Subtype-selectivity of labelled ligand: The discovery of differences in affinity of some agents in the interaction with the same class of receptor in different tissues led to the subdivision of receptors. Problems can arise when using the subtype-nonselective labelled ligand for the determination of a subtype-selective ligand. For instance, muscarinic antagonists exert binding selectivities towards muscarinic receptors in different tissues, according to which the subdivision of this receptor into M_1–M_3 was introduced. When a nuvenzepine (M_1-selective) receptor assay was performed by competition with non-selective ^{3}H-*N*-methylscopolamine, the slope of competition curve was less steep than when the M_1-selective labelled ligand ^{3}H-pirenzepine was used [35]. In the latter case, the assay provided maximum specificity and sensitivity, together with higher accuracy. The specificity of assay was achieved by selective labelling of the M_1 muscarinic receptor whereas the receptor material used contained a heterogenous receptor population (rat cerebral cortex).

(B) Selectivity of labelled ligand based on its physico-chemical properties: In addition to the subtype selectivity, several reports have appeared about the binding heterogeneity of ligands based on their different physico-chemical properties. For instance, subtype-nonselective muscarinic antagonists like *N*-methylatropine and *N*-methylscopolamine (quaternary compounds) in competition experiments with their tertiary analogues (atropine, scopolamine) are able to distinguish two binding sites in rat brain, and in some other tissues, whereas the tertiary analogues recognize only one site. One of the possible explanations may be the existence of a portion of the muscarinic receptors located in a hydrophobic membrane environment and thus less accessible for the charged quaternary ligands [36]. As a result, the competition curve of labelled tertiary and unlabelled quaternary ligand will be shallow or even biphasic, which has a detrimental effect on the sensitivity of the receptor assay (Fig. 7).

Also the lipophilic or hydrophilic properties of the labelled ligand can cause heterogeneity

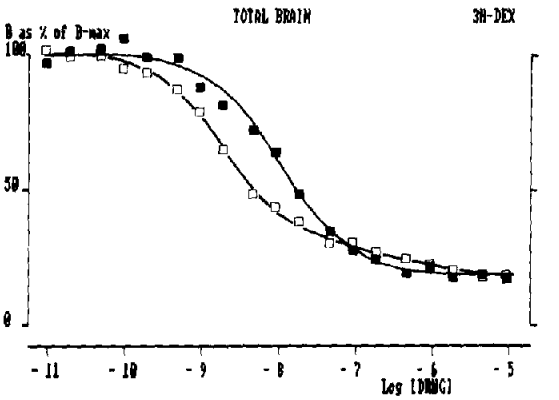

Figure 7
Inhibition of ^{3}H-dexetimide binding to bovine brain membranes (total brain minus cerebellum) by scopolamine (■) and *N*-methyl-scopolamine (□). Solid lines are computer-fitted curves according to a one-site (scopolamine) or a two-site (*N*-methylscopolamine) binding model. Typical experiment performed in duplicate (from [36]).

of binding, which is of a different character than the heterogeneity based on subtype selectivity. The lipophilic nature of a labelled ligand can be responsible for its binding to non-defined binding sites which can be displaceable, but which do not exert specificity and stereoselectivity. Examples are ^{3}H-dihydroalprenolol and ^{3}H-CGP 12177. Both ligands are nonselective beta-adrenergic antagonists with approximately the same affinity towards the beta-adrenergic receptors. The lipophilic nature of ^{3}H-DHA is responsible for its binding to additional non-beta-adrenergic high affinity binding sites on several types of tissue [37]. ^{3}H-CGP 12177 has hydrophilic properties and labels only cell surface beta-adrenergic receptors [38].

Characterization of receptor–ligand system. When the choice of the receptor preparation and labelled ligand has been made, this receptor–ligand system has to be characterized by binding studies. It is necessary to demonstrate that the bound labelled ligand is attached to the pharmacological receptor if *in vitro* receptor assay results are meant as an estimation of the pharmacological potency of analyte. In practice, this means that:

(a) The saturability of the labelled ligand binding to the receptor preparation has to be confirmed.

(b) The labelled ligand binding should be reversible. The bound labelled ligand should dissociate from the receptor preparation after

either dilution or addition of ligand in excess. The second approach is used to determine the nonspecific binding (to the non-receptor proteins). The bound fraction of labelled ligand which is left after the addition of the excess represents the nonspecific binding.

(c) The specificity of binding (desired receptor class is labelled) has to be assessed with ligands from different receptor classes in the displacement of labelled ligand from the receptor preparation.

(d) The distribution of *in vitro* ligand binding in various tissues should parallel the known distribution of receptors derived from *in vivo* studies with intact cells.

The labelled ligand binding to the receptor preparation should show saturation since this is inherent to specific binding. The nonspecific binding is a linear function of the free concentration of labelled ligand. The specific binding is measured indirectly; it is calculated as the difference between the total binding and the nonspecific binding. In parallel assays, labelled ligand is incubated without (total binding) and with (nonspecific binding) an excess of unlabelled ligand (Fig. 8(a)).

From the saturation curve of specific binding, which represents the dependence of the bound amount of labelled ligand on its free concentration at a fixed receptor protein concentration, the number of binding sites (B_{max}) and the affinity of labelled ligand (K_d^*) towards the receptor preparation can be determined. Usually, the analysis of a labelled ligand binding proceeds with the construction of a Scatchard plot, i.e. the plot of the ratio of Bound over Free ligand versus Bound ligand concentration (B/F vs B), (Fig. 8(b)). From this plot by fitting a straight line to the data, the affinity of labelled ligand can be determined. The intercept with the Bound axis estimates the number of binding sites and the slope is equal to $-1/K_d^*$. A general strategy and a versatile computer program for analysis of data from ligand-binding experiments has been developed by Munson and Rodbard [39].

Another way to evaluate the dependence of the Bound radioligand on its free concentration is the graphical presentation of B versus log F as proposed by Bjerrum [40].

Another approach, that has not been widely used until now, but one which is rather useful when dealing with heterogenous binding sites is the evaluation of binding data by affinity spectra [41]. An affinity spectrum is defined as a plot of the number of binding sites against corresponding dissociation constants. The advantage of this method in comparison to nonlinear regression analysis is the fact that no starting values and mathematical models have to be supplied and that the statistical assessment of the results is straightforward from a detailed graphical display of a likelihood function. The parameters to be estimated, i.e.

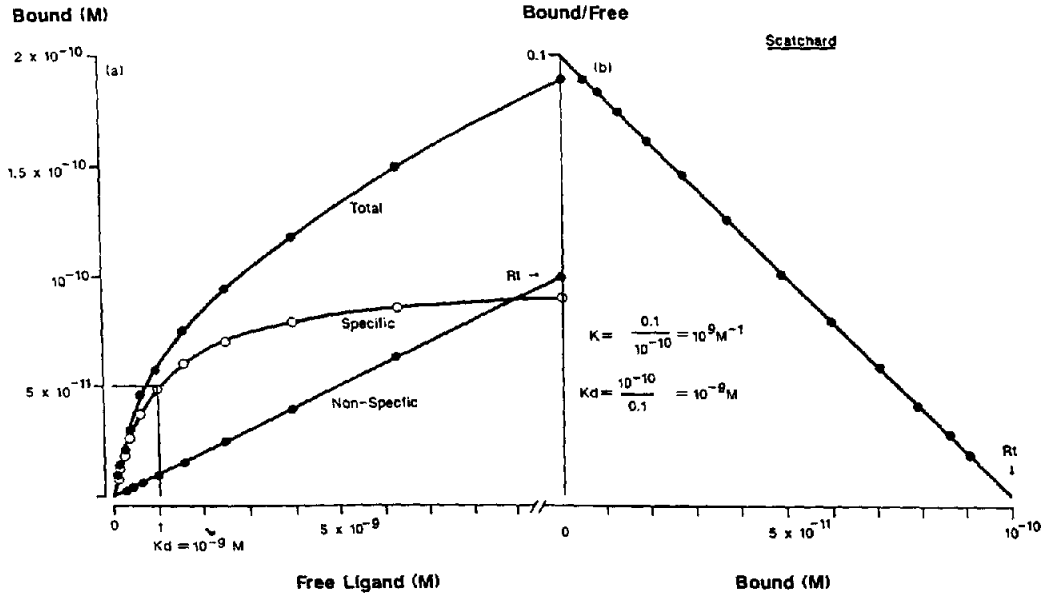

Figure 8
(a) Labelled ligand saturation curve showing total, nonspecific and specific binding. (b) Scatchard plot of specific binding, showing calculation of initial estimates of K_d and B_{max} (from [74]).

the number of binding sites (B_{max}) and the dissociation constant (K_d) are obtained visually from the plots, or as the results of calculations that are based on the principle of least-square errors. Such an approach has been applied to the studies on drug interaction with plasma proteins (the evaluation of racemic propranolol binding to human alpha$_1$-acid glycoprotein, [42]) and recently also to the evaluation of the binding of labelled ligands to the receptor (^{3}H-DHA and ^{3}H-CGP 12177 binding to beta$_2$-adrenoceptors of rat reticulocytes, [38]).

Estimation of nonspecific binding: Some additional remarks have to be made about the properties of the ligand used for the determination of the nonspecific binding. It is important to choose a suitable unlabelled ligand and an appropriate concentration. In order to determine the concentration of unlabelled ligand, competition experiments with labelled and unlabelled ligands are performed separately from the receptor assay. The lowest concentration of unlabelled ligand which completely inhibits the labelled ligand occupancy of receptors is considered appropriate for the determination of nonspecific binding. In practice, a concentration of 1000 × K_d of unlabelled ligand is sufficient. The use of a structurally different compound than the labelled ligand for this purpose is emphasized. This requirement is supported by the observation that some labelled ligands (lipophilic compounds) bind to the 'acceptor sites' present in the receptor preparation. These sites can be of lower affinity than the 'true receptors' (thus, they will be included in the value of nonspecific binding) and the binding of labelled ligand to them can be also saturable [29]. When the same compound as the labelled ligand (but unlabelled) is used to define the nonspecific binding, this could inhibit the labelled ligand binding also from these non-receptor sites and the nonspecific binding will be underestimated (and hence, the specific binding, as the difference between the total and nonspecific binding, will be overestimated). For instance, when a lipophilic labelled ligand is used in a receptor assay, the use of an excess of a hydrophilic unlabelled ligand is suggested or vice versa. Thus, the combination of ^{3}H-DHA and propranolol, as unlabelled ligand, seems to be unsuitable for a receptor assay of beta-blockers, because the similarity in binding properties of these ligands (both are lipophilic) can lead to a wrong estimation of specific binding. This was confirmed by the use of affinity spectra. When the 'specific' binding of ^{3}H-DHA was determined by the addition of an excess of propranolol and evaluated by affinity spectra, 43.6% of this binding could not be considered as the 'true' receptor binding (the specific binding was overestimated) [38]. When hydrophilic ^{3}H-CGP 12177 and propranolol were used the specific binding was estimated correctly.

Sample preparation. RA is usually applied to body fluids such as plasma, serum, urine, saliva or cerebrospinal fluid. In some cases the measurements have to be carried out in tissue extracts (brain, liver, etc.).

Adequate sample preparation may be necessary with the following aims:

(1) Elimination of endogenous compounds which affect binding of labelled ligand in a nonspecific manner, e.g. depletion of labelled ligand by proteins present in biological sample (plasma proteins) or modulation of receptor binding properties by certain anions or cations (e.g. urine).

(2) To isolate a particular analyte (parent drug or a particular active metabolite) from the biological matrix in order to obtain adequate specificity and sensitivity.

(A) Elimination of matrix interference: Unextracted samples can be assayed directly if the biological matrix does not effect the assay and the given selectivity and sensitivity are in line with the aim of the analysis. In case that the concentration of analyte is high enough a small volume of biological fluid which does not interfere with the assay or a diluted sample may be assayed directly.

The problem of interference concerns both the labelled ligand binding as well as the binding of ligand being assayed. As we have seen above, the physico-chemical properties of the ligand (lipophilic versus hydrophilic) play an important role.

The impact of the interference on the analytical characteristics of a receptor assay is shown in Fig. 9: (A) A high protein binding of the analyte reduces its free concentration in biological fluid. This is responsible for the apparent loss of potency of the analyte. (B) Labelled ligand binding to plasma proteins will result in the change of the amount of labelled ligand bound to the receptor and the sensitivity of assay will be lower. (C) When both labelled

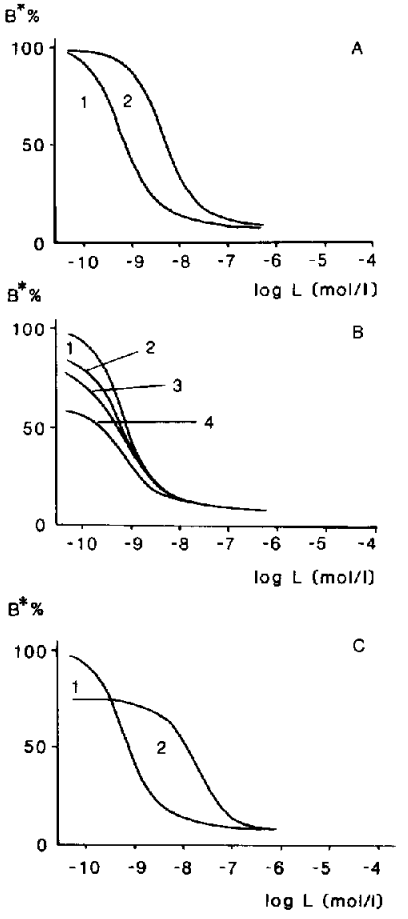

Figure 9
The influence of plasma on labelled and unlabelled ligand binding to the receptor in RRA. (A) Impact of protein binding of the analyte on the displacement curve in RA: 1 = without proteins; 2 = with proteins. (B) Impact of protein binding of the labelled ligand on the displacement curve in RA: 1 = control experiment without plasma; 2 = 2–10 μl of plasma; 3 = 20 μl of plasma; 4 = 50 μl of plasma. (C) Impact on the displacement curve if both the analyte and the labelled ligand bind to proteins in RA: 1 = without proteins; 2 = with proteins.

ligand and analyte bind to plasma proteins, it has impact on the affinity of analyte as well as on the sensitivity.

To avoid the binding of a labelled ligand to plasma proteins, a more hydrophilic ligand may be used. Receptor binding of a lipophilic labelled ligand was found to decline with increasing plasma volumes in the assay, leading to a rightward shift of the apparent K_d*-value. Yet, receptor binding of a hydrophilic ligand (^{3}H-CGP 12177) was not affected by the presence of plasma and therefore was more appropriate for the receptor assay of beta-blockers in plasma [1]. These phenomena are shown in Fig. 10.

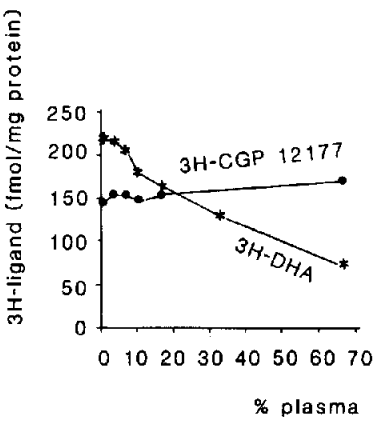

Figure 10
The influence of plasma on the receptor-binding of a lipophilic (^{3}H-DHA) and a hydrophilic labelled ligand (^{3}H-CGP 12177) (from [1]).

In the case when an analyte does not bind to matrix constituents, the measured concentration corresponds to its total concentration in a biological sample. When binding of analyte to matrix constituents is high, its free concentration will be measured. In order to separate this free fraction from the matrix, the biological sample can be subjected to dialysis or ultrafiltration. Another possibility is to extract the analyte from the biological sample by liquid–liquid extraction or solid-phase extraction. In the latter two cases the measured concentration will correspond to the total concentration of analyte. However, its free fraction determines its effect *in vivo*. Extraction of an analyte with a high protein binding in plasma may lead to erroneous interpretations with respect to the anticipated effect if this protein binding is not taken into account. Furthermore, plasma protein binding of parent drugs, active metabolites or enantiomers may be different [43]. Extraction of a plasma sample containing the parent drug and metabolites may disturb a concentration ratio between these compounds and may make a deduction of the effect in man from a RA result complicated, if not impossible. Therefore, the use of unextracted sample may be advantageous in certain situations. An example can be the measurement of native plasma samples of beta-blockers which gave a good agreement between the RRA results and the effects observed in man [1].

The development of a quantitative radioreceptor assay for beta-blockers in plasma samples illustrated that the character (solubilized versus particulate receptor) and source of

receptor preparation (different animal tissues) influenced the inhibition effect of plasma on ligand binding. The rationales of these effects, however, still remain to be explained. In RRA of propranolol, the addition of the same plasma volumes to lung membranes produced a lower inhibition than with cerebral cortical membranes [44]. In addition to this, both intra- and inter-assay variations were also lower in the former case. Thus, using lungs as the source of receptors was preferable. Plasma interference with the assay performed with cerebral cortical membranes was the reason for the separation of propranolol from plasma proteins by dialysis [8]. The use of solubilized receptor may in some cases be preferable, since the interference of plasma with the assay with solubilized receptor was lower than in the case of crude membrane homogenate [45]. When the sensitivity of an assay is high, dilution of the sample can avoid plasma interference, so that the relative impact of the interference becomes negligible [46, 47].

Liquid–liquid extraction (LLE) represents a classical sample preparation method. In recent years sample preparation by solid-phase extraction (SPE) has gained widespread acceptance as a viable alternative [48]. Solid-phase extraction is based on the principles of liquid chromatography [49]. However, the aims of SPE are to isolate and concentrate the relevant compound from a sample and at the same time removing unwanted, interfering substances, whereas the aim of liquid chromatography is to separate compounds from each other. SPE involves a solid phase (the sorbent which is usually silica or bonded silica) and a liquid phase (eluent). Advantages of SPE over LLE are the high selectivity, clean extracts, no emulsions, more reproducible results, reduced solvent usage and higher throughput through automation.

A solid-phase extraction procedure followed by a quantitative receptor assay provides an efficient procedure for therapeutic drug monitoring and pharmacokinetic studies of a.o. anticholinergics [50], the immunosuppressant agent FK-506 [51], digoxin [52] and opioid peptides [53]. The analyte, eluted from the column by an organic solvent is determined by the receptor assay. The extraction solvent should be without influence on the receptor binding of analyte. If this is not the case, the organic solvent may be evaporated and the dry residue redissolved in an appropriate aqueous buffer. However, complete redissolution of the analyte may not always be easy, and furthermore, care should be taken that the analyte is not being co-evaporated or sublimated.

(B) Isolation of some particular analytes: Besides the above mentioned extraction procedures, some special approaches to the sample preparation can be mentioned. Pretreatment with antibodies adsorbed onto a solid support, was used in the receptor assay of human interleukin (IL-1) [54]. IL-1 is composed of two polypeptides (isoforms) termed IL-1 alpha and IL-1 beta. These two proteins have similar molecular masses, however, the IL-receptor shows distinctly different binding properties for them: the binding of IL-1 beta is of higher affinity (LOD = 1×10^{-11} M). In order to selectively quantitate IL-1 alpha with a lower affinity (LOD = 3.7×10^{-9} M) in the presence of LI-1 beta, plasma samples were pretreated with an anti-human IL-1 beta antibody adsorbed onto Pansorbin. This pretreatment selectively removed IL-1 beta isoform without any cross-reactivity with IL-1 alpha. On the other hand, the low sensitivity of the receptor assay for IL-1 alpha enabled an almost selective assay for IL-1 beta without pretreatment. In this way, the ratio of the two IL-1 isoforms in the sample could be determined.

In the case of RRA for the platelet activating factor (PAF), a lipid fraction containing the compound of interest was first extracted from saliva with methanol–chloroform–water, acetylated and purified by HPLC and determined in the HPLC eluate [55].

The RRA of 1,25-dihydroxyvitamin D required the elimination of lipid interference with the assay. For this purpose a HPLC purification step prior to the assay was introduced [56].

Main directions in the improvement of receptor assays. The development of a receptor assay includes the following steps: (1) choice of a receptor preparation; (2) choice of a labelled ligand; (3) choice of an unlabelled ligand for the determination of nonspecific binding; (4) characterization of the receptor–ligand system, evaluation of binding data and optimization of assay conditions; (5) sample preparation; (6) incubation of labelled ligand and analyte with the receptor preparation at optimum conditions; (7) separation of the bound and free

fractions; (8) measurement of the bound or free fractions; (9) evaluation of the results.

Considering this scheme and keeping in mind the demands for the specificity, sensitivity and accuracy, two main areas for improvements may be defined:

(1) Improvement of the receptor preparation by enrichment of specific receptor sites (higher ratio specific/nonspecific binding) or the improvement of stability and homogeneity of the receptor preparation.

(2) Improvements in the analytical procedure of the RA, by addressing factors such as (i) automation/standardization of the pipetting; (ii) use of multi-well plates or other incubation devices which are amenable to automation of the whole assay; (iii) use of receptor material immobilized on solid matrices (multi-well plates, tubes, beads, etc.); (iv) innovation of counting technology of radiolabelled support samples in the microplate format, solid or liquid scintillation counting of samples; (v) introduction of non-isotopic receptor assays, etc.

The improvement of receptor preparation. The importance of a high ratio specific/nonspecific binding has already been outlined. From this point of view, the character of the receptor preparation is important.

Another important point is the use of crude membrane homogenate introducing batch-to-batch variations. Furthermore, aggregation and sedimentation of such receptor preparations can cause a non-uniform distribution of receptors in the assay vials which may result in poor precision. These factors may represent serious limits for the application of a receptor assay.

A good example of the importance of the choice of the appropriate receptor source is the digoxin receptor assay developed by Bednarczyk *et al.* [57], in which the different sources of digoxin receptor — porcine heart, dog kidney and human heart tissue were compared. The highest concentration of receptor binding sites per mg of tissue was found in the crude membrane homogenate from human heart. The use of this receptor preparation resulted in an improvement of precision and sensitivity as well as specificity (the interference by endogenous steroids was lower than with other tissues).

Fractionation of the crude membrane homogenate may increase the number of specific binding sites per mg of protein (a higher specific/nonspecific ratio).

The purification procedure involves the solubilization of the crude membrane homogenate by a detergent and the purification of the solubilizate by HPLC or by affinity chromatography.

(A) Solubilized receptor: Receptors are transmembrane glycoproteins embedded in the lipid bilayer of cell membrane. The first step in the purification of the individual proteins is the dispersion of the bilayer and the separation of the obtained components so that they can be fractionated. For this purpose an appropriate detergent is to be used. Detergents are amphophilic molecules, for example, phospholipids. They possess a hydrophilic head and hydrophobic tail. Due to these properties they will compete with the endogenous phospholipids and cholesterol molecules in the binding to integral membrane proteins. Detergents present in concentrations above their critical micelle concentration form micelles in the aqueous phase. With increasing detergent concentrations penetration into the membrane takes place so that mixed protein–detergent–phospholipid micelles are formed. In the whole process the detergent-to-phospholipid ratio is important. A crucial point is the choice of an appropriate detergent which is able to solubilize the receptor while retaining their biological activity. There are many detergents available, which can be subdivided into ionic (sodium deoxycholate, phosphatidylcholine, cetyltrimethylammonium bromide), nonionic (digitonin, TRITON series, Lubrol) or zwitterionic substances (CHAPS) can be chosen. The strategy for solubilization and purification of different receptor types is determined by the structure of the receptor rather than by the ligand specificity. Thus, a classification of receptors according to some common structural features can be useful. To this end, receptors may be classified into three groups [58]:

(1) Receptors with a single transmembrane segment (epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), insulin-like growth factor-2 receptor (IGF_2R), atrial natriuretic peptide receptor (ANPR), low-density lipoprotein receptor (LDLR), insulin receptor (IR), interleukin-2 receptor (IL_2R), nerve growth factor receptor (NGFR)).

(2) Oligomeric receptors incorporating both

ligand-binding sites and an ion channel (nicotinic acetylcholine receptor (nAChR), γ-aminobutyric acid receptor (GABAR), glycine receptor (GlyR)).

(3) Receptors which exert their effects by coupling to GTP-binding proteins (G-proteins) (muscarinic acetylcholine receptor (mAChR), β-adrenergic receptor (βAR), α-adrenergic receptor (αAR), substance K receptor (SKR), 5-hydroxytryptamine receptor (5HTR), angiotensin 2 receptor (A_2R)).

Receptors belonging to the first group are best solubilized with non-ionic detergents such as the TRITON series. Detergent binds preferably to the hydrophobic transmembrane part, while the ligand binding domain situated extracellularly is preserved from possible harmful effects of detergent. Thus, this group of receptors is relatively easily accessible to solubilization. The second group of receptors can be solubilized in the active form with sodium cholate or deoxycholate, in some cases by TRITON X-100 (nAChR). However, addition of exogenous phospholipids is necessary to preserve receptor binding properties.

For the third group of receptors common detergents such as TRITON X-100 and sodium cholate cannot be used since the ligand binding activity is completely lost. The reason is probably that the extracellular domains are smaller than those of the previous groups and that the transmembrane segments also participate in the ligand binding process. The transmembrane segment is the place of action for the detergent, thus the ligand binding activity is not recovered after solubilization. The most reliable detergent for this group is digitonin, sometimes supplemented with sodium cholate due to its mild solubilization properties.

On the basis of the above mentioned classification of receptors and their accessibility in the solubilization, it has to be assessed if the solubilization results in a stable and active receptor preparation in a quantity which is sufficient for a substantial number of receptor assays.

In some cases solubilization without further purification may result in a more homogeneous preparation which provides substantially better accuracy and precision.

Solubilized receptor may also give a substantial improvement by reduction of plasma interferences. Protein binding of labelled and unlabelled ligand is reduced by detergent. Furthermore, the variations in plasma interference with ligand binding, observed when crude homogenate was used and caused by batch-to-batch variations in such a receptor material, can be overcome by using the more homogeneous soluble preparation [59–62]. However, in other cases, the affinity of solubilized receptor was reduced in comparison with receptors imbedded in the cell membrane so that intact cells were preferable over the solubilized receptor [63].

(B) Purified receptor: The solubilized receptor can be processed further through purification by HPLC or affinity chromatography. The use of purified receptors may provide a further improvement in the receptor assay. For instance, the use of the receptor for atrial natriuretic peptide (ANP), purified by affinity chromatography, markedly improved the sensitivity and reproducibility of the assay. Moreover, the standard purified material was stable even after storage and repeated thaw and freeze cycles [64]. However, receptors occur in living species in extremely low concentrations. The source material for the purification contains microgram or nanogram amounts of active proteins and hence the yield of receptors is often very low. The purification step usually results in very low quantities of receptors which only allow further analysis of the receptor protein (partial sequence analysis, etc.) but these quantities are insufficient for routine assays.

Thus, when trying to obtain solubilized and highly purified receptors, the following criteria should be kept in mind:

(1) The simplicity of the purification step — the purification step should be as simple as possible, rapid and relatively inexpensive.

(2) The yield of purified receptor should be high enough to warrant quantitative receptor assays and the purified receptor should maintain its original binding properties.

(3) Possible detrimental effects of detergents and chemicals used should be determined and reduced to an acceptable level.

(C) Recombinant receptor: Rapid advances have been recently made in our understanding of the structure and function of receptors. This progress allows the preparation and isolation of cDNA clones, which can produce soluble proteins with binding properties of the intact receptor molecule after expression in cultured cells. Therefore, recombinant receptors are interesting and offer a novel perspective in obtaining standardized receptor material. It

can be produced in a sufficient quantity with uniform binding properties. For instance, cDNA encoding for the extracellular domain of the interleukin receptor (ILR) possessing ligand binding properties was used to produce the soluble murine IL-1 receptor by expression in HeLa cells. This receptor fragment (expressed in recombinant form) is secreted into the medium as a soluble protein which retains the IL-1 binding properties of the intact receptor. Such a receptor material was coupled to the IL-1RM5 antibody coated plates (analogy with ELISA) and used for the measurement of IL-1 activity (solid-phase RA) [61].

The use of recombinant receptors in receptor assays has been discussed earlier.

(D) Improvement of the stability of receptor preparation: Adequate stability of a receptor preparation is a prerequisite for its use in a receptor assay. This stability has to be ensured after storage of receptor material for longer periods of time.

In most cases, crude membrane homogenates used as receptor sources can be stored frozen (at −20°C or −80°C) without changes in the binding properties. However, multiple thaw and freeze cycles can lead to losses in binding sites and in homogeneity of the preparation. From this point of view, lyophilization of crude membrane homogenates is advisable resulting in material which may bring an improvement in the sensitivity and precision of the method as has been reported by several authors for anticholinergics and benzodiazepines [65–68].

Generally, with the introduction of purification steps, the stability of receptor material will decrease. This is connected with the loss of the natural surroundings of the receptor and the increased accessibility for proteases. Thus, purification and preferably also binding studies have to be performed at low temperatures in the presence of protease inhibitors. On the other hand, the stability of a purified receptor preparation may be improved by immobilization on solid matrices.

The improvement of the performance of receptor assay. In this paragraph the improvement of performance of receptor assays achieved by the use of receptors immobilized on solid matrices will be discussed.

Generally, receptor assays can be performed with:

(1) Membrane-bound receptor material distributed freely in the assay vial, e.g. crude membrane homogenate.

(2) Soluble receptor material distributed freely in the assay vial, e.g. solubilized receptor, purified receptor or recombinant receptor.

(3) Receptor immobilized on solid matrices, such as nitrocellulose membranes, tubes, microtitre plates, beads, etc. (solid-phase RA).

(A) Solid-phase receptor assay: Solid-phase receptor assays have been developed in analogy with solid-phase immunoassays. The ability of proteins to adsorb on plastic surfaces and membranes like nitrocellulose is employed. Plastic tubes or microtitre plates have been used as reaction vials and this permits rapid separation of the bound and free labelled ligand simply by washing the tube, plates, etc. Subsequently, numerous modifications of this basic principle, involving different plasticware, membranes and beads have emerged, together with covalent binding of the protein to the solid phase. This approach can overcome limitations of solid-phase assays caused by the small surface area to which proteins can be adsorbed.

The use of immobilized receptor was prompted by demands for sensitive and rapid methods to study receptors during their solubilization and purification. Since the stability of receptor in the process of solubilization and purification decreases, the idea of using nitrocellulose (NC) as a transfer medium for proteins appeared. Immobilized receptor can then be used for studying ligand and antibody binding. When antibody binding to the solubilized receptor is studied, it can be difficult to separate free labelled antibody from the antibody–receptor complex. The free antibody is also precipitated by polyethylene glycol, which is commonly used as a precipitation method. By using immobilized solubilized receptor, the antibody–receptor complex is retained and measured for bound labelled antibody while the free fraction is removed in the washing procedure [69].

The advantages of solid-phase RA over conventional solution-phase RA can be defined as follows:

(1) Solid-phase RA is a fast method for the simultaneous testing of large numbers of (biological) samples.

(2) The characterization and the binding properties of the solubilized receptors can be more easily studied by immobilizing the

extracted receptor proteins on solid phase and then probing them with labelled ligands or antibodies.

(3) Biological fluids, such as plasma, urine, etc., do not interfere with the binding characteristics of the immobilized receptor (unextracted plasma samples can be used).

(4) The solid phase with adsorbed protein can be used in some cases without significant loss of sensitivity and specificity for at least 1 month at room temperature or 0°C.

(5) The solid-phase receptor assay is simple and reproducible.

Yet, the application of this approach to quantitative receptor assays requires some additional comments.

Solid-phase supports can be divided into low capacity (polystyrene, polyvinylchloride, Nylon, glass, etc.) and high capacity (agarose, Sephadex, cellulose, nitrocellulose, etc.) supports. The low capacity supports are usually in the form of a continuous surface (walls of tubes and plates), while the high capacity supports are used as particles. The former, especially microtiter plates, offer the advantage of automation, which is an important advantage for quantitative assays in routine practice. For instance, the quantitative receptor assay of endothelin was performed on crude membrane homogenate immobilized on a 96-well microtitre plate. The assay was performed in 100 μl volumes with only 10–20 μg of membrane proteins. Endothelin concentrations as low as 10^{-10} M could be reliably determined directly in serum samples and the 96-well assay format allowed the rapid quantitation of a large number of samples [70]. However, the amount of proteins which can be immobilized is very low, which can be the reason for lower sensitivity for some assays. In the case of endothelin, this problem about the low capacity of solid support could be overcome by using a labelled ligand with high specific activity (^{125}I-endothelin) which enabled the use of such a small amount of unpurified receptor.

Another possibility is to use more purified receptor preparations enriched in specific binding sites. For instance, the immobilization of solubilized prolactin receptor on NC-membranes was sufficient for screening purposes [69]. Yet, a more purified receptor material (by affinity chromatography) together with a labelled ligand with a high specific activity (^{125}I-oPRL) was necessary in the quantitative solid-phase receptor assay of prolactin [71]. Because of the limited amount of protein that could be incorporated in the NC-membrane, the ConA-purified receptor was immobilized in order to obtain sufficient radioactivity for counting. Thus, the improvement of the performance of receptor assay goes hand in hand with the improvement of receptor preparation.

Some applications of solid-phase receptor assay are shown in Table 1.

Conclusions

Quantitative receptor assays can be seen as a modern application of the classical receptor assays used to establish the pharmacological

Table 1
Applications of solid-phase receptor assays

Receptor material	Methodology of the assay	Reference
Crude membrane homogenate of hGHR	Immobilized on 96-well microtitre plates, performed in the same plate	[72]
Solubilized mAChR	Performed on 96-well microtitre plates, precipitation method, cell harvester and filtration device used	[73]
Intact cells IL-1 alpha R	Immobilized on antibody coated 96-well microtitre plates, vacuum filtration manifold used	[61]
Solubilized IL-1 alpha R type II	Immobilized on NC-membrane filters, performed in microtitre plates, multichannel pipetting system	[54]
Solubilized prolactin R	Immobilized on NC-membrane filters, performed on filtration device	[69]
Prolactin R purified by affinity chromatography	Immobilized on NC-membrane filters, performed in tubes	[71]
Crude membrane homogenate endothelin R	Immobilized and performed in 96-well microtitre plates	[70]

properties of substances such as hormones, neurotransmitters and drugs. Thus, the potential of these assays goes hand in hand with the progress in basic knowledge on the receptor itself and the identification of biologically active chemical entities (pharmacological screening). On the other hand, quantitative receptor assays require their own methodology, which may differ substantially from that used in pharmacological studies. Receptor assays (up until now, known almost exclusively in the form of radioreceptor assays) possess the advantage over chemical detection in that they measure the total biological activity of a sample and that they are simple, specific and sensitive. Depending on the class of compounds under assay, a meaningful correlation can be derived between plasma concentrations and the therapeutic effect elicited.

The various limitations of RRA, which follow from the biological material involved in assay, can be overcome by using more standardized receptor preparations (solid-phase RRA with immobilized receptor) and by automation of the whole procedure.

Future advances may be dependent on the introduction of fluorescence- and enzyme-labelled ligand instead of radioactive-labelled ligand, in order to overcome the problems connected with the handling of radioactive material and to further improve the sensitivity of receptor assays.

References

[1] A. Wellstein, in *Determination of Beta-blockers in Biological Material* (V. Marko, Ed.), Chpt. 6.2. Elsevier, Amsterdam (1989).

[2] P. Gorden and B.D. Weintraub, in *Williams Textbook of Endocrinology* (J.D. Wilson and D.W. Foster, Eds), Chpt. 6, pp. 133–146. Philadelphia (1985).

[3] M. Blecher and R.S. Bar, in *Receptors and Human Disease* (Williams and Wilkins, Eds), pp. 237–257. Baltimore (1981).

[4] R. Lefkowitz, J. Roth and I. Pastan, *Science* **170**, 633–635 (1970).

[5] S.J. Enna and S.H. Snyder, *J. Neurochem.* **28**, 1121–1124 (1977).

[6] J.W. Ferkany, L.A. Smith, W.E. Seifert, R.M. Caprioli and S.J. Enna, *Life Sci.* **22**, 2121–2128 (1978).

[7] I. Creese and S.H. Snyder, *Nature* **270**, 180–182 (1977).

[8] R.B. Innis, S.B. Bylund and S.H. Snyder, *Life Sci.* **23**, 2031–2038 (1978).

[9] R.B. Innis, L. Tune, R. Rock, R. De Paulo, D.C. U'Prichard and S.H. Snyder, *Eur. J. Pharmacol.* **58**, 473–479 (1979).

[10] J.W. Ferkany, Symposium on Analysis of Neurotransmitters, Stockholm, Sweden (1987).

[11] R.J. Gould, K.M. Murhy and S.H. Snyder, *Life Sci.* **33**, 2665–2672 (1983).

[12] R. Zaczek, K. Koller, R. Cotter, D. Heller and J.T. Coyle, *Proc. Nat. Acad. Sci.* (USA) **80**, 1116–1119 (1983).

[13] R. Simantov and S.H. Snyder, *Life Sci.* **18**, 781–788 (1976).

[14] R. Simantov, S.R. Childers and S.H. Snyder, *Br. Res.* **135**, 358–367 (1977).

[15] B.L. Brown, R.P. Elkins and J.D.M. Albano, *Adv. Cycl. Nucl. Res.* **2**, 25–40 (1972).

[16] R.A.J. Challis, I.H. Batty and S.R. Nahorski, *Biochem. Biophys. Res. Commun.* **157**, 684–691 (1988).

[17] K. Ensing, K.G. Feitsma, D. Bloemhof, W.G. in't Hout and R.A. de Zeeuw, *J. Biochem. Biophys. Meth.* **13**, 85–96 (1986).

[18] D. Rodbard, *Anal. Biochem.* **90**, 1–12 (1978).

[19] S.V. Zaitsev, M.G. Sergeeva, S.D. Varfolomeev, *Bioorganic-zeskaja Chemija* **11**, 370–379 (1985).

[20] K. Ensing, Bioanalysis of Anticholinergics with Muscarinic Receptors in Relation with Chronic Obstructive Lung Diseases. Ph.D. thesis, University of Groningen (1984).

[21] K. Ensing and R.A. de Zeeuw, *Anal. Lett.* **17**, 1674–1678 (1984).

[22] T.C. Chen, A.K. Turner and M.F. Holick, *J. Nutr. Biochem.* **1**, 320–327 (1990).

[23] K.A. Frey, R.L.E. Ehrenkaufer, S. Beaucage and B.W. Agranoff, *J. Neurosci.* **5**, 421–428 (1985).

[24] M.M. Illondo, M. Vanderschueren-Lodeweyckx and P. de Meyts, *Horm. Res.* **36**, 21–26 (1991).

[25] E. Stefanini, A.M. Ortu, F. Vernaleone and G.L. Gessa, *Pharmacol. Res. Commun.* **19**, 777–791 (1987).

[26] R.C. Eastman, M.A. Lesniak and J. Roth, *J. Clin. Endocrinol. Metabol.* **49**, 262–267 (1979).

[27] K. Ensing and R.A. de Zeeuw, *Trends Anal. Chem.* **3**, 102–106 (1984).

[28] M. Huang, L. Metherell and O.P. Rorstad, *J. Rec. Res.* **8**, 831–838 (1988).

[29] P.G. Strange, in *Critical Reports on Applied Chemistry*, Vol. 24 (*Radiochem. Biomed. Res.*) pp. 56–93. Blackwell, London (1988).

[30] F. Pekkonen and B.D. Weintraub, *J. Biol. Chem.* **255**, 8121–8127 (1980).

[31] P. Crevat-Pisano, C. Hariton, P.H. Rolland and J.P. Cano, *J. Pharm. Biomed. Anal.* **4**, 697–716 (1986).

[32] S. Costagliola, S. Swillems, P. Niccoli, J.E. Dumont, G. Vassart and M. Ludgate, *J. Clin. Endocrinol. Metab.* **75**, 1540–1544 (1992).

[33] R. Quirion and P. Gaudreau, *Neurosci. Biobehav. Rev.* **9**, 413–420 (1985).

[34] J. Šmisterová, L. Šoltés and Z. Kállay, *J. Label. Comp. Radiopharm.* **27**, 481–484 (1989).

[35] G. Caselli, M.P. Ferrari, G. Tonon, G. Clavenna and M. Borsa, *J. Pharm. Sci.* **80**, 173–177 (1991).

[36] A.F. Roffel, K. Ensing, W.G. in't Hout, R.A. de Zeeuw and J. Zaagsma, *Arch. Int. Pharmacodyn.* **314**, 90–104 (1991).

[37] S.R. Nahorski and A. Richardson, *Br. J. Pharmacol.* **66**, 469–470 (1979).

[38] Z. Kállay, J. Šmisterová, L. Šoltés and V. Marko, *J. Rec. Res.* **11**, 909–917 (1991).

[39] P.J. Munson and D. Rodbard, *Anal. Biochem.* **107**, 220–239 (1980).

[40] I.M. Klotz, *Science* **220**, 979–981 (1983).

[41] H.J. Tobler and G. Engel, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **322**, 183–192 (1983).

[42] L. Šoltés, B. Sébille and A. Fügedi, *BioScience* **8**, 13–17 (1989).

[43] E.J. Ariens, *Eur. J. Clin. Pharmacol.* **26**, 663–668 (1984).

[44] D.B. Barnett, M.I. Batta, B. Davies and S.R. Nahorski, *Eur. J. Clin. Pharmacol.* **17**, 349–354 (1980).
[45] D.B. Barnett, N. Cook, K.E.J. Dickinson and S.R. Nahorski, *Br. J. Clin. Pharmacol.* **13**, 284P (1982).
[46] R.P. Elkins, J.F. Kelly and B.J. Rosenberg, **32**, 180–183 (1986).
[47] S.R. Nahorski, M.J. Batta and D.B. Barnett, *Eur. J. Pharmacol.* **52**, 393–396 (1978).
[48] X.-H. Chen, J.-P. Franke and R.A. de Zeeuw, *Forensic Sci. Rev.* **4**, 147–159 (1992).
[49] M. Zief and R. Kiser, *Solid-Phase Extraction for Sample Preparation*. J.T. Baker, Phillipsburg, NJ (1988).
[50] N.M. Cintron and Y. Chen, *J. Pharm. Sci.* **76**, 328–332 (1987).
[51] J.N. Murthy, Y. Chen, V.S. Warty, R. Venkataramanan, J.G. Donnelly, A. Zeevi and S.J. Soldin, *Clin. Chem.* **38**, 1307–1310 (1992).
[52] E.L. Manchester, E. Giesbrecht and S.J. Soldin, *Ther. Drug Monit.* **9**, 61–66 (1987).
[53] G.H. Fridland and D.M. Desiderio, *Life Sci.* **41**, 809–812 (1987).
[54] G. Scapigliati, P. Bossu, S. Censini, A. Tagliabue, D. Boraschi and P. Ghiara, *J. Immunol. Meth.* **138**, 31–38 (1991).
[55] C. Tiberghien, L. Laurent, M.P. Junier and F. Dray, *J. Lipid Mediators* **3**, 249–266 (1991).
[56] T.A. Reinhardt, R.L. Horst, J.W. Orfan and B.W. Hollis, *J. Clin. Endocrinol. Metab.* **58**, 91–98 (1984).
[57] B. Bednarczyk, S.J. Soldin, I. Gasinska, M.D. Costa and L. Perrot, *Clin. Chem.* **34**, 393–397 (1988).
[58] T. Haga, K. Haga and E.C. Hulme, in *Receptor Biochemistry — A Practical Approach* (E.C. Hulme, Ed.), Chpt. 2. Oxford University Press, Oxford (1990).
[59] P. Brodsted, M. Luthman, L. Wide, S. Werner and P. Roos, *Acta Endocrinol.* **122**, 241–248 (1990).
[60] H. Ong, A. De Lean and C. Gagnon, *Clin. Chem.* **34**, 2275–2279 (1988).
[61] J. Slack, J.E. Sims, A.M. Pitt and S.K. Dower, *Biotechniques* **7**, 1132–1138 (1989).
[62] F. Riske, R. Chizonite, P. Nunes and A.S. Stern, *Anal. Biochem.* **185**, 206–210 (1990).
[63] S. Radoff, Z. Makita and H. Vlassara, *Diabetes* **40**, 1731–1738 (1991).
[64] T. Mizuno, K. Uchida, M. Shimonaka, M. Akita, S. Hirose, T. Yukimura, M. Saitoh, F. Ikemoto and K. Yamamoto, *Biomed. Res.* **11**, 29–34 (1990).
[65] K. Ensing, W.G. in't Hout and R.A. de Zeeuw, *Anal. Lett.* **20**, 489–502 (1987).
[66] J. Lund, *Scan. J. Clin. Lab. Invest.* **41**, 275–280 (1981).
[67] M.G. Sergeeva, I.N. Kurochkin, O.A. Sklyankina, S.V. Zaitsev and S.D. Varfolomeev, *Neurochem.* **5**, 15–23 (1989).
[68] S.J. Dencker and G. Johansson, *Psychopharmacology* **97**, 561–562 (1989).
[69] S. Sakai and H. Hurakami, *Anal. Biochem.* **167**, 406–410 (1987).
[70] J.S. Nichols, H. LeVine, G.F.H. Smith, D.M. Wypij and J.S. Wiseman, *J. Biochem. Biophys. Meth.* **25**, 173–184 (1992).
[71] M. Suzuki, K. Kohmoto and S. Sakai, *Anal. Biochem.* **200**, 42–46 (1992).
[72] M. Bortolussi, O. Selmin and A. Colombatti, *J. Immunoassay* **8**, 219–235 (1987).
[73] A. Ahmad, R.K. Gordon and P.K. Chiang, *Feb.* **214**, 285–290 (1987).
[74] E.C. Hulme, in *Receptor Biochemistry — A Practical Approach* (E.C. Hulme, Ed.), p. 311. Oxford University Press, Oxford (1990).

[*Received for review 25 July 1993; revised manuscript received 5 October 1993*]