Journal of Pharmaceutical & Biomedical Analysis
Vol. 4, No. 6, pp. 697–716, 1986
Printed in Great Britain

0731–7085/86 $3.00 + 0.00
Pergamon Journals Ltd.

Fundamental aspects of radioreceptor assays*

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Abstract: Significant advances in understanding the actions of hormones, neurotransmitters and drugs resulted from the development of new analytical procedures. Among these advances the discovery that, for a given drug (or hormone or neurotransmitter), there exists a receptor site on membranes to which the substance must attach in order to evoke a biological response, led to the setting up of the radioreceptor assays.

Thus ligand-binding techniques can be used as assays for measuring drug levels in biological tissues and fluids. Radioreceptor assays are based on the principle of competitive protein-binding methods; when a radioligand and an unlabelled ligand are present together with a specific receptor preparation, the amount of radioactive ligand bound to the receptor is a quantitative function of the amount of unlabelled ligand present in the incubation medium.

The technology of the radioreceptor assays is simple and rapid to perform when optimal conditions for binding are determined. Radioreceptor assays are highly sensitive, reliable, precise and accurate. Their chief limitation relates to specificity as any substance having an appreciable affinity for the receptor displaces the specifically bound radioligand. Paradoxically this lack of specificity may be in some cases advantageous in that it allows for the detection of the parent compound and active metabolites in proportion to their affinity for the specific receptor.

During recent years, radioreceptor assays have been applied to quantitative determination of hormones, neurotransmitters and drugs. Among these, the radioreceptor assay of benzodiazepines is a typical example. In addition to drug analysis and drug monitoring, radioreceptor assays have been found to be versatile tools for basic studies on the receptor itself, on the endogenous ligand(s) for the receptor and on the discovery and the identification of biologically active chemical entities (pharmacological screening). The future of radioreceptor assays in the pharmacological sciences appears to be promising.

Keywords: *Receptor–ligand-binding; hormones; neurotransmitters; drugs.*

Introduction

Receptor-binding assays are based on the fact that, for a given biologically active substance or ligand, there exists a receptor site in tissues to which the substance must attach in order to evoke a corresponding response [1]. This attachment is reversible, specific and saturable. If the ligand is radiolabelled, one can talk about radioreceptor

*Presented at the "Second International Symposium on Drug Analysis", May 1986, Brussels, Belgium.

binding assays or radioreceptor assays (RRAs). The relationship between receptor, radioligand and complex is shown in equation (1) where (R), (L) and (RL^*) are the concentration of receptor, radioligand and complex, respectively, and k_{-1} and k_{+1} are the dissociation and association rate constants:

$$(R) + (L^*) \rightleftharpoons (RL^*). \quad (1)$$

Their ratio, the so-called dissociation constant Kd, is inversely proportional to the affinity of the radioligand for the receptor. Equation (1) obeys the law of mass action so that at equilibrium Kd may be calculated from the following relationship:

$$Kd = (RL^*)/(R).(L^*).$$

If an unlabelled ligand (L) is introduced into the system, we obtain equation (2) where two receptor complexes are generated:

$$(R) + (L^*) + (L) \rightleftharpoons (RL^*) + (RL). \quad (2)$$

A representation of the receptor–radioligand interactions is given in Fig. 1. When a measured quantity of radioligand is added to specific receptors the radioligand binds to form a labelled complex. If unlabelled ligand is introduced it will compete for the binding sites, displacing the radioligand. At equilibrium, the bound radioactivity remaining is a function of the amount of cold ligand added. It is possible to determine unknown quantities of this unlabelled ligand by comparing the bound radioactivity displaced by an unknown sample with the amount displaced by a series of solutions of known concentration. Binding assays have numerous uses which can be divided into two classes. First, they are versatile tools for basic studies on the receptor itself, e.g. distribution of the receptors, identification of receptor subtypes, and of physiological, pharmacological or pathological modifications of binding parameters. Second, binding assays have been applied to the discovery of endogenous ligands and to the screening of new compounds, although the intrinsic activity of these compounds cannot be predicted. RRAs can be considered as analytical applications of binding studies.

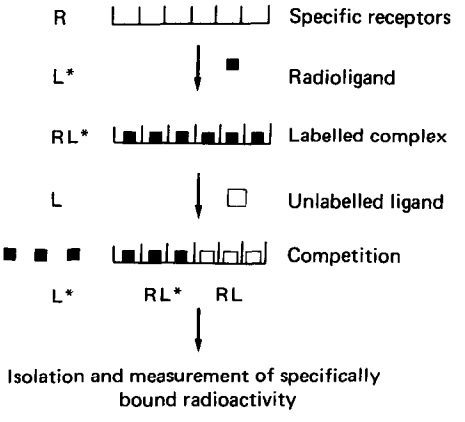

Figure 1
Basic scheme of a radioreceptor assay.

After these general considerations the fundamental aspects of RRAs will be discussed together with the general methodology and the analytical characteristics of RRAs. Finally, some RRAs that are performed routinely and their applications will be considered.

Methodology of Radioreceptor Assays

Basic principles

Before any RRA can be developed there are four parameters that must be evaluated: the choice of the appropriate radioligand, the identification of the receptor, the determination of the linearity of the binding and the evaluation of optimal binding conditions. These will be illustrated for benzodiazepine receptor binding.

The choice of the appropriate radioligand. This radioligand should have the same chemical properties as the unlabelled ligand, i.e. the labelling should not change the specificity for the receptor. For this reason tritiated compounds are preferred. Second, the labelled ligand must have a high specific activity (Ci $mmol^{-1}$) in order to ensure maximum sensitivity and to decrease nonspecific binding. This nonspecific binding is due to the irreversible attachment of the radioligand to biological components which are not receptors. These nonspecific binding sites have a relatively low affinity and their numbers far exceed those of the specific receptor sites, so that the nonspecific binding is apparently nonsaturable.

With a radioligand of high specific activity it is possible to perform the incubation with a low radioligand concentration. Under these conditions the radioligand will bind primarily to the specific high affinity sites. In order to estimate the nonspecific binding (Fig. 2), incubation is performed with a large excess of unlabelled ligand which will

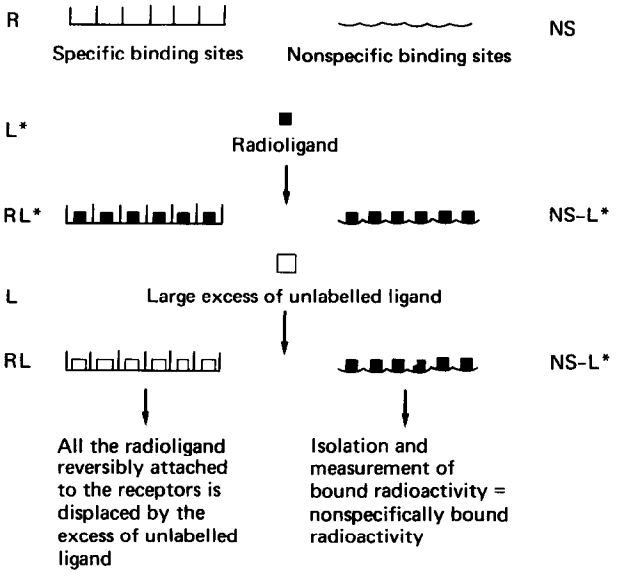

Figure 2
Determination of nonspecifically bound radioactivity.

displace all the specifically attached radioligand. The bound radioactivity thus measured will be due to nonspecific binding only. Specifically bound radioactivity is defined as the difference between the amount of radioligand bound in the absence of, or presence of, a large excess of unlabelled ligand. A RRA cannot be developed until a radioligand of high specific activity has been prepared. Several tritiated benzodiazepines are available for RRA, all having a specific activity ranging from 70–90 Ci $mmol^{-1}$. Some radioiodinated compounds are used as labels, particularly for hormone RRAs, and have very high specific activities (1000–2000 Ci $mmol^{-1}$).

The identification of the receptor. It is necessary to demonstrate that the bound radioligand is attached to the appropriate receptor before an RRA can be developed. This is indicated by: the saturability of the receptor preparation; the specificity of the binding; and the heterogeneous distribution of the specific receptors in a given tissue.

Saturability implies the existence of a limited number of specific binding sites. This number of binding sites is estimated by the saturation curve, which plots the bound radioactivity versus increasing quantities of radioligand added to constant volumes of receptor preparation. Figure 3 shows the saturation curve of the benzodiazepine receptors by ^{3}H-flunitrazepam [2]. The number of binding sites corresponds to B_{max} where the curve reaches a plateau. It is expressed in fmol mg^{-1} protein. This curve also allows the determination of *Kd* for the labelled compound, which corresponds to the radioligand concentration at half-saturation of receptor sites.

More accurate values for these parameters can be obtained from a linear plot of the saturation curve. The Eadie–Hofstee representation (Fig. 4) plots the bound radioactivity versus the bound to free ratio (B against B/F). The value for B_{max} is found from the intercept of the line on the y axis, and the slope of the line is equal to $-1/Kd$. Another theoretically linear plot is the Scatchard representation which plots B/F versus B.

The most important criterion that must be fulfilled is the specificity of the binding; thus compounds which are supposed to physiologically interact with a given receptor would inhibit the binding of the radioligand. Compounds which are known to be biologically inactive at this site are likely to be much weaker inhibitors of binding. The affinity of these substances for the receptor can be estimated by their displacement curve which plots the bound radioactivity against increasing concentration of ligand added to constant quantities of radioligand and receptors. Figure 5 shows the displacement curve of ^{3}H-flunitrazepam by diazepam (unpublished data). The amount of ligand which displaces 50% of the bound radioligand is called the IC_{50} value. This value is inversely proportional to the affinity of diazepam for the receptor. Attempts have been made to correlate IC_{50} and pharmacological effect. Mohler *et al.* [3] have correlated the IC_{50} values of various benzodiazepines (Fig. 6) with the minimum effective dose necessary to induce a muscle relaxant effect in the cat.

Further evidence for the specificity of ^{3}H-diazepam binding to rat brain membranes was indicated by the inability of numerous drugs such as tricyclic antidepressants and neuroleptics to inhibit the binding [4]. The authors have obtained similar results (unpublished data) with some pharmacologically inactive benzodiazepine metabolites such as 7-aminoflunitrazepam (Fig. 7).

Another indication that specific binding occurs at biologically relevant receptors can be obtained by studying the regional distribution of the specific binding in the animal. All known hormone, neurotransmitter and drug receptors are heterogeneously distributed

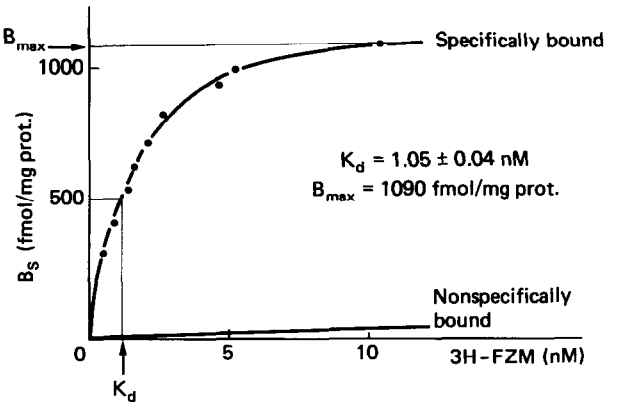

Figure 3
Saturation curve of specifically bound ^{3}H-flunitrazepam on synaptosomal fraction from rat cerebral cortex (from [2]).

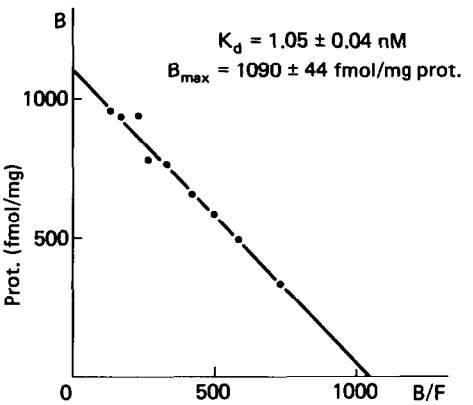

Figure 4
Eadie–Hofstee analysis of the saturation curve of ^{3}H-flunitrazepam binding on synaptosomal membranes from rat cerebral cortex (from [2]).

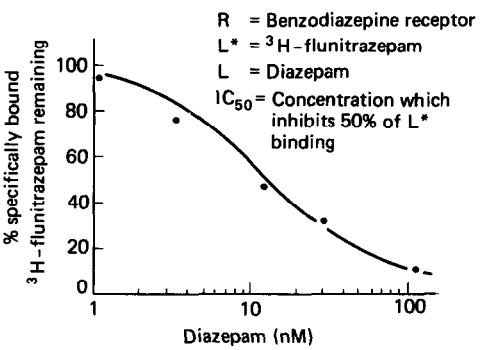

Figure 5
Displacement curve of ^{3}H-flunitrazepam by diazepam.

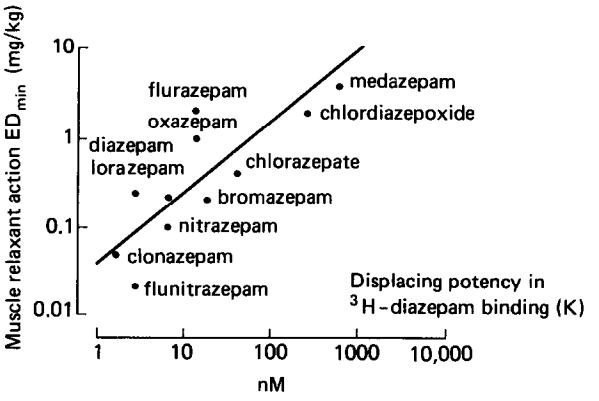

Figure 6
Correlation between affinity and pharmacological potencies of some benzodiazepines (from [3]).

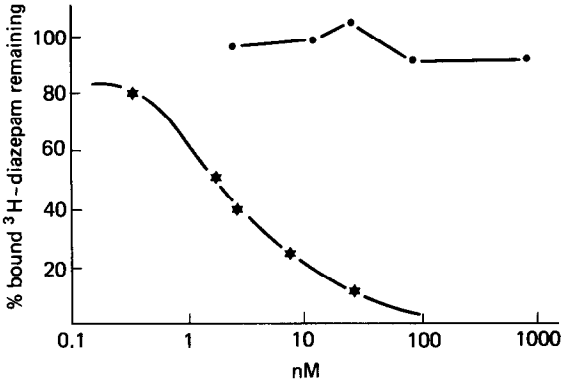

Figure 7
Displacement curve of ^{3}H-diazepam by 7-aminoflunitrazepam (•–•) and flunitrazepam (⋆–⋆). Experiments were performed on synaptosomal membranes from rat cerebral cortex.

throughout a given area whereas nonspecific binding should be uniformly distributed. Hariton *et al.* [2] have shown that ^{3}H-flunitrazepam binds preferentially to cerebral cortical and hippocampal membranes of rat brain.

The determination of linearity of binding. The binding of the radioligand to the receptor must be shown to be linear with respect to increasing receptor concentration. For example, (Fig. 8) ^{3}H-diazepam binding is linear up to 3 mg protein per assay [5].

Evaluation of optimal binding conditions. It is necessary to determine the optimum conditions of pH, temperature, incubation time and incubation buffer for binding before a RRA may be developed. With regard to ^{3}H-diazepam binding (Fig. 9), the respective values of these parameters are pH 7–7.4, 4°C and 15–60 min [5]; the incubation buffer used is generally Tris–HCl or Krebs–Tris–HCl. When it has been demonstrated that the radioligand bound to the tissue is attached to the appropriate receptor and when the

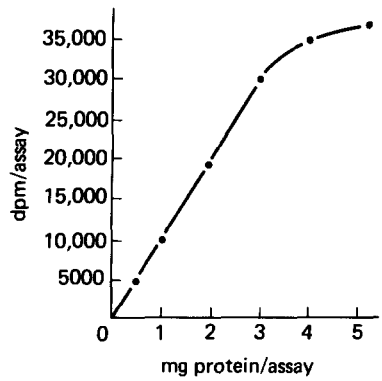

Figure 8
Linearity of ^{3}H-flunitrazepam binding with increasing concentrations of synaptosomal membranes from rat cerebral cortex (from [3]). (Experiments were performed on synaptosomal membranes from rat cerebral cortex.)

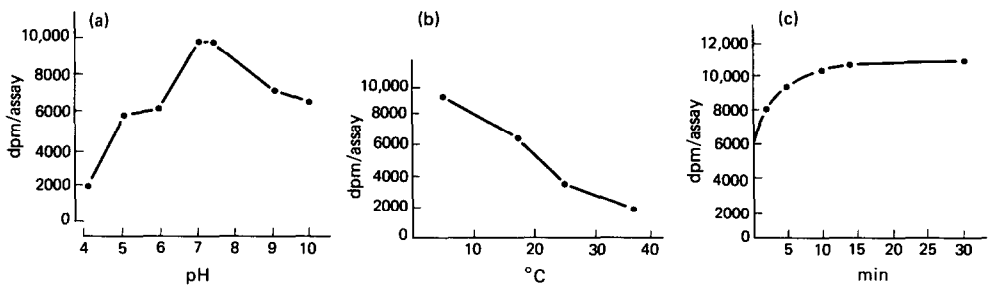

Figure 9
(a) pH Dependancy of ^{3}H-diazepam binding; (b) temperature dependancy of ^{3}H-diazepam binding; (c) time necessary to reach equilibrium. Experiments were performed on synaptosomal membranes from rat cerebral cortex (from [5]).

optimal binding conditions have been determined, a RRA can be developed as an offshoot of these receptor radioligand studies.

Before the development of RRAs is considered it is necessary to cover the basic principles of multiple binding site interactions. It is well known that the ligand can demonstrate high affinity for more than one class of receptor. When a radioligand which binds to 2 classes of sites with equal affinity is displaced by a ligand which displays more affinity for one of these classes, the displacement curve will be shallow. This is the case (Fig. 10) when ^{3}H-flunitrazepam is displaced by CL 218872, a triazolopyridazine, structurally unrelated to the benzodiazepines [6]. The saturation curve of the benzodiazepine receptors by ^{3}H-CL 218872 is biphasic and the corresponding Eadie–Hofstee plots are curvilinear. The curvilinear plots can be resolved into 2 lines, corresponding, respectively to each class of site, so that the B_{max} and the *Kd* can be calculated (Fig. 11).

Thus it seems that two distinct types of high affinity benzodiazepine receptor exist in the central nervous system: the so-called benzodiazepine alpha I and benzodiazepine alpha II subsites (Fig. 12). In addition, Weissmann *et al.* [7] have demonstrated a third class of site, the benzodiazepine beta subsite, corresponding to the peripheral benzodiazepine receptors which bind selectively to ^{3}HRO 5-4864 or chlorodiazepam.

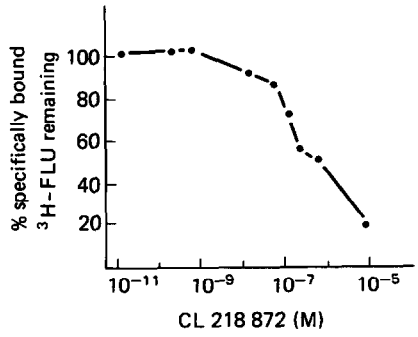

Figure 10
Displacement curve of ^{3}H-flunitrazepam by CL 218 872 (from [6]). Experiments were performed on slide-mounted tissue sections.

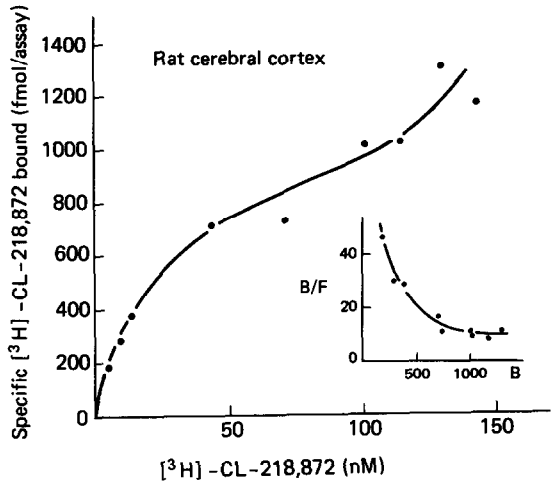

Figure 11
Saturation curve and Scatchard analysis (inset) of ^{3}H-CL 218 872 binding to rat cerebral cortical homogenates.

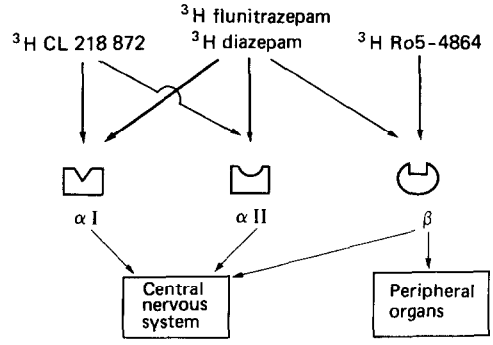

Figure 12
Benzodiazepine receptor subtypes.

For a RRA for benzodiazepines on rat brain membranes, all of these sites are represented in the preparation, but the radioligands and the unlabelled compound do not distinguish between them. Standard curves generated for RRAs with classical benzodiazepines indicate the presence of a single homogeneous class of binding sites.

Development of RRAs

There are four stages in the development of RAA: the receptor preparation; the sample preparation; the standard curve preparation; and the isolation and measurement of the bound radioactivity.

Receptor preparation. The receptor preparations used are homogenates containing, among others, the receptors to be studied. The sources of these homogenates are animal tissues. The choice of the tissue is dependent not only on the substance to be analysed but also on its availability and receptor concentration. The preparation of receptor suspensions always entails the homogenization and centrifugation of the chosen tissue but no single procedure is suitable for all RRAs. The procedure depends on the nature of the tissue and on the localization of the receptors in the target cells. Since most RRAs utilize neurotransmitter or drug receptor binding, attention will be concentrated on how receptor preparations from cell membranes are obtained, ignoring the cytosolic binding sites for steroid hormones. To illustrate the preparation of membrane receptor suspensions the procedures used for benzodiazepine binding studies are described [8].

Three methods are described for the preparation of receptor suspensions from brain tissue, but these methods can be applied to any peripheral organ or particular brain area bearing the benzodiazepine receptors. In the first method (Fig. 13) rat brain is rapidly removed after decapitation, homogenized in 20 volumes of 0.32 M sucrose and centrifuged at 1000 **g** for 10 min. The resulting crude *nuclear* pellet, or P1 fraction, contains cellular material such as nuclei or cell debris. The supernatant is centrifuged at 20,000 **g** for 20 min. The resulting crude mitochondrial pellet is washed twice with 50 mM tris–HCl buffer at pH 7.4. This pellet, or P2 fraction, can be divided by sucrose gradient centrifugation into myelin, mitochondria and synaptosome fragments [9]. A microsomal P3 fraction and a ribosomal S3 fraction may be obtained from the supernatant S2. Marangos [10] has shown that ^{3}H-diazepam binds preferentially to the synaptosomes, and ^{3}H RO 5-4864 to the P1 fraction and to the mitochondria (Fig. 14). The P1 fraction was therefore used for the studies on beta receptors and the P2 fraction for the studies on alpha receptors and for RRAs.

The second way of preparing membranes uses a "total or crude membrane preparation" which is obtained by homogenization of the brain tissue in the assay buffer, followed by a single centrifugation at 48,000 **g** for 10 min, which causes the sedimentation of virtually all membraneous elements. The resulting pellet is resuspended in buffer and stored at −30°C without washing. This crude unwashed membrane preparation can be used directly for binding assays, but contains endogenous brain GABA which may interfere with benzodiazepine binding.

In the third method of membrane preparation GABA is removed by extensive washing of the membranes with the assay buffer. Crude washed membranes are used by most investigators. They contain all the benzodiazepine subsites and it is possible to change the specificity of the assay, simply by using different radioligands. Marangos *et al.* [10] have studied the tissue distribution and the brain regional localization of ^{3}H RO 5-4864 and ^{3}H-diazepam binding with crude washed membranes.

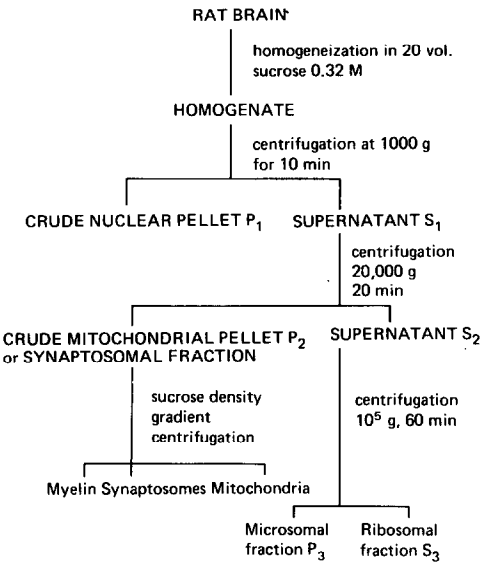

Figure 13
Subcellular fractionation (from [9]) preparation of synaptosomal membranes from rat cerebral cortex.

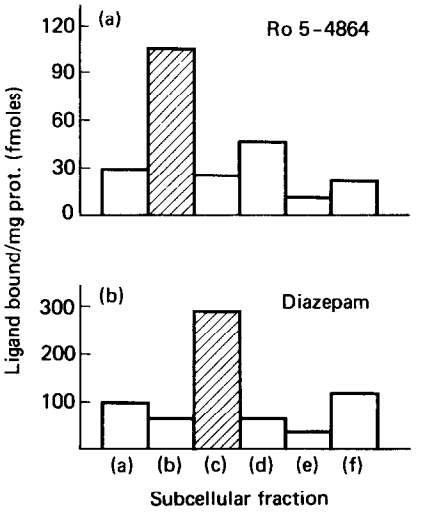

Figure 14
Subcellular localization of (^{3}H) Ro 5-4864 and (^{3}H) diazepam binding sites in brain.

The receptor preparations used for benzodiazepine RRA contain several irrelevant binding sites which may alter the specific binding. Asano [11] gives a method for the solubilization and purification of the benzodiazepine binding sites with nonionic detergents, but this procedure has not been used for RRA development.

The receptor preparation procedures for certain RRAs differ slightly from the method described above; e.g. for tissues containing fibrous components, such as blood vessels, it may be necessary to filter the homogenate. It is sometimes necessary to perform the analysis with freshly prepared membranes, such as for measuring serotonin binding, because freezing the receptor preparation can cause a decline in specific binding.

Sample preparation. To study the tissue or fluid concentration of a given substance by RRA, two possibilities must be considered. First, in many cases, assays can be performed on unextracted plasma or other biological fluids. Second, when this simple procedure is impossible or when the sample is a tissue, it is necessary to extract the ligand into a suitable *vehicle.*

Direct addition of biological fluid into the incubation medium can produce significant interference when relatively large volumes are assayed without extraction since many ligands bind avidly to proteins present in the sample. Hunt *et al.* [12] have tested the influence of increasing volume of plasma on ^{3}H-diazepam binding (Fig. 15). This interference varies with respect to the receptor preparation used and requires careful preliminary evaluation for each assay. In many cases, use of a small volume of sample (10–20 μl) and adding the same volume of the drug-free body fluid to the standard curve, is all that is required to eliminate this interference. For example, to measure plasma levels of benzodiazepines, the authors and Dorow *et al.* [13] have successfully used this procedure for the determination of diazepam levels after high-dose therapy. Likewise, to measure GABA in cerebrospinal fluid, Enna [14] has shown that an aliquot can be assayed directly.

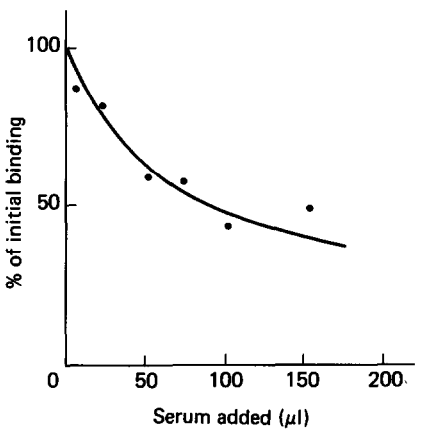

Figure 15
Effect of added serum on the binding of ^{3}H-diazepam [12].

In some cases, extraction of the ligand from the sample is necessary. It is therefore important to determine if the *vehicle* used for sample extraction has any effect on ligand binding. If a significant effect is noted, standard curves should be generated in an equivalent volume of the *vehicle.*

Extraction should be performed: when the interference of plasma protein is too strong; when the concentration of the substance in body fluid is so low that it would not be detectable by direct addition of 10–20 μl into the incubation medium; and when the sample is a tissue.

The extraction techniques used are: deproteinization; organic solvents extraction; and homogenization.

For example, benzodiazepines can be extracted from biological fluids by organic solvents such as butylacetate or diethylether at an appropriate pH. Then an aliquot of the organic layer is evaporated to dryness and the incubation is performed on the residue. Later it will be described how this technique was tested for midazolam plasma determination. Hunt *et al.* [15] reported a similar procedure for clobazam determination.

When the sample is a tissue, the RRA can sometimes be performed directly on the homogenate. For example, to measure brain GABA [1] the tissue is disrupted in water and the homogenate centrifuged at 48,000 **g** for 10 min. Virtually all the GABA is extracted in the aqueous phase so that an aliquot of the supernatant can be used in the assay. Finally, further purification may be necessary if more than one compound of appreciable affinity for the receptor is present in the sample. For example, to measure adrenal medullary norepinephrine content by RRA using a ^{3}H-norepinephrine label, one must first separate epinephrine from the tissue extract since it is very similar to norepinephrine in its ability to displace ^{3}H-norepinephrine [16].

In summary, as with all analytical procedures, sample preparation for RRA depends on the nature of the compound to be analysed and the source of the sample.

Standard curve preparation. The amount of unlabelled ligand in an unknown sample is calculated by comparing the percentage decrease in specifically bound radioactivity, after the addition of the sample, to a standard curve of radioligand displacement by known amounts of unlabelled compound. Because the apparent affinity of a ligand for a receptor is dependent, to some extent, on the ligand and receptor concentrations, and because these two factors may vary slightly, standard curves should be generated for each experiment. Standard curves are presented as plots of the percentage of specifically bound radioactivity against increasing concentration of the unlabelled ligand (Fig. 16).

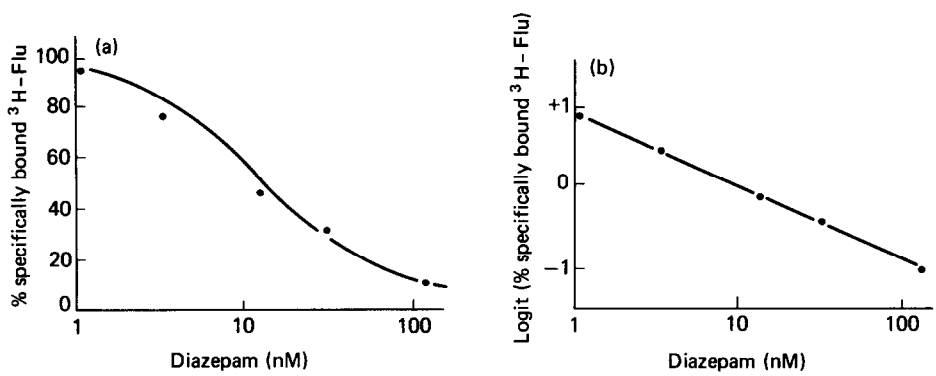

Figure 16
Standard curve for RRA of benzodiazepines.

Using semi-logarithmic plots these curves are sigmoidal and can be converted to a linear form by using a logit–log transformation [17].

Generally four or five concentrations of standard, covering a range ten-fold higher to ten-fold lower than the affinity of the ligand for the receptor, are sufficient. The central position of the standard curve, corresponding to 20–80% of bound radioactivity, is the most accurate area as variability is greatest at the extremes. Accordingly, the amount of the unknown sample added to the incubation medium should be such that 20–80% of the specifically bound ligand is displaced.

Isolation and measurement of bound from free radioactivity. The method used must fulfil certain criteria. Firstly, no modification of the equilibrium between receptor and ligand must occur. Second, the separation should be both complete and rapid, and the

method should be suitable for automization. Two methods are currently used: vacuum filtration; and centrifugation.

During filtration, the unbound radioactivity passes through the filter, while the membrane containing the bound labelled ligand is trapped on the filter. Glass fibre filters are especially suitable for this purpose. The filter is washed with incubation buffer after filtration to remove unbound radioligand and reduce nonspecific binding.

During centrifugation of the receptor preparation, a pellet containing the radioactivity is formed. The supernatant which contains the free labelled ligand is discarded. This centrifugation technique is used for GABA–RRA. The filtration technique is used for many drug RRAs (e.g. benzodiazepine RRAs).

When the receptor is soluble, the method of separation can be derived from RIA procedures, i.e. adsorption of the unbound ligand or fixation of the receptor on an inert, solid matrix. The isolated bound radioactivity is always measured by liquid scintillation counting.

Analytical Characteristics of RRAs

Data analysis

Sensitivity. The lower limit of sensitivity for RRAs is usually defined as the amount of ligand that displaces 20% of the specifically bound radioligand. The sensitivity of a RRA depends upon 3 factors.

(a) The relative receptor affinity of the ligand being assayed; the higher this affinity, the smaller the amount that can be measured. The affinity of the radioligand has to be similar to that of the substance being measured [18].

(b) The incubation volume: the smaller the volume used, the greater the sensitivity. The reason for this increase in sensitivity with decreasing incubation volume is that, although the concentrations necessary to displace 20% of the specifically bound radioligand remain the same, the mass of ligand necessary to cause this displacement will become smaller as the volume decreases (Fig. 17).

(c) The specific activity of the radioligand: the higher the specific activity, the greater the sensitivity (Tables 1 and 2). The specific activity of the radioligand is the main limiting factor of the decrease in incubation volume. A two-fold increase in specific activity permits a reduction in assay volume by 50% (Fig. 17).

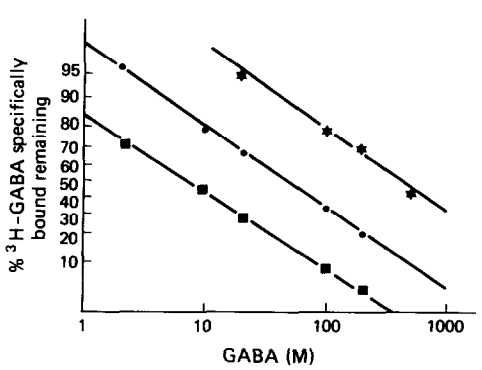

Figure 17
Influence of the incubation volume on the sensitivity of GABA radioreceptor assay (from [1]). ■–■, 0.2 ml incubation volume; ●–●, 2 ml incubation volume; ★–★, 20 ml incubation volume.

Table 1
Radioreceptor assays for hormones

Hormone assayed	Receptor preparation used	Radioligand used
ACTH	Membranes from rat adrenals	^{125}I-ACTH
hCG	Membranes from bovine corpore lutea	^{125}I-hCG
LH	Membranes from bovine corpore lutea	^{125}I-hCG
LH	Membranes from mature rat testis	^{125}I-LH
FSH	Membranes from bovine testis	^{125}I-FSH
Insulin	Solubilized receptors from placental membrane	^{125}I-insulin
	Rat liver plasma membranes	^{125}I-insulin
Prolactin	Membranes from mouse mammary tissue	^{125}I-prolactin

Table 2
Radioreceptor assays for neurotransmitters

Neurotransmitters assayed	Membrane preparation used	Radioligand used
Serotonin	From rat brain	^{3}H-serotonin
Dopamine	From rat corpus striatum	^{3}H-dopamine
Norepinephrine	From calf brain	^{3}H-norepinephrine
Glycine	From rat brain	^{3}H-strychnine
Aminobutyric	From rat brain	^{3}H-GABA
Angiotensin II	From mammalian brain	^{125}I-angiotensin
Opioid peptides	From mammalian brain	^{3}H-naloxone

Precision. In addition to being sensitive, RRAs are also precise. Precision is higher for concentrations of substance displacing 20–80% of bound radioligand; it is a function of the technique used to separate bound from free radioactivity. Within a given experiment, results obtained from multiple analysis of the same sample should vary by less than 5%.

Accuracy. Accuracy is tested by comparing the RRA results with those of a physico–chemical method for a given ligand. For example, correlation between gas–liquid chromatography and RRA determination of samples spiked with midazolam shows a correlation coefficient of 0.998 (unpublished data). For greater accuracy all the determinations of unknowns were done in duplicate or triplicate.

Specificity. Because measurements of the displacement of specifically bound radioligand is the basis of the RRA, any substance that can interfere in a consistent manner with the binding is included in the calculated value. The interference may be due to the active metabolites of the unchanged ligand or to endogenous or exogenous compounds unrelated to the ligand being assayed. If these interfering substances have the same intrinsic activity as the ligand being measured, the RRA will estimate a biological or pharmacological activity which includes all of these compounds. The result will be expressed as weight equivalents of the ligand measured. If the interfering substances act at the receptor in an antagonistic fashion, the result will be uninterpretable. An example

of this problem is given by the binding of ^{3}H-serotonin in the presence of neuroleptics, LSD or ergot alkaloids. These interferences can be ruled out if the sample preparation includes a selective extraction step. The specificity of the RRA appears to be the chief limitation of the procedure because one must demonstrate that the only substance in the sample preparation that will interfere with radioligand binding is the compound being analysed. In certain drug binding assays, this specificity can become an advantage because the RRA acts as a "bioassay" which estimates all active species, and metabolites as well as parent compound, in proportion to their affinity at the receptor. The specificity can be evaluated by comparing the values obtained with RRA to those obtained using a physico–chemical technique, which determines selectively the parent drug. In this respect two possibilities have to be considered.

First, if the metabolite is inactive, or if a poorly active metabolite is present at low concentration in the sample, the same result will be found using the two analytical methods. For example, when samples containing midazolam and its less active, hydroxylated metabolite in low concentration are analysed using RRA and gas–liquid chromatography, virtually the same values are obtained (correlation coefficient = 0.991, [19]).

Second, if the metabolite is active and present in relatively large amounts in the sample, the results found by RRA will be dramatically different from those obtained by the physico–chemical techniques which determine selectively the parent drug. For example, when samples containing diazepam and its desmethyl and/or hydroxylated active metabolites are analysed using the two analytical methods, the correlation coefficient is 0.730 [20]. In such cases, results of the RRA must be expressed as "weight equivalents" of the compound which is used to generate the standard curve (e.g. diazepam equivalents). Binding interferences are not a molecular *entity* but a biological or pharmacological activity. Moreover the levels of RRA active material in a biological sample seem to correlate better with clinical observations than do the levels of the parent drug alone.

Advantages and disadvantages

The chief advantages of RRA are sensitivity, simplicity and cost. As mentioned above, RRAs which use tritiated labels are able to detect pmol quantities of a compound and, in situations where iodinated radioligands can be used, they are able to readily detect fmol quantities. This range of sensitivity is superior to that of most presently available analytical methods.

RRAs are simple and inexpensive; thus, once a binding assay has been perfected, the technical expertise to analyse samples by RRA can be mastered within a few days. The assays are rapid since the time necessary to reach binding equilibrium is generally short (less than 1 h). Finally, since common laboratory equipment and supplies are used and since the assay can be performed without extensive training, RRAs are inexpensive relative to more sophisticated techniques.

The primary disadvantage of RRAs lies paradoxically with the specificity in that any substance having an appreciable affinity for the receptor will interfere with the binding. These interferences are numerous but easily anticipated and sometimes eliminated or allowed for in the calculated value, in order to estimate a biological or pharmacological activity. Finally, the problems associated with the use of radioisotopes must be considered a disadvantage. These problems could be overcome if a fluorescent-labelled ligand was used in place of a radioligand.

Applications of RRA Techniques

Some of the analytical applications of RRAs in the determination of hormone, neurotransmitter and drug levels in biological tissues or fluids will be demonstrated.

RRAs for hormones

RRAs for hormones (Table 1) are not often used to determine hormone levels, but to study the pharmacological or pathological modifications of the binding parameters. Nevertheless, some RRAs have been described including ACTH [21], LH [22–24], hCG, FSH [25], GH [26] and prolactin [27]. The radioligands used are iodinated and the membrane preparations are obtained from various tissues such as testis or ovary. The RRA for insulin is useful for the determination of the activity of synthetic insulins and their analogues; it also gives information on the possible role played by the insulin receptors in some pathological states such as obesity, where ^{125}I insulin binding is decreased [28]. Finally, RRA for insulin can be used to detect the antireceptor antibodies present in the plasma of some patients showing carbohydrate intolerance. Despite the fact that the preparation of receptors for steroid hormones has not been discussed, consideration will be given to the determination of oestrogen, progesterone and *androgen* receptors in tumours of women present with metastatic breast carcinoma. The binding of these steroids to the neoplastic tissue could predict the patients' response to cancer hormonotherapy. Recently, Grill [29] has reported a double labelling assay for the simultaneous determination of oestrogen and progesterone receptors using a ^{125}I-labelled oestrogen and a tritiated progesterone.

RRAs for neurotransmitters

The main neurotransmitters measured by RRAs are (Table 2): serotonin [30], dopamine [31], norepinephrine [32, 33], glycine [34] and GABA [35]. RRAs for angiotensin II [16] and for opioid peptides have also been performed. The levels of these transmitters can be determined easily in tissues such as brain and in biological fluids such as blood or cerebrospinal fluid. This is of interest because the levels of these transmitters can be modified during certain diseases or by the administration of certain drugs. For example, Enna *et al.* [14] related the central nervous system content of GABA determined by RRA, with various neurological and psychiatric disorders. Blood GABA levels have been shown to be related to the biochemical effectiveness of therapeutically active GABA-ergic agonists [18].

RRAs for neurotransmitters are useful tools to study the effect of drugs on the binding of the neurotransmitters. For example, benzodiazepines increase the affinity of the GABA on the benzodiazepine–GABA–ionophore Cl receptor complex [36]. Also, it has been demonstrated that this potentialisation was mediated by the specific benzodiazepine receptor subunit and was reciprocal. RRAs for neurotransmitters therefore allow the study of drug and disease effects on the biological levels and on the binding of a given neurotransmitter.

The receptor preparations used are obtained from the rat or calf whole brain or from certain brain areas, such the corpus striatum for dopamine binding. The radioligands are all tritiated except for ^{125}I-angiotensin II.

RRAs for drugs

Drug levels can be estimated in all biological tissues or fluids such as saliva, urine or cerebrospinal fluid although plasma levels are more often measured (Table 3). The list of

Table 3
Radioreceptor assays for drugs

Drug	Membrane preparation	Radioligand	Refs
Tricyclic antidepressants	From rat brain (cholinergic receptors)	^{3}H-quinuclidinyl benzylate	[39]
	From rat platelets (serotonin receptors)	^{3}H-serotonin	[40]
	From human platelets (imipramine receptors)	^{3}H-imipramine	[41]
Beta-blockers	From mammalian lung	^{H}-dihydroalprenolol	[47]
	From rat cerebral cortex	^{3}H-dihydroalprenolol	[37]
	From turkey erythrocytes	^{125}I-hydroxybenzylpindolol	[50]
	Solubilized receptors from rat lung	^{3}H-dihydroalprenolol	[49]
Neuroleptics	From rat corpus striatum	^{3}H-spiroperidol ^{3}H-haloperidol	[38]
Benzodiazepines	From rat or calf brain	^{3}H-diazepam	[15, 42 and 43]
	From rat brain (solubilized receptors)	^{3}H-flunitrazepam	[53]

the drugs measured by RRA include beta-blockers [33, 37], neuroleptics [38], tricyclic antidepressants [39–41] and benzodiazepines [15, 42, 43]. Recently, Aaltonen *et al.* [44] have published a comparison between RRA and RIA for atropine. These drug RRAs represent a breakthrough in the clinical monitoring of certain treatments as they determine total levels of receptor active substances including active metabolites.

Some of the receptor preparations proposed for tricyclic antidepressant assays do not make use of specific tricyclic antidepressant binding sites and so do not seem to be well suited for the assay of these compounds. The RRAs for these drugs exploit some of these heterogeneous interactions with muscarinic cholinergic receptors [34] in mammalian brain or with serotonin receptors on rat platelets [40]. A possibly more suitable RRA for tricyclic antidepressants exploits the presence of high affinity ^{3}H-imipramine binding sites on human platelets [41] since some authors [45] have reported a decrease of this ^{3}H-imipramine binding in depressed patients. Specific tricyclic antidepressant receptors have been demonstrated in rat brain [46] but no correlation has been found between binding data and clinical effects.

Many RRAs for beta-blocker drugs have been reported using different receptor preparations and radioligands [37, 47–50]. These assays do not require prior extraction of sample. Plasma levels of circulating catecholamines contribute to overall receptor activity but are always less than 10% even in phaeochromocytoma. The use of solubilized receptors [48, 49] seems to reduce plasma interference. Clinically, plasma beta-blocking activity determined by RRA correlates well with measurements of physiological cardiac *beta-blocker* in normal human volunteers. But the general clinical applicability of the determination of beta-blocker plasma levels is debatable as direct correlation between clinical effects and plasma levels is not possible for all actions of these drugs.

Neuroleptics are thought to exert their therapeutic antischizophrenic actions by blocking brain dopamine receptors; therefore neuroleptic RRAs will measure "total dopamine blocking activity" by both the unchanged drug and its active metabolites. The

membranes are prepared from rat or calf corpus striatum and the radioligands used are ^{3}H-haloperidol or ^{3}H-spiroperidol [38]. Therapeutic response to neuroleptics in schizophrenic patients may be successfully predicted by the measurement of all neuroleptic active compounds in the plasma [51]. This RRA can also be used for neuroleptic drug monitoring [52].

RRAs for benzodiazepine drugs have been reported [15, 42, 43, 53] using the displacement of ^{3}H-diazepam or ^{3}H-flunitrazepam bound on rat cerebral cortex. These techniques estimate the "total benzodiazepine activity" present in the sample including the active metabolites. Despite the relative simplicity and ease of use of the RRA, few clinical studies employing this method have been described [13, 54, 55]. For example, in order to establish a well adapted drug administration, the authors [19, 20] have monitored with RRA the plasma levels of "benzodiazepine activity" during midazolam conducted anaesthesia and during high-dose diazepam therapy for tetanus.

Clinical monitoring of treatments using RRA is not yet common practice, although biological levels of RRA active material seem to correlate better with therapeutic effects than does the concentration of the parent compound alone. In addition to simple concentration effect correlations, pharmacokinetic studies can be performed with RRAs and the apparent elimination half-life of receptor active material is in good agreement with the duration of the clinical effect. Two possibilities can be considered (Fig. 18).

First, when the metabolites are nonexistent or inactive, or when they are active but present in very low amounts, kinetics of the "RRA active pool" will be similar to those of the parent drug. For example, during midazolam managed anaesthesia, the half-life of midazolam from the results of gas–liquid chromatography is the same as that for RRA active material, because the main metabolite, hydroxymidazolam, is less active than the parent drug and its levels never exceed 10% of the midazolam concentration.

Second, when the metabolites are active and present in sufficiently large amounts, the kinetics of the parent drug will be different from that of the "RRA active pool". For instance, during diazepam-managed tetanus therapy, gas–liquid chromatographic estimation of diazepam half-life is much lower than the half-life calculated from RRA results. This discrepancy is due to the long acting desmethyl metabolite whose levels exceed those of diazepam and whose IC50 value approaches that of diazepam. The determination of the pharmacokinetic parameters of RAA active material could allow

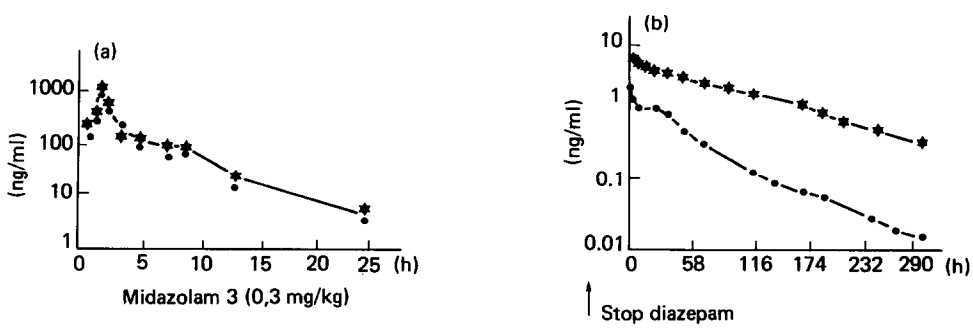

Figure 18
Plasma levels and elimination half-lives of RRA active material (★–★) and of unchanged drug (●–●) determined selectively by GLC (from [19, 20]). (a) ★–★, $t^{1/2}$ RRA = 2.95 h; ●–●, $t^{1/2}$ GLC = 3.24 h. (b) ★–★, $t^{1/2}$ RRA = 52.6 h; ●–●, $t^{1/2}$ GLC = 21.2 h.

the simultaneous *modelization* of the kinetics of receptor occupancy and of the intensity of the effect, performed using *in vivo* binding techniques [56].

Conclusion

In conclusion, the present availability of radioactively labelled receptor ligands, coupled with well established conditions for binding assays, now allows the development of RRAs for hormones, neurotransmitters and drugs. These techniques are simple and specific and can be performed at relatively low cost. They possess considerable advantage over chemical analysis since they provide an assay of the "bioactivity" of the sample analysed. In addition to being analytical tools, RRAs have been found to be powerful instruments for basic studies on the receptor itself, on its endogenous ligand(s) and on the discovery of active compounds. The authors can predict an increase in the use of RRAs in a variety of clinical pharmacology studies and pharmacological problems. Other advances may include the introduction of fluorescent-labelled ligands to replace radioligands and thus to circumvent the problems associated with handling radioactive substances (Fig. 19).

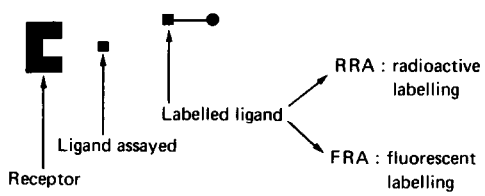

Figure 19
Evolution of radioreceptor assays: fluorescent receptor assays.

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[*Received for review 10 June 1986*]