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A PARTIALLY AUTOMATED RADIOLIGAND BINDING ASSAY SYSTEM FOR USE IN CLINICAL AND PHARMACEUTICAL RESEARCH

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ABSTRACT

Using a Tecan robotic sample processor and IBM compatible PCs we have developed a flexible, partially automated radioligand binding assay system. It handles pipetting parameters of up to 16 saturation or competition experiments at a time with up to 24 radioligand- or competitor-concentrations in a range over 4 orders of magnitude per experiment. The system provides enough flexibility so that all pipetting parameters including different tube-, rack-sizes, sample volumina and pipetting sequences may be easily adapted to the large variety of experimental requirements in binding assays. It rationalizes and increases assay throughput (up to 70% spare of working time), improves reliance and reproducibility of results. Radioactive exposure is minimized to the time preparing the radioligand working solution and transferring the sample tubes to and from the sample processor. The system has proven effective in various investigations on binding interactions, as well as in clinical studies on receptor expression under physiologic, pathological and therapeutic conditions.

INTRODUCTION

Binding assays with radiolabeled drugs are used to measure and characterize the interactions of ligands with a variety of receptors for hormones, neurotransmitters and drugs. Over the past two decades radio-

ligand binding techniques have become increasingly important in clinical research and in the pharmaceutical industry as an essential part in drug discovery and evaluation. In clinical investigations receptor expression is studied in tissue samples from healthy, sick, untreated or treated subjects with the aim of correlating changes in their characteristics to the pathophysiology and pharmacotherapy of diseases. In the process of drug discovery the technique allows in vitro targeting of a distinct receptor thought to be involved in the pathophysiology of a given disease which thus may yield important information regarding selectivity, specifity and/or affinity of novel compounds intended for use as therapeutic drugs (1).

A wide range of assays have been developed. Applications in clinical research often involve the study of variations in the expression of various receptors in dynamic situations where receptor properties may alter along a timescale and profile measurements are necessary, for example in drug-response or circadian studies. In order to measure these alterations effectively, it is necessary to take samples for assay at set intervals over a certain time period, e.g. before and after drug application. In most cases this means that many assays have to be performed within a short time for each patient, thus necessitating rapid sampling and assay techniques which generally involves a variety of experimenters working on the same system. In the drug development process many thousands of compounds have to be screened before one can be introduced as new drug.

Valid results may only be obtained under well defined, standardized experimental conditions which in turn may differ largely depending on various experimental protocols. The scatter of data points is a most critical experimental parameter and validity therefore highly depends on the reproducibility of the results which in turn relies upon the accurancy and precision of the assay steps involved. Ligand binding assays are tedious procedures involving a large number of pipetting steps combined with radioactive exposure of the lab personnel. Manual pipetting techniques have considerable potential for dispensing imprecision and error associated with liquid carry over and contamination. In addition, when several workers are involved in a study, the variation in pipetting skills leads to additional error.

There has been considerable interest in the automation of such procedures but because of the variety of assays, it has not previously been

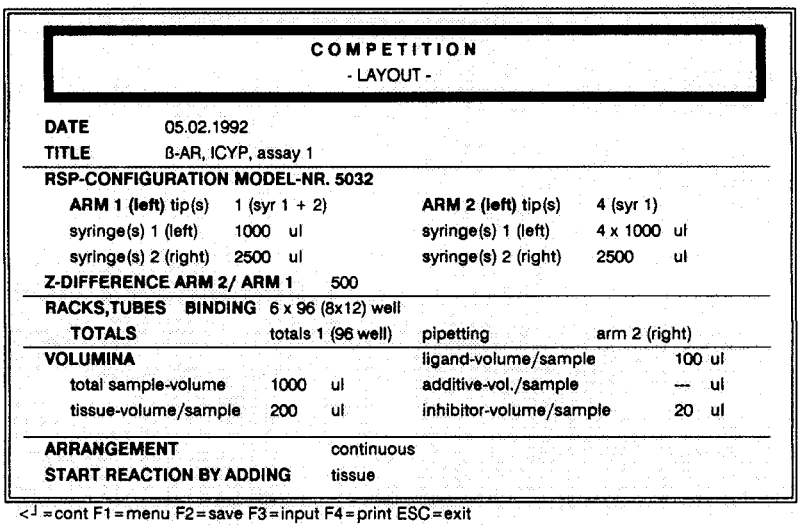
possible to develop a flexible and satisfying system. Using a robotic sample processor and a personal computer (PC) we have developed a partially automated radioligand binding assay system which rationalizes and increases assay throughput, improves reliance and reproducibility of results, reduces radioactive exposure and frees lab workers from time-consuming pipetting jobs.

ASSAY SYSTEM

We have designed a software (EBSS = equilibrium binding studies software) for IBM compatible PCs controling a Tecan Robotic Sample Processor Model 5032 (dual arm system). The system handles pipetting parameters of two types of experiments, saturation or competition experiments (up to 16 single assays at a time). Up to 24 concentrations of a radioligand or competing compound in a range over 4 orders of magnitude may be defined per experiment. An additive (e.g. antioxidans) may be supplemented to all samples uniformly. Pipetting may be performed alternatively in 1 of 3 different tube or rack types, respectively, with total sample volumes between 200 and 2000 ul. Depending on the desired pipetting volumina, the syringes of the sample processor are adapted to achieve maximal pipetting precision ($\pm$ 2 % of nominal value). The pipetting sequence can be altered, automatic pipettes may be switched on or off so that certain pipetting steps may be performed by hand or at a later point of time (e.g. tissue pipette, if samples are prepared for an experiment which is to be started later by tissue addition).

All parameter inputs are menu controlled. A hardcopy of the screens with three main input menus defining assay parameters (for the example described later) are shown in FIG. 1 - 3. Depending on substance-specific constants (molecular weights, specific activity) and experiment-specific variables (volumes, concentrations) required reagent-dilutions (radioligand, competing compounds, additive) and titertube arrangements are displayed. The pipetting steps in performance are displayed and may be printed on a hardcopy device together with exact time course informations.

The system provides enough flexibility so that all pipetting parameters can easily be adapted to the large variety of experimental requirements

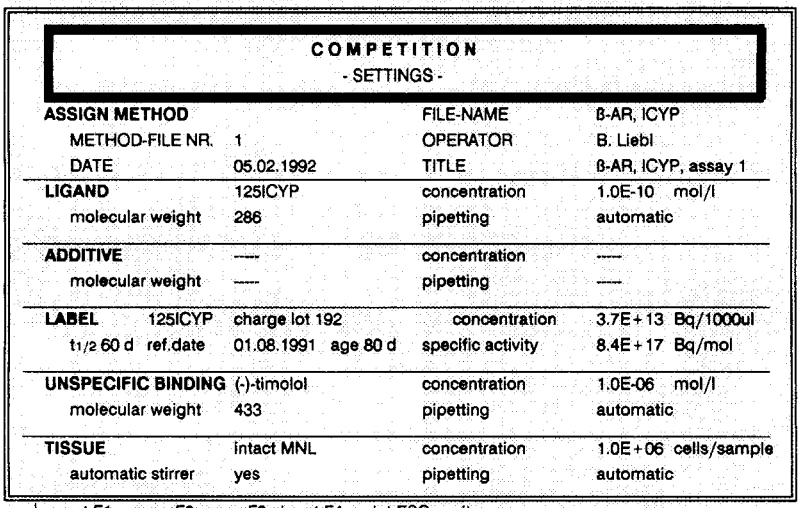


COMPETITION
- LAYOUT -

DATE	05.02.1992				
TITLE	ß-AR, ICYP, assay 1				
RSP-CONFIGURATION MODEL-NR. 5032					
ARM 1 (left) tip(s)	1 (syr 1 + 2)		**ARM 2 (left)** tip(s)	4 (syr 1)	
syringe(s) 1 (left)	1000	ul	syringe(s) 1 (left)	4 x 1000	ul
syringe(s) 2 (right)	2500	ul	syringe(s) 2 (right)	2500	ul
Z-DIFFERENCE ARM 2/ ARM 1	500				
RACKS,TUBES BINDING	6 x 96 (8x12) well				
TOTALS	totals 1 (96 well)		pipetting	arm 2 (right)	
VOLUMINA			ligand-volume/sample	100	ul
total sample-volume	1000	ul	additive-vol./sample	---	ul
tissue-volume/sample	200	ul	inhibitor-volume/sample	20	ul
ARRANGEMENT	continuous				
START REACTION BY ADDING	tissue				

<↵ = cont F1 = menu F2 = save F3 = input F4 = print ESC = exit

FIG. 1: Screen-hardcopy of input menu defining processor configuration, material and liquid handling parameters.



COMPETITION
- SETTINGS -

ASSIGN METHOD			FILE-NAME	ß-AR, ICYP	
METHOD-FILE NR.	1		OPERATOR	B. Liebl	
DATE	05.02.1992		TITLE	ß-AR, ICYP, assay 1	
LIGAND	125ICYP		concentration	1.0E-10	mol/l
molecular weight	286		pipetting	automatic	
ADDITIVE	-----		concentration	-----	
molecular weight	-----		pipetting	-----	
LABEL 125ICYP	charge lot 192		concentration	3.7E+13	Bq/1000ul
$t_{1/2}$ 60 d ref.date	01.08.1991	age 80 d	specific activity	8.4E+17	Bq/mol
UNSPECIFIC BINDING	(-)-timolol		concentration	1.0E-06	mol/l
molecular weight	433		pipetting	automatic	
TISSUE	intact MNL		concentration	1.0E+06	cells/sample
automatic stirrer	yes		pipetting	automatic	

<↵ = cont F1 = menu F2 = save F3 = input F4 = print ESC = exit

FIG. 2: Screen-hardcopy of input menu defining substance- and tissue-specific parameters.

Method-File-Nr.1: ß-AR, ICYP/B.Liebl

COMPETITION
- EXPERIMENT NR. 3 -

DATE	05.02.1992		
TITLE	ß-AR, ICYP, assay 1		
COMPETITOR	isoprenaline	MOLECULAR WEIGHT	248
CONCENTRATION RANGE	LOWEST POSSIBLE CONCENTRATION		1.0E-09 mol/l
	HIGHEST POSSIBLE CONCENTRATION		1.0E-01 mol/l
REPLICATIONS	triplicates		
INTERMEDIATE DILUTIONS	automatic		

CONCENTRATIONS 21

1.	5.0E-09 mol/l	7.	5.0E-07 mol/l	13.	5.0E-05 mol/l	19.	5.0E-03 mol/l
2.	1.0E-08 mol/l	8.	1.0E-06 mol/l	14.	1.0E-04 mol/l	20.	1.0E-02 mol/l
3.	2.5E-08 mol/l	9.	2.5E-06 mol/l	15.	2.5E-05 mol/l	21.	1.0E-01 mol/l
4.	5.0E-08 mol/l	10.	5.0E-06 mol/l	16.	5.0E-04 mol/l		
5.	1.0E-07 mol/l	11.	1.0E-05 mol/l	17.	1.0E-03 mol/l		
6.	2.5E-07 mol/l	12.	2.5E-05 mol/l	18.	2.5E-03 mol/l		

<J =cont SH<J =back F1=menu F2=save F3=input F4=print F9=get,F10=save exp. ESC=exit

FIG. 3: Screen-hardcopy of input menu describing one competition experiment. Up to 16 different experiments are possible per run.

in binding assays. All parameters can be saved in so-called method files and loaded bypassing the input functions, they may be copied, deleted or revised and saved anew. Other options allow a parameter-listing or the generation of an ASCII-format file which may be later combined with radioactivity measurements for data analysis.

If separation of bound from unbound radioactivity is performed by centrifugation, the system may be additionally used for removal of the supernatant.

APPLICATIONS

The advantages of the system is demonstrated in the following by a set of competition experiments which were performed in our lab for characterization of binding sites for ^{125}iodocyanopindolol (^{125}ICYP) in human peripheral mononuclear leucocytes (MNL) (2-5).

Using the sample processor three competition experiments (in triplicates) with 8, 24 and 95 pmol/l ^{125}ICYP, respectively, and 21 concentrations of isoprenaline in the concentration range of 10^{-10} - 10^{-1} mol/l were prepared. Total and unspecific binding (in duplicates) were determined in parallel samples with and without 10^{-6} mol/l of (-)-timolol. Alltogether 70 specimens per experiment (210 alltogether) were prepared with varying volumes of buffer, various isoprenaline dilutions, a constant volume of radioligand- (100 ul) and timolol-solution (20 ul) as well as 200 ul of cell-suspension to achieve a total reaction volume of 1000 ul. FIG. 1 - 3 show hardcopies of the input screens for assay-parameter definition for one of these experiments.

Before introduction of the assay system in our lab one person was employed for at least 4 hours for sample preparation for such a set of experiments, while another lab worker was occupied with cell preparation. Using the processor sample preparation could be achieved by a single experimenter parallel to preparation of MNL by density gradient centrifugation. After parameter definition, working time for sample preparation was reduced to arranging the titer tubes (1 ml Biorad^{R} in microtiterplate-sized racks) and preparing stock solutions for radioligand and competing compounds (isoprenaline, timolol) according to instructions from the program. Automatic pipetting of one experiment took about 10 minutes.

Experiments were started by adding 200 ul pMNL suspension in a concentration of 5 x 10^{6} cells/ml to each of the 210 specimens. After incubation in a waterbath (2 h, 37°C) the sample-racks were centrifuged at 5000 g for 10 minutes and transferred back to the lab robot. Bound radioactivity was separated from the unbound ligand by removing the supernatant by the machine. Compared to the conventional rapid filtration-method over glass fiber filters this procedure saved material (no filters necessary), time and radioactive exposure of the experimenters.

The radioactive counts were evaluated using a PC. Due to the pipetting precision of the sample robot scatter of data points was in the range of $\pm$ 4 % of the mean value. To determine the binding parameters (binding capacity $Bmax_1$ and $Bmax_2$ for high and low affinity binding sites, the respective equilibrium dissociation constants Ki_1 / Ki_2 for isoprenaline and Kd_1 / Kd_2 for ^{125}ICYP)(2) data were mathematically fitted to a binding

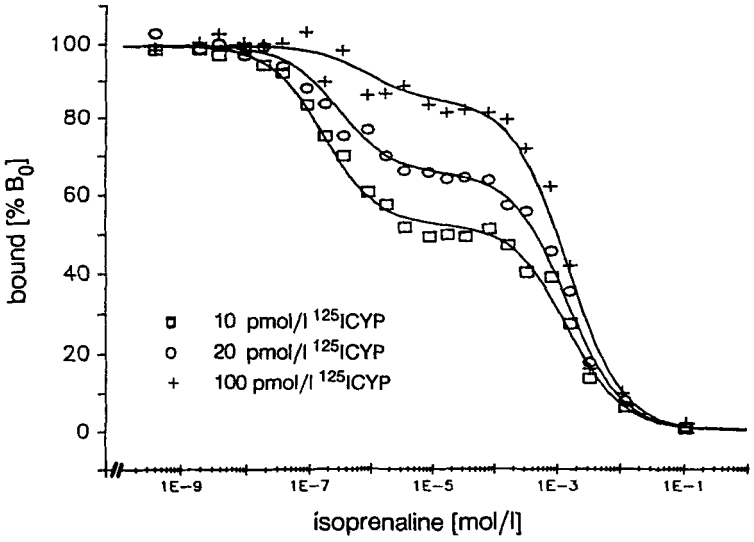


FIG. 4: Competition curves for isoprenaline with 3 ^{125}ICYP-concentrations. B_0: specific binding, $Bmax_1$: 510 sites/cell, $Bmax_2$: 9180 sites/cell; Ki_1: 2.6×10^{-7}, Ki_2: 4.2×10^{-3} (isoprenaline), Kd_1: 1×10^{-11} mol/l, Kd_2: 2.9×10^{-9} (^{125}ICYP).

equation for two classes of binding sites using a computer-based nonlinear iteration procedure (FIG. 4).

A summarized comparison of time consumption and exposure to radioactivity with and without use of sample processor and PC is given in TABLE 1. Alltogether 70 % of working time was saved (84 min compared to 302 min). Radioactive exposure was minimized to the time of transferring the sample racks from the robot to the waterbath, to the centrifuge and back to the robot. After preparing the radioligand working solution no further direct contact with radioactivity was necessary.

The assay system has been applied in several clinical studies (e.g. circadian and drug-response studies) on receptor expression under physiologic, pathological and therapeutic conditions. Results from these investigations have been published elsewere (6-11). They implied multiple satu-

TABLE 1.
Comparison of time consumption and exposure to radioactivity (- = none, + = low, ++++ = high) with and without use of sample processor and PCs for 3 competion experiments (21 concentrations in triplicates each).

TASK	WITHOUT SAMPLE-PROCESSOR AND PC total time (min)	working time (min)	radioact. exposure	WITH SAMPLE-PROCESSOR AND PC total time (min)	working time (min)	radioact. exposure
cell preparation by density gradient centrifugation	120	30	-	120	30	-
inscription of tubes	30	30	-	-	-	-
arrangement of tubes in racks	10	10	-	5	5	-
calculation of dilutions	20	20	-	-	-	-
prepare stock dilutions	15	10	++	15	15	++
prepare intermediate dilutions	15	15	-	-	-	-
pipetting 60 tubes (radio-ligand,buffer,displacer,cells)	90	90	++++	30	5	-
transfer to waterbath, incubation	120	5	+	120	5	+
transfer to, load centrifuge centrifugation, unload centrifuge	12	2	+	10	1	+
separation bound from free radioactivity	20	20	+++	10	3	-
load radioactivity-counter and counting	125	5	-	125	5	-
raw data analysis	60	60	-	5	5	-
nonlinear iterativ curve fit	not possible		-	10	10	-
SUM	637	**302**		450	**84**	

ration experiments, for example at 4 hour intervals and 30, 60, 120, 180, 240 and 360 minutes after drug application. Up to 14 experiments with 12 radioligand concentrations per experiment had to be performed within 36 hours for each subject (8, 10). Without the lab robot 2 persons were busy for 1 day preparing the assays for such a study (inscription of titer tubes, arranging them in racks, making reagent dilutions, pipetting jobs). By employing the lab robot preparation time was reduced to about 4 h and could be achieved by a single technician parallel to other tasks in the course of the study.

The system has also proven effective in the research department of a major German pharmaceutical company. It is applied there for the screening of novel compounds for possible binding interaction using the option of performing multiple competition experiments (up to 16 per run) with a small number of concentrations (1 to 4) per substance to be investigated. The responsible scientists report that the same number of experiments which previously afforded 3 technicians is performed now by a single person in half of the time.

CONCLUSIONS

Radioligand binding assays are a well established technique in pharmacological research. They are technically simple to perform, but time-consuming and prone to error if performed manually. Valid information is only obtained if conditions are carefully controlled. Using a robotic sample processor and IBM compatible PCs a flexible, partially automated assay system has been developed which rationalizes and increases assay throughput, improves reliance and reproducibility of results and minimizes radioactive exposure of experimenters.

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