PRINSIP FISIOLOGIS BERBAGAI MACAM ESAI HORMON

Kuliah 4

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Hormones

1. Hormones are chemical messengers that enter the blood, which carries them from endocrine glands to the target cells
2. Hormone action in the target cells/organ
3. Hormone metabolism
4. Hormone removal or clearance

Hormone Concentrations in Blood

- Most hormones are present in blood in extremely minute quantities, some in concentrations of pg / ml (1 pg = 1 billionth of mg)
- Almost impossible to measure by usual chemical means
- Extremely sensitive methods: radioimmunoassay

Hormones Removal From Plasma

- Hormones are cleared from plasma in several ways:
  1. Metabolic destruction by tissues
  2. Binding with tissues
  3. Excretion by liver into bile
  4. Excretion by kidneys into urine
- A decreased Metabolic Clearance Rate of hormone causes excessively high concentration of the hormone in body fluids

Metabolic Clearance Rate of Hormones

- **Two factors can affect hormone concentration in blood:**
  - **rate of secretion**
  - **rate of removal which is called metabolic clearance rate**

    This is expressed in terms of the number of ml of plasma cleared of the hormone per minute

Hormones Measurement

1. Measurement of hormone secretion rate
2. Measurement of circulating hormones concentration
3. Measurement of hormone action
4. Measurement of hormone metabolite product
5. Measurement of hormone excretion

Measurement of Hormone Secretion Rate

- **A simple method for estimating hormone secretion is:**
  - **Measuring the concentration of natural hormone in plasma by means of a radioimmunoassay procedure (C)**
  - **Measuring metabolic clearance rate (MCR)**
  - **By multiplying C x MCR, one derives a value that is equal to steady-state of hormone production/ hormone secretion**

Measurement of Hormone Secretion Rate.....

- However, hormone production often increases or decreases rapidly
- In such case, one can measure the changing rate of secretions only by:
  - collecting samples of arterial blood entering the gland (AB) and samples of venous blood leaving the gland (VB)
  - measuring rate blood flow through the gland (BF)
  - by multiplying BF x (VB-AB), one can derive the instantaneous secretion rate

Measurement of Hormones Concentration

- Physiologically variable that fluctuates each day with a cyclical periodically
- Measurements of particular variable are usually obtained at a single time of day
- In certain types of hormonal diseases, plasma concentration of the hormone may be normal at one time, but higher at other time

Measurement of Hormones Concentration…..

- Thus, if the hormone in the blood was measured at only one time of day, the disorder might be missed
- To avoid this problem is to obtain repeated measurements of the hormone over a 24-hour period
- Ideally, repeated blood measurements could be drawn to provide as complete a profile as possible of the minute-to-minute changes in circulating hormone level.
- But it is not practical in practice

Measurement of Hormones Concentration…..

- A simpler method is to obtain a 24-hour cumulative urine sample
- Metabolites of many hormones appear in the urine as part of the daily process of clearing excess hormones from the blood
- The more hormones in the blood, the more it or its metabolites appear in the urine
- A-24 hour measurement will provide information on the integrated, or summed, amount of hormone produced during the day and night.
- So that it is a time-averaged mean

Measurement of Hormones Concentration…..

- In fact that time-averaged means reveal nothing of the countless small (sometimes large) fluctuations in circulating hormone concentration that occurred during that time
- It reveals whether or not abnormally low or high total amounts of hormone were produced

Measurement of Hormones Through Its Action

- Different hormone has different effects on target organ, so that the effects of the hormones on the target organ may reflect the hormone secretion or production
- For examples: Basal Metabolic Rate (BMR) may reflect thyroid hormones

Measurement of hormones through its action…..

- Hormones has pharmacological effects on target organs so that it also may reflect the concentration of that hormone
- For examples: measurement of cardiovascular parameters may reflect sympathetic neurohormones

Measurements of Metabolic Clearance Rate of Hormones

- To calculate Metabolic Clearance Rate (MRC), one makes following 2 measurements:

1. Rate of disappearance of the hormone from plasma per minute (D)
2. Concentration of the hormone in each ml plasma (C)

$$MCR = \frac{D}{C}$$

How to Measure

- **Quantitative:**
  - **Blood samples : hormones**
  - **Urine samples : metabolites product hormone excretion**
- **Qualitative:**
  - **direct effect on target organ**
  - **pharmacological effect**

How to Measure.....

- **Blood samples: hormones**

Most hormones are unstable, so that need appropriate approach:

- **before assaying**
  - > **drawing samples**
  - > **transportation: temperature**
  - > **storing: temperature and long life**
- **during assaying:**
  - > **direct assessment**
  - > **indirect / through appropriate process: derivatization process**

How to Measure…..

- **Valid and reliable**

  on method, tools, competencies

  - **Intra-assay validation (intra-day validation)**
  - **Inter-assay validation (inter-day validation)**
  - **Standard Curve**

Guideline on Bioanalytical Method Validation

(Committee for Medicinal Products for Human Use / CHMP, 2011)

Method Validation

- The main objective of method validation is to demonstrate the reliability of a particular method for the determination of an analyte concentration in a specific biological matrix, such as blood, serum, plasma, urine, or saliva
- If an anticoagulant is used, validation should be performed using the same anticoagulant as for the study samples
- Generally a full validation should be performed for each species and matrix concerned

Method Validation…..

- **Main characteristics of bioanalytical method that are essential to ensure the acceptability of the performance and the reliability of analytical results are:**
  - **Selectivity**
  - **Lower limit of quantification (LLOQ)**
  - **the response function and calibration range (calibration curve performance / standard curve)**
  - **Accuracy**
  - **Precision**
  - **Matrix effects**
  - **Stability of the analytes in biological matrix**
  - **Stability of the analytes and of internal standard (IS) in the stock and working solutions and in extracts under the entire period of storage and processing conditions**

Method Validation…..

- During method validation and analysis of study sample, a blank biological matrix will be spiked with the analytes of interest using solutions of reference standards to prepare calibration, standards quality control samples and stability samples
- In addition, suitable internal standards (IS) can be added during sample processing in chromatographic method

Selectivity

- The analytical method should be able to differentiate the analytes of interest and internal standard (IS) from endogenous components in the matrix or other component in the sample
- Selectivity should be proved using at least 6 individual sources of the appropriate blank matrix, which are individually analysed and evaluated for interference
- Normally, absence of interfering components is accepted where the response is < 20% of the lower limit of quantification for the analyte and 5% for the internal standard (IS)

Lower Limit of Quantification (LLOQ)

- LLOQ is the lowest concentration in a sample which can be quantified reliably, with an acceptable accuracy
- LLOQ is considered being the lowest calibration standard
- The analyte signal of LLOQ sample should be at least 5 times the signal of blank sample
- LLOQ should be adapted to expected concentrations and to the aim of study: for bioequivalence studies LLOQ should be not higher than 5% of Cmax
- LLOQ may be not necessary for exploratory pharmacokinetic studies

Calibration Curve Performance

- Before carrying out the validation of the analytical method, it should be known what concentration range is expected
- The range should be covered by calibration curve range, defined by LLOQ being the lowest calibration standard and the upper limit of quantification (ULOQ) being the highest calibration standard
- A minimum of 6 calibration concentration levels should be used, in addition to the blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS)
- Each calibration standard can be analysed in replicate

Calibration Curve Performance…..

- The calibration curve parameters should be reported (slope and intercept in case of linear fit)
- The back calculated concentrations of the calibration standards should be presented together with the calculated mean accuracy values
- All the available (or acceptable) curves obtained during validation, with a minimum of a 3 should be reported

Calibration Curve Performance…..

- The back calculated concentrations should be within ±15% of the nominal value, except for LLOQ for which it should be within ±20%
- At least 75% of calibration standards, with a minimum of 6 calibration standard levels, must fulfill this criterion
- In case replicates are used, the criteria (within ±15% or ±20% for LLOQ) should also be fulfilled for at least 50% of calibration standards tested per concentration level
- In case a calibration standard does not comply with these criteria, this calibration standard sample should be rejected

Standard Curve

- A standard curve is a type of graph used as a quantitative research technique.
- A graphic plot of tracer binding versus the known concentration of test substances in a set of standards usually prepared by serial dilution or incremental addition.
- Multiple samples with known properties are measured and graphed
- So then allows the same properties to be determined for unknown samples by interpolation on the graph.

Accuracy

- **Within-run accuracy**

  Determined by analysing in a single run a minimum of 5 samples per level at a minimum 4 concentration levels which are covering the calibration range.
  The mean concentration should be within 15% of nominal values, except for LLOQ should be 20% of the nominal value

- **Between-run accuracy**

  For the between-run accuracy, LLOQ, low, medium and high QC samples from at least 3 runs analysed on at least 2 different days should be evaluated
  The mean concentration should be within 15% of the nominal values for the QC samples, except for LLOQ which should be within 20% of the nominal value)

Precision

- **Within-run precision**

  For the validation of the within-run precision, there should be a minimum of 5 samples per concentration level at LLOQ, low, medium and high QC samples in a single run
  The within-run CV value should not exceed 15% for QC samples, 20% for LLOQ

- **Between-run precision**

  For the validation of the between-in run precision, LLOQ, low, medium and high samples from at least 3 runs analysed on at least 2 different days should be evaluated
  The between-run CV value should not exceed 15% for QC samples, 20% for LLOQ

Matrix Effects

- Matrix effects should be investigated when using mass spectrometric methods
- Using at least 6 lots of blank matrix from individual donors
- For each analyte and IS, matrix factor should be calculated for each lot of matrix, by calculating the ratio of peak area in the presence of matrix (measured by analysing blank matrix spiked after extraction with analyte), to the peak area in absence of matrix (pure solution of analyte)
- The IS normalized MF should also be calculated by dividing the MF of analyte by the MF of the IS
- The CV of IS normalized MF calculated from 6 lots of matrix should not be greater than 15%
- This determination should be done at a low and at a high level of concentration (maximum of 3 times LLOQ and close to ULOQ)

Stability

- Evaluation of stability should be carried out to ensure that every step taken during sample preparation and sample analysis, as well as the storage conditions used do not affect the concentration of analyte
- The following stability tests should be evaluated:
  - stability of the stock solution and working solutions of the analyte and internal standard (IS)
  - freeze and thaw stability of the analyte in the matrix from freezer storage conditions to room temperature or sampling processing temperature
  - short term stability of the analyte in matrix at room temperature or sampling processing temperature
  - long term stability of analyte in matrix stored in freezer
  - on-instrument/ autosampler stability of the processed sample at injector or autosampler temperature

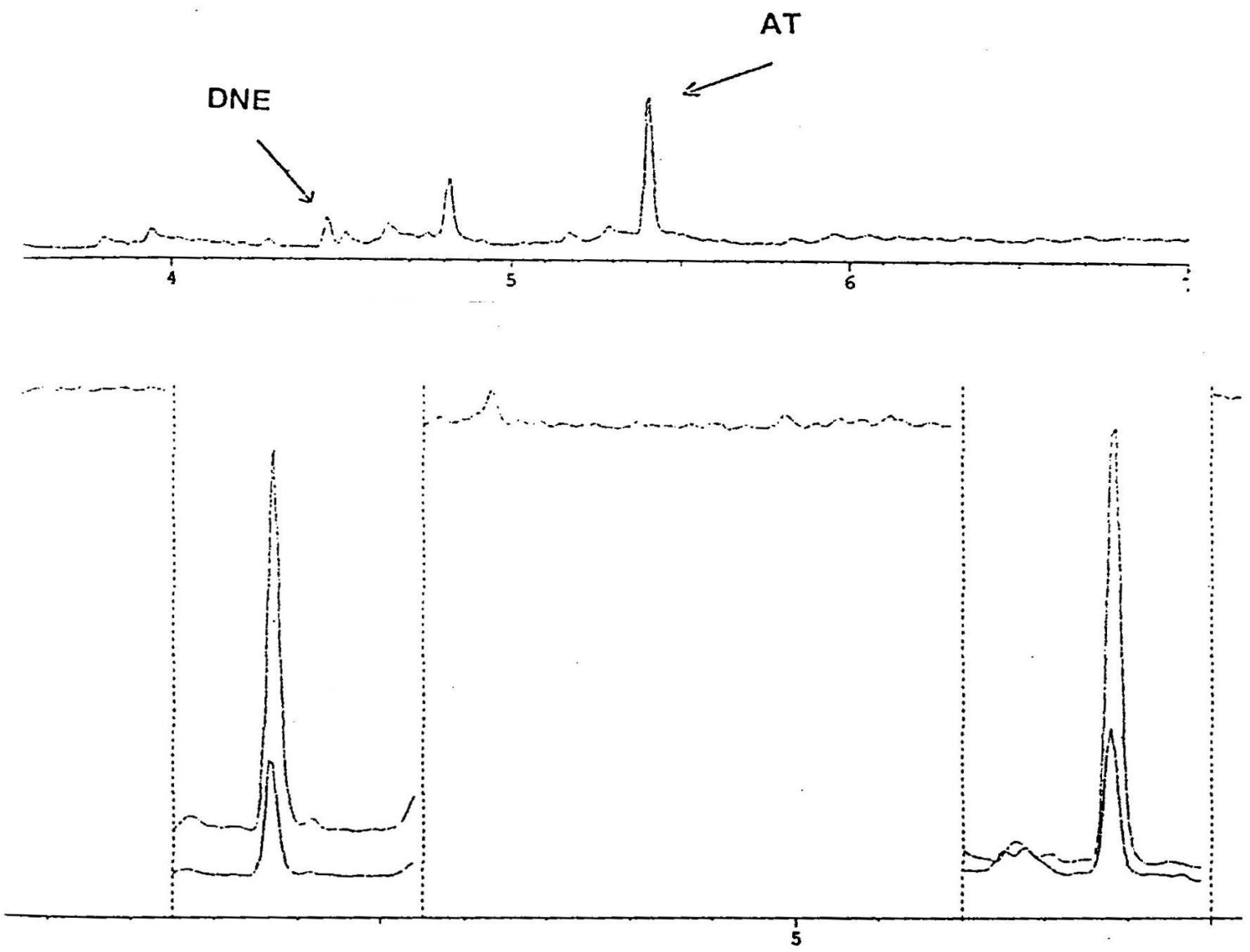
Percentage recoveries of internal standard amitriptyline

Concentration of amitriptyline	Pure AT (height in chrom.)	Extracted AT (height in chrom.)	Recovery (%)
20000pg/ml	31485	28922	91.86
	25358	20594	81.21
	28832	26866	93.18
	31115	29189	93.81
	27719	22865	82.49
	28899	21839	75.57
	32143	25846	80.41
Mean ± SD: 85.50 ± 7.31			

Percentage recoveries of derivatized NE

Concentration of NE	Pure NE (height in chrom.)	Extracted NE (height in chrom.)	Recovery (%)	Mean ± SD
16000 pg/ml	103455 109876 83387 104638	89075 90632 69863 88943	86.10 82.49 83.78 85.00	84.34±1.56
8000 pg/ml	53549 56791 42013 52995	44232 44345 35294 45046	82.60 78.08 84.01 85.00	82.42±3.06
4000 pg/ml	25711 28903 20876 26894	21698 24346 17745 23675	84.39 84.23 85.00 88.83	85.41±1.78
2000 pg/ml	12894 13764 11143 13547	10521 11230 8971 11693	81.59 81.59 80.51 86.31	82.50±2.59
1000 pg/ml	6690 6902 5621 6854	5429 5885 4525 5713	81.15 85.27 80.50 83.35	82.57±2.18
500 pg/ml	3112 3487 2876 3269	2471 2959 2386 2879	85.83 84.86 82.96 88.07	85.43±2.13
250 pg/ml	1615 1775 1313 1656	1265 1473 1089 1334	78.33 82.99 82.94 80.56	81.20±2.23

Mean ± SD of all concentrations: 83.44 ± 2.59



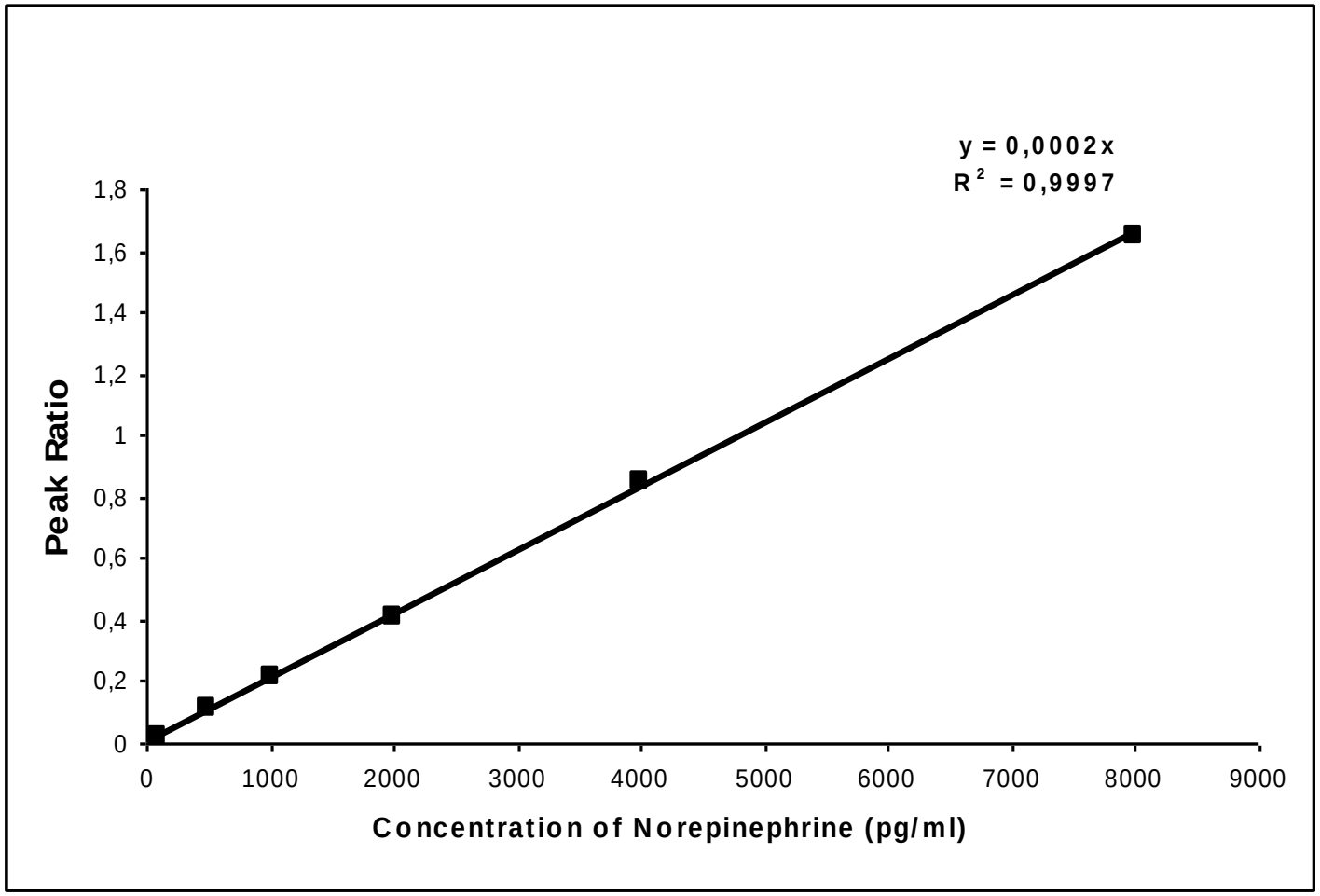
Above: Scanning chromatogram of derivatized extracted NE (DNE) and extracted amitriptyline (AT)

Below: Selected ion monitoring (SIM) chromatogram of derivatized extracted NE (left); extracted amitriptyline (right)

Peak ratio of derivatized NE and amitriptyline (for standard curve)

Concentration of NE (pg/ml)	Peak ratio				Mean ± SD
	1st run	2nd run	3rd run	4th run	
8000	1.59	1.64	1.72	1.67	1.65 ± 0.05
4000	0.86	0.90	0.81	0.83	0.85 ± 0.04
2000	0.39	0.40	0.41	0.43	0.41 ± 0.02
1000	0.21	0.22	0.19	0.20	0.21 ± 0.01
500	0.117	0.111	0.107	0.099	0.11 ± 0.008
100 (LLOQ)	0.019	0.024	0.026	0.021	0.023 ± 0.003

LLOQ, lower limit of quantitation



The standard curve based on peak ratio of derivatized NE and internal standard amitriptyline. The concentrations of NE were from 8000 pg/ml to 500 pg/ml and 100 pg/ml, the lower limit of quantitation (LLOQ).

Accuracy and precision of the assay for NE with amitriptyline as internal standard (n=4)

Conc of NE	Intra-assay variation (Intra-day variation)						Inter-assay variation (Inter-day variation)					
	Peak ratio				Mean ± SD	CV (%)	Peak ratio				Mean ± SD	CV (%)
	1st run	2nd run	3rd run	4th run			1st run	2nd run	3rd run	4th run		
8000	1.67	1.62	1.70	1.65	1.66 ± 0.03	1.81	1.67	1.59	1.68	1.72	1.66±0.05	3.01
4000	0.90	0.86	0.82	0.88	0.87 ± 0.03	3.45	0.90	0.82	0.86	0.83	0.85±0.04	4.71
2000	0.41	0.42	0.44	0.40	0.42 ± 0.02	4.76	0.41	0.40	0.43	0.44	0.42±0.02	4.76
1000	0.22	0.20	0.19	0.20	0.20 ± 0.01	5.00	0.22	0.20	0.19	0.21	0.21±0.01	4.76
500	0.121	0.118	0.129	0.124	0.123 ± 0.005	4.07	0.121	0.117	0.119	0.127	0.121±0.004	3.31
100 (LLOQ)	0.024	0.028	0.026	0.021	0.025 ± 0.003	12.00	0.024	0.026	0.027	0.020	0.024 ± 0.03	12.50

CV, Coefficient of variation; LLOQ, lower limit of quantitation; Conc, concentration

Radioimmuno-Assay

Radioimmuno-Assay

- **For hormones assessment**
- **Principle of radioimmuno-assay:**

  Antibody (globulin) which is specific for the hormone that will be assessed must be produced from the animal in a great amount (commercially available)
- **The antibody then mixed with:**
  - **animal serum which contain hormone to be assessed (h)**
  - **pure standard hormone which has been labeled by radioisotop (hsr) (with known amount)**

Radioimmuno-Assay.....

- Antibody and hormone will be bound (ab-h & ab-hsr)
- Hormone to be assessed and hormone labeled by radioisotope competitively binds the antibody
- Concentration of ab-hsr then measured, soon after the binding has reached equilibrium, using radioactive-counting technique

Radioimmuno-Assay.....

- To make “assay highly quantitative”, radioimuno-assay must also be applied for “standard” solution from pure un-labeled hormone with some levels of concentration
- The results then will be arranged in a **Standard Curve**

Thank You