Instruction

EURIA-CCK

Cholecystokinin radioimmunoassay

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For Research Use Only. Not for use in diagnostic procedures.

REF **RB 302 RUO**

Σ 100

INTRODUCTION

Cholecystokinin (CCK) is one of the classical gut hormones. It is believed to be a major regulator of gall bladder contraction and pancreatic enzyme secretion.
CCK occurs in many different molecular forms. In 1971, Mutt and Jorpes isolated a 33-amino-acid polypeptide which exhibited the properties ascribed to CCK (1). Later a CCK-39 with a further 6 amino acid residues linked to the NH_2 terminus of the triacontatriapeptide was described (2). An identical sequence of the c-terminal octapeptide (CCK-8) has been found in mammals except for the guinea pig and the chinchilla, where valine substitutes methionine at position 6 from the C-terminus. The structures of CCK in the small intestine have been determined for peptides of 58 amino acids from the dog, 25 and 18 amino acids from the dog, 22 and 8 amino acids from the rat and guinea pig and 7 and 5 amino acids from the dog.
The structure of a preproCCK has been determined. It consists of 115 amino acids in man (3). The C-terminal sulphation (Tyr ^{27} in CCK-33) and the C-terminal amidation are important for the biological activity (4). CCK shares an identical sequence with gastrin in the 5 C-terminal amino acids. Definite physiological actions of CCK that occur during the intravenous infusion of CCK are:

1/ Stimulation of gall bladder contraction (5, 6)

2/ stimulation of pancreatic enzyme secretion (7)

3/ enhancement of secretin-induced water and bicarbonate secretion from the exocrine pancreas (7)

4/ inhibition of gastric emptying (8, 9).

There are three main problems in the development of specific radioimmunoassays for CCK (21, 22, 23).

First, the homology of the antigenic C-terminal penta-peptide with gastrin requires an antiserum with very low cross-reactivity towards gastrin. CCK in plasma and in intestinal tissue is heterogeneous. Thus, the antiserum used should ideally bind all biological active forms with equimolar potency.

Second, the radioiodination of CCK requires special, mild labelling since oxidation of the methionine residues occurs in oxidative labelling methods.

Third, plasma concentrations of CCK are very low which makes it necessary to have a very highly sensitive assay system. The Euro-Diagnostica CCK radioimmunoassay is based on an antiserum with very low cross-reactivity to gastrin-17, sulphated gastrin. The assay system has been optimized to a very high sensitivity: 0,3 pmol/L.

PRINCIPLE OF THE METHOD

The intended use of these reagents is for assay of CCK in plasma. CCK is extracted from plasma by an ethanol extraction method. CCK in extracts is assayed by a competitive radioimmunoassay using an antiserum raised against CCK-8 sulphate N-terminally conjugated to bovine albumin. CCK in standards and samples compete with ^{125}I-CCK8 sulphate in binding to the antibodies. ^{125}I-CCK-8 sulphate binds in a reverse proportion to the concentration of CCK in standards and samples. The assay is standardized against CCK-8 sulphate. Antibody-bound ^{125}I-CCK-8 sulphate is separated from the unbound fraction using double antibody solid phase. The radioactivity of the bound fraction is measured in a gamma counter. For professional use within a laboratory.
The result shall not be used for clinical diagnosis or patient management.

PHYSIOLOGICAL CONSIDERATIONS

Decreased CCK secretion has been found for individuals with celiac disease. Those individuals had an impaired gallbladder contraction after meal (10,11,12). CCK secretion and gallbladder contraction returns to normal in individuals on a gluten-free diet with a normal jejunal mucosa (11,12). Increased plasma CCK concentrations in individuals with chronic pancreatitis have been reported (13,14). Recent investigations with specific and sensitive CCK assays have shown normal fasting levels in individuals with chronic pancreatitis (15, 16).
Elevated CCK concentrations have been reported in individuals with hepatic cirrhosis (17). In a later study no difference was observed between cirrhotics and controls (18). Increased postprandiol CCK response has been observed in non-insulin-dependent diabetes (19). High amounts of CCK have been found in pituitary adenomas of individuals with Nelson´s syndrome and some individuals with Cushing´s disease (20). Increased basal concentration of sulphated CCK was found in plasma of one unoperated individual with Nelsons´s syndrome (20).

Normal fasting level of CCK: $\leq$1.12 pmol/L.

PRECAUTIONS

For research use only – not for use in diagnostic procedures

As the regulations may vary from one country to another, it is essential that the person responsible for the laboratory is familiar with current local regulations, concerning all aspects of radioactive materials of the type and quantity used in this test.

This kit contains ^{125}I (half-life: 60 days), emitting ionizing X (28 keV) and γ (35.5 keV) radiations. Steps should be taken to ensure the proper handling of the radioactive material, according to local and/or national regulations. Only authorized personnel should have access to the reagents.

The following precautions should be observed when handling radioactive materials:

- Radioactive material should be stored in specially designed areas, not normally accessible to unauthorized personnel.

- Handling of radioactive material should be conducted in authorized areas only.

- Care should be exercised to prevent ingestion and contact with skin and clothing. Do not pipette radioactive solutions by mouth.

- Drinking, eating or smoking should be prohibited where radioactive material is being used

- Hands should be protected by gloves and washed after using radioactive materials.

- Work should be carried out on a surface covered by disposable absorbing material.

- Spills of radioactive material should be removed immediately, and all contaminated materials disposed as radioactive waste. Contaminated surfaces should be cleaned with a detergent.

This kit contains components of human origin. They have been tested by immunoassay for hepatitis B surface antigen, for antibodies to HCV and for antibodies to HIV-1 and HIV-2 and found to be negative. Nevertheless, all recommended precautions for the handling of blood derivates, should be observed.

- Material safety data sheets for all hazardous components contained in this kit are available on request from Euro Diagnostica.

Warning

REAG	A	Ab	
REAG	E	CAL	50

REAG	F	CONTROL
REAG	G	CONTROL

Contains ProClin 300:
Reaction mass of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC no. 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC no. 220-239-6] (3:1)

H317:	May cause an allergic skin reaction.
P264:	Wash hands thoroughly after handling.
P280:	Wear protective gloves/protective clothing/eye protection/face protection.
P302+352:	IF ON SKIN: Wash with plenty of soap and water.
P333+313:	If skin irritation or rash occurs: Get medical advice/attention.

COMPOSITION OF THE REAGENT KIT

The reagents provided in each kit are sufficient for 100 tubes.

1. Anti-CCK-8 (Reagent A)

Rabbit antiserum to synthetic cholecystokinin 26-33, sulphate. The antiserum is diluted in and lyophilized in 5.0 mL 0.5 M phosphate buffer, pH 7.4, with 2.5% human serum albumin and 0.5% ProClin 300. Color: Yellow.
Reconstitution in 52 mL distilled water.

2. ^{125}I-CCK-8 (Reagent B)

Activity: 0.75 μCi or 28 KBq. Produced by Bolton and Hunter labelling of cholecystokinin 26-33, sulphate. HPLC-purified, monoiodinated. Specific activity: 62-77 Mbq/nmol (1700-2100 μCi/nmol).
Lyophilized in 5.0 mL 0.5M phosphate buffer, pH 7,4 with 2.5 % human serum albumin, 0.2% Merthiolate and 5000 KIU Trasylol/mL. Color: Blue
Reconstitution in 52 mL distilled water.

3. Double antibody solid phase (Reagent C)

Anti-rabbit-Ig coupled to cellulose particles in 0.01 M phosphate buffer pH 6.8 with 0.25% Human serum albumin, 0.045% NaCl, 0.05% NaN_3, 0.185% EDTA and 0.05% Tween 80.
11 mL suspension.

4. Diluent (Reagent D)

Assay buffer to be used for preparation of CCK-8 working standards and for reconstitution of sample extracts.
50 mL of 0.05M phosphate buffer, pH 7.4 containing 0.25% human serum albumin and 0.02% Merthiolate.

5. CCK-8 standard (Reagent E)

Concentration: 50 pmol/L of cholecystokinin 26-33, sulphate.
Volume: 5.00 mL standard after reconstitution.
Lyophilized in 0.05M phosphate buffer, pH 7.4, with 0.25% human serum albumin and 0,05% ProClin 300.

6. Controls (Reagent F-G)

Lyophilized controls with two different levels of CCK-8. 2.00 mL of each control after reconstitution. The CCK-8 concentrations of the controls are given on the label of the vials.
Contains 0.05% ProClin 300.

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

Distilled water.
Ethanol, 96%.
Disposable test tubes 11-13x70mm of glass. Disposable test tubes 11x13x55 mm of polystyrene.
Pipettes with disposable tips: 100, 200, 500 and 1000 μL.
Volumetric pipettes: 1,00 and 5.00 mL.
Equipment for evaporation in vacuum.
Vortex-mixer.
Centrifuge, refrigerated.
Gammacounter.
Magnetic stirrer.

REAGENT PREPARATION AND STORAGE

Store all reagents at 2-8 °C before reconstitution and use. The water used for reconstitution of the lyophilized reagents should be distilled in an all-glass apparatus or be of corresponding purity. Dissolve the contents in the vial by gentle inversion, avoid foaming. The stability of the reagents is found on the labels of the vials. For lyophilized reagents the expiry date is valid for the unreconstituted reagents. Reconstituted reagents are stable for 10 weeks or to the expiry date on the vial when stored as described below.

Reagent A: Anti-CCK-8
Reconstitute with 52 mL distilled water.
Store at 2-8°C.

Reagent B: ^{125}I-CCK-8
Reconstitute with 52 mL distilled water.
Store at - 20° or lower if reused.

Reagent C: Double antibody solid phase
Ready for use. Stir continuously during pipetting this reagent. Store at 2-8°C.

Reagent D: Diluent
Ready for use. Store at 2-8 C.

Reagent E: CCK-8 standard
Reconstitute with 5.00 mL distilled water.
Store at -20 °C or lower if reused.

Reagent F-G: Controls
Reconstitute with 2.00 mL distilled water.
Store at -20° C or lower if reused.

SPECIMEN COLLECTION

Subjects should be fasting at least ten hours prior to sample collection.
Vein blood is collected in tubes containing EDTA and Trasylol ® (5000 KIU Trasylol in a 10 mL vacutainer). The sample is cooled in an ice-bath immediately. Plasma is separated by centrifugation (refrigerated centrifuge).The plasma should be frozen within 2 hours and stored at -20°C or lower until assayed. Repeated thawing and freezing must be avoided.

ASSAY PROCEDURE

The assay is performed in 2 steps:

A. Extraction of plasma samples

B. Radioimmunoassay of extracts

A. Extraction procedure

In order to eliminate non-specific interference from plasma proteins extraction of CCK from plasma is necessary. It is recommended to control the recovery under the users own experimental conditions.

Performance

1. Thaw the samples immediately before starting the extraction. Store the thawed samples in an ice-bath.

2. Transfer 1.00 mL plasma to an 11x70mm glass tube.

3. Add 2.00 mL 96% ethanol and vortex-mix vigorous for 10 seconds.

4. Allow the tubes to stand on the bench for 10 minutes.

5. Centrifuge at 1700xg for 15 minutes.

6. Decant the supernatant containing CCK in another 11x70 mm glass tube.

7. Evaporate to dryness in vacuum.
   The evaporation may be performed in a Speed Vac Concentrator at 37°C. An alternative is evaporation in a freeze drier over night.

8. Dissolve the dry extracts in 1.00 mL diluent (Reagent D). Vortex-mix and let the tubes stand on bench for 30 minutes before assay in the RIA procedure.
   If the sample extracts are not assayed the same day as extracted store at -20°C until assayed.

Recovery control:

It is important to determine the recovery in the extraction procedure under the users own experimental conditions.
To estimate the extraction recovery add 200 µL of CCK-standard 50 pmol/L to 800 µL blood donor plasma. The concentration will be 10 pmol/L. Handle the recovery sample by the same extraction procedure as the unknown samples. Perform in duplicate. Extract also the same blood donor plasma after adding 200 µL diluent (Reagent D) to 800 µL plasma. Analyse the extracts in the radioimmunoassay.

$$\% \text{ recovery} = \frac{(\text{pmol/L found with addition} - \text{pmol/L found without addition}) \times 100}{10}$$

B. Radioimmunoassay of extracts
Reconstitute the reagents as specified .
Reagents should be brought to room temperature prior to use. Accuracy in all pipetting steps is essential.
All tests (standards, controls and samples) should be performed in duplicate.
A complete assay includes:

Standards (St-tubes): 7 different concentrations:
0, 0.78, 1.56, 3.12, 6.25, 12.5, 25 pmol/L

Controls (C-tubes): For recovery control (C_{REC}) and radioimmunoassay control (C_{RIA}).

Samples (P-tubes):
Tubes for the determination of the ***non-specific binding*** without antiserum ***(NSB-tubes)***.
Tubes for the determination of the ***total radioactivity*** added ***(TOT-tubes)***.
For an overview see table 1 on page 13.

PERFORMANCE

1. Reconstitute the reagents according to the instructions on page 6.

2. Prepare the CCK-8 working standards by dilution of the CCK-8 standard 50pmol/L (Reagent E) with diluent (Reagent D) according to the following:
   a. 1.00 mL standard 50 pmol/L + 1.00 mL diluent = 25 pmol/L
   b. 1.00 mL standard 25 pmol/L + 1.00 mL diluent = 12.5 pmol/L
   c. 1.00 mL standard 12.5 pmol/L + 1.00 mL diluent = 6.25 pmol/L
   d. 1.00 mL standard 6.25 pmol/L + 1.00 mL diluent = 3.12 pmol/L
   e. 1.00 mL standard 3.12 pmol/L + 1.00 mL diluent = 1.56 pmol/L
   f. 1.00 mL standard 1.56 pmol/L + 1.00 mL diluent = 0.78 pmol/L
   g. Diluent = 0 pmol/L.
   Store the standards at -20°C or lower if reused

3. Pipette 200µL of standards (0-25 pmol/L), controls and samples in their respective tubes.

4. Pipette 200µL of the zerostandard (diluent) in the NSB tubes.

5. Pipette 500µL anti-CCK-8 (Reagent A) in all tubes except the NSB-tubes and TOT-tubes.
   Vortex-mix.

6. Pipette 500µL diluent (Reagent D) in the NSB-tubes.

7. Incubate for 2 days at 2-8° C, (44-50 hours).

8. Pipette 500µL ^{125}I-CCK-8 (Reagent B) in all tubes. Vortex-mix. The TOT-tubes are Sealed and kept aside.

9. Incubate for 4 days at 2-8°C, (92-100 hours).

10. Pipette 100µL Double antibody solid phase (Reagent C) in all tubes except the TOT-tubes. This reagent should be stirred continuously with a magnetic stirrer during pipetting.
    Vortex-mix carefully.

11. Incubate for 30-60 minutes at 2-8°C.

12. Centrifuge for 15 minutes at +4°C (minimum 1700xg)

13. Decant the supernatants.

14. Count the radioactivity in all tubes in a gamma counter.
    Counting time: 2-4 minutes.

Alternative incubation time
The incubation time can be shortened to 4 days (92-100 hours) with equilibrium assay conditions with retained precision of the assay.
The lowest detectable concentration will then increase with a factor of approximately 1.5.
This performance may be used if the samples to be assayed contain CCK at levels, which do not require optimal sensitivity.

Performance

1. Pipette 200 µL of standards (0-25 pmol/L), controls and samples in their respective tubes.

2. Pipette 200 µL of the zerostandard (diluent) in the NSB-tubes.

3. Pipette 500 µL ^{125}I-CCK-8 (Reagent B) in all tubes. The TOT-tubes are sealed and kept aside.

4. Pipette 500 µL anti-CCK-8 (Reagent A) in all tubes except the NSB-tubes and TOT-tubes.

5. Pipette 500 µL diluent (Reagent D) in the NSB-tubes. Vortex mix.

6. Incubate for 4 days (92-100 hours) at 2-8° C.

7. Pipette 100 µL Double antibody solid phase (Reagent C) in all tubes except the TOT-tubes. This reagent should be stirred continuously with a magnetic stirrer during pipetting. Vortex mix carefully.

8. Incubate for 30-60 minutes at 2-8° C.

9. Centrifuge for 15 minutes at +4° C (minimum 1700 x g).

10. Decant the supernatants.

11. Count the radioactivity in all tubes in a gamma counter. Counting time: 2-4 minutes.

CALCULATION

1. Subtract the average count rate (CPM) of the NSB-tubes from the count rate (CPM) of the standards, controls and samples.

2. A standard curve is generated by plotting the bound fraction CPM or B/TOT against the concentrations of the CCK-8 standards. An example of a standard curve is given on page 14.

3. Interpolate the CCK-concentrations of the controls and samples from the generated standard curve. Correct the observed concentrations in the samples with respect to the recovery in the extraction procedure. It is important that each laboratory estimates the recovery under its own experimental conditions.

4. The standard curve and the calculation of the CCK-concentrations in the samples can also be done by a computer method.

ASSAY CHARACTERISTICS

Sensitivity
The lowest detectable concentration is 0.3 pmol/L. The figure corresponds to a decrease in binding of 2xSD of the bound radioactivity in the zero-concentration standard.

Accuracy
A mean recovery of 80% was obtained when known amounts of CCK-8 sulphate were added to plasma samples.

Precision
Intra assay variation

Level	Coefficient of variation (CV)
4.4 pmol/L	5.5%
20.6 pmol/L	2.0%

Inter assay variation (total variation)

Level	Coefficient of variation (CV)
4.2 pmol/L	13,7%
20.6 pmol/L	4.1%

Specificity
The following cross-reactions have been found:

Peptide	Cross-reaction
Cholecystokinin 26-33, sulphate	100.0%
Cholecystokinin-33, sulphate	134.0%
Cholecystokinin 26-33, non-sulphated	<0.01%
Cholecystokinin 30-33	<0.01%
Gastrin-17, sulphate	0.5 %
Gastrin-17, non-sulphated	<0.01%

Interference
Samples displaying cloudiness, hemolysis, hyperlipemia or containing fibrin may give inaccurate results.

QUALITY CONTROL

In order for the laboratory to completely monitor the consistent performance of the radioimmunoassay there are some important factors, which must be checked.

1. Observed concentrations of controls
The found concentrations of the controls (Reagent F and G) should be within the limits given on the labels of the vials.

2. Recovery control
The recovery should be at least 50% for a valid assay. It is important that the recovery is controled under the user´s own experimental conditions.

3. Total counts
Counts obtained should approximate the expected CPM when adjusted for counter efficiency and radioactive decay. The content of ^{125}I-CCK-8 in this kit will give a total counts in the assay (TOT) of 10500 CPM (-5, +20 %) at the activity reference date (counter efficiency = 80%).

4. Maximum binding (Bo/TOT)
Calculate for each assay the % bound radioactivity of the zero-standard $\left(\frac{Bo}{TOT} \times 100\right)$

$\frac{Bo}{TOT} \times 100$ is generally 30-45% at the activity reference date

$\frac{Bo}{TOT} \times 100$ may have decreased a few % at the expiry date of the kit.

5. Non-specific binding (NSB/TOT)
Calculate for each assay the non-specific binding $\left(\frac{NSB}{TOT} \times 100\right)$

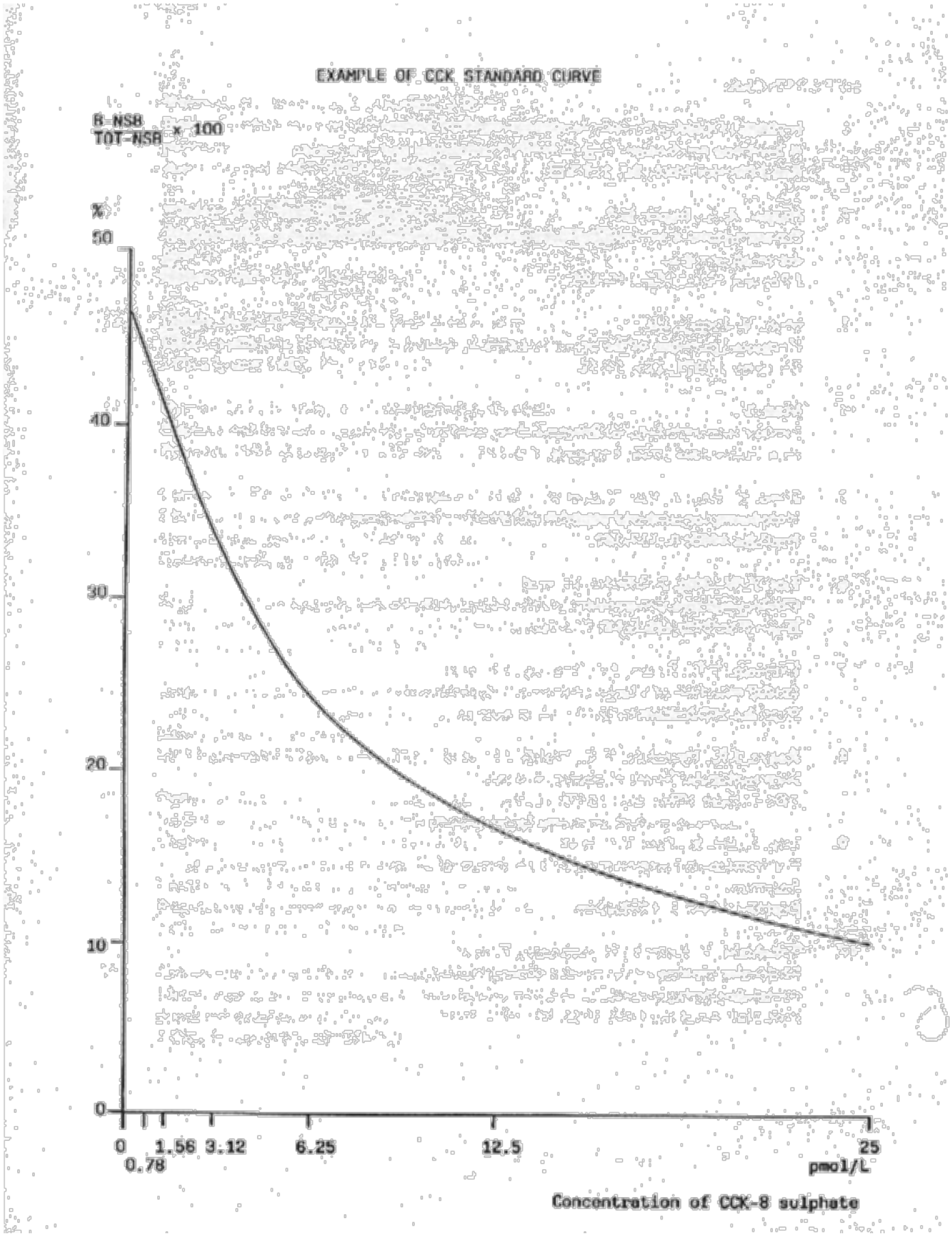
$\frac{NSB}{TOT} \times 100$ is less than 5% if decanting is made properly.

6. Shape of standard/curve
For example, monitor the 80, 50 and 20% points of the standard line for run-to-run reproducibility.

OUTLINE OF THE RIA PROCEDURE

Type of tubes	Tube no	Standard sample or control	Anti-CCK-8 (A)	Diluent (D)		^{125}I-CCK-8 (B)		Double antibody solid phase (C)	
TOT	1- 2	-	-	-	Vortex-mix and incubate for 2 days at 2-8°C, (44-50 hours).	500 µL	Vortex-mix and incubate for 4 days at 2-8°C, (92-100 hours).	-	Vortex-mix and incubate for 30-60 min. at 2-8°C. Centrifuge 15 min. at 1700 x g at +4° C. Decant and count the radioactivity of the pellets.
NSB_{St}	3- 4	200 µL	-	500		500 µL		100 µL	
Stand 0	5- 6	200 µL	500 µL	-		500 µL		100 µL	
Stand 0.78	7- 8	200 µL	500 µL	-		500 µL		100 µL	
Stand 1.56	9-10	200 µL	500 µL	-		500 µL		100 µL	
Stand 3.12	11-12	200 µL	500 µL	-		500 µL		100 µL	
Stand 6.25	13-14	200 µL	500 µL	-		500 µL		100 µL	
Stand 12.5	15-16	200 µL	500 µL	-		500 µL		100 µL	
Stand 25	17-18	200 µL	500 µL	-		500 µL		100 µL	
$Control_{RIA}$ (F)	19-20	200 µL	500 µL	-		500 µL		100 µL	
$Control_{RIA}$ (G)	21-22	200 µL	500 µL	-		500 µL		100 µL	
$Control_{REC}$	23-24	200 µL	500 µL	-		500 µL		100 µL	
$Control_{REC}$	25-26	200 µL	500 µL	-		500 µL		100 µL	
Sample 1	27-28	200 µL	500 µL	-		500 µL		100 µL	
Sample 2 etc.	29-30	200 µL	500 µL	-		500 µL		100 µL	

Table 1.


EXAMPLE OF CCK STANDARD CURVE
B-NSB
TOT-NSB x 100
%
50
40
30
20
10
0
0
0.78
1.56
3.12
6.25
12.5
25
pmol/L
Concentration of CCK-8 sulphate

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SYMBOLS USED ON LABELS

LOT	Batch code.
REF	Catalogue number.
	Use by date.
	Temperature limit.
	Date of manufacture.
	Contains radioactive substances.
	Biological risks.
	Consult instructions for use.
	Manufacturer.
 100	Contains sufficient for 100 tests.
	Warning

REAG A Ab	Anti-CCK-8
REAG B Ag ^{125}I	^{125}I-CCK-8
REAG C DASP	Double antibody solid phase
REAG D DIL	Diluent
REAG E CAL 50	CCK-8 standard 50 pmol/L
REAG F CONTROL	Control, level 1
REAG G CONTROL	Control, level 2

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