we are honored to have dr andrew
pharmd degree from utrecht university in
the netherlands where he also completed
a phd focused on clinical
pharmacokinetics of herb drug
interactions and oncology
andrew is also a clinical pharmacologist
certified by the dutch society of
clinical pharmacology and biopharmacy
after receiving his phd andrew completed
a threeyear postdoctoral fellowship in
my lab at the national cancer institute
where he conducted research spanning the
entire drug development pipeline from
drug discovery via target identification
and validation to preclinical
development and ultimately clinical
evaluation
during a second postdoctoral fellowship
in the netherlands he specialized in the
field of pharmacogenetics and oncology
in 09 he joined roswell park
comprehensive cancer center in buffalo
new york as an assistant professor in
the department of pharmacology and

therapeutics were excited to have him

give todays lecture

hello everyone my name is andrew gui

today ill talk about the chemical

analysis of drugs and metabolize in

biological systems

this also called bioanalysis

one hour is way too short to cover this

for this field in full detail but i hope

that after this lecture youll have a

flavor of the basic concepts and

applications of bioanalysis

so first ill discuss with you

the definition of bioanalysis and its

main applications

then

i will discuss with you how

chromatographic assays and ligand

binding essays are developed

during the next section

i will guide you through the validation

process of these essays

and at the very end ill show you a real

life example

of a method that we developed and

validated at the bioanalytical

metabolomics and pharmacokinetic shared

resources at russell park

now the definition of bioanalysis is

shown over here

its a subdiscipline of analytical

chemistry that deals with the

quantitation of xenobiotics such as

drugs

and the dungeness compounds in

biological systems

like humans or animals

measuring drug levels is important in

various areas

for example in drug development

when youre doing preclinical studies

you very often need to characterize the

metabolism of the drug in for example

human liver microsomes

or in animals

but also

later on in clinical testing

drugs the pharmacokinetics of drugs also

need to be characterized in humans in

phase one pharmacokinetic studies

also in clinical practice drug assays

are needed for example when therapeutic

drug monitoring needs to be carried out

in which

you will assess if the patient is

receiving the optimal dose

and forensic science and antidoping are

also other areas for which drug ashes

are needed

now before a bio analytical essay can be

used for any of these applications it

needs to go through the stages of method

development and validation

during method development

you will optimize

the processes that

concern extraction separation and

detection of the analyte

entering method validation you need to

prove that the method is suited to the

analysis of your study samples

so let me first talk about

chromatographic

sa development

a chromatographic essay

consists of several

stages sample preparation analyte

separation and analyte detection

before starting

method development its important to

consider several factors for example

what information do you have on your

analytes

are you dealing with a small molecule or

a large molecule

what are the physical chemical

properties

for example how powder is a drug is it

charged or not

how stable is it under high temperatures

also it is always good to know if there

is information on the metabolism of the

drug for example if there are any active

metabolites then these should also be

included in your essay

information on the expected

concentration in your study samples is

also very handy because this will

determine the

concentration range of your assay which

runs from the lower limit of

quantitation up to the upper limit of

quantitation

the

matrix is also an important factor

do you need to measure the drug in

plasma serum or urine

because some matrices contain more

components that may be interfering with

your detection method

therefore the type of matrix will

dictate which sample preparation method

you should use

sample volume is another

important factor and you should also

always do literature research to see if

there are any other published methods on

your analyte because this could give you

a good starting point on

you know how and where to start your

essay development

then there are also practical factors to

consider

for example um on the workload uh how

many samples do you expect to be run

within you know within an analytical run

if you have higher number of samples

then a 9 well blade format

needs to is very handy otherwise you can

just use the microcentrifuge tubes

how frequent will you receive samples

and also throughput and turnaround time

is important factor especially

for an essay used mechanical practice

where very often the turnaround time

needs to be very fast

so

therefore the sample preparation method

and also the chromatographic runtime

should also be as short as possible

then

your lab resource are also

is also an important factor what type of

equipment

do you have in your lab

do you have uv detectors or do you have

mass spectrometers

are there any automatic automated liquid

handling devices available

and

also a very important factor was the

expertise and the experience of the

people in the lab because this often

dictates which sample preparation method

is being used

now as said

chromatographic essays consists of the

stages listed over here

in the following section ill discuss

versus sample preparation methods that

are out there

sample preparation is needed because

very often you cannot simply inject your

biological sample into

your parmetographic system because if

you do this you will plug the

chromatography column

and you will leave lots of matrix

deposits on your mass spectrometer

so therefore you need to remove matrix

components such as proteins

phospholipids and red blood cells

so this will lead to a lower background

signal

improve sensitivity of your assay and

also reduce variation in signal and this

will all lead to a better essay

the preferred sample preparation method

is simple

fast inexpensive and reliable

and in the next slide i

have listed four of the most commonly

used sample preparation methods

so its diluted shoot

protein precipitation

liquid liquid extraction and solid phase

extraction

there are multiple commercial

variants available

for these methods but over here ill

just discuss the basic concepts of

of each of these methods

now diluted shoot and protein

precipitation

are the most simple and cheapest methods

that are out there

but these are also relatively dirty

compared with the other extraction

methods

because those methods will remove more

matrix components and it will leave you

with a cleaner sample

another benefit of the extraction

methods

is the ability to concentrate your

sample

because you can evaporate the sample and

reconstitute it in a lower volume and

this will increase the sensitivity of

your essay

extraction methods however are also

more time consuming

and especially solid phase extraction is

relatively more costly

than

dilution and protein precipitation

so lets take a closer look at each of

these methods when your matrix has low

protein content then its relatively

clean

and so you can apply the to do the shoot

method

heres an example of an essay

that was used to measure tmao and its

metabolite tma

in human urine conducted over hour

time period

so it starts here with 0 microliters of

urine sample

that is mixed

with 990 microliters of internal

standard solution ill discuss later

where the internal standard is

but after mixing this solution it

is injected directly into

the lc lcms system

and the analytes are detected

so this is a very simple and fast

straightforward sample preparation

technique

when a matrix contains

more proteins then

protein precipitation is a better method

um

example of matrices with high protein

content

is serum plasma or whole blood

protein presentation works by mixing the

sample with three to five times the

volume with organic solvents

such as methanol or acetonitrile

and these organic solvents will

precipitate proteins in your sample

so this in this example

methanol is added to a plasma sample

after vortexing and centrifuging

a protein pellet is formed at the very

bottom

of your tube

and then you will be able to isolate

the supernatant which contains the

analyte

and inject the supernatant into the lc

system

when you need a cleaner sample or you

need a more sensitive assay

then liquid liquid extraction can be

used

liquid liquid extraction involves the

partitioning of the analyte

from an aqueous biofluid such as plasma

into water immiscible organic solvent

and this is all based on polarity

and this works well with neutral and

nonpolar small molecules

so here in this example

the analyte is indicated by stars

and a water immiscible organic solvent

is added

such as ethioacetate

typically you would use organic solvent

with a density thats lower than water

so the organic layer will stay on top

because of

the affinity

of the analyte for the organic phase

it will move to the organic layer

then you can freeze

your samples so the water phase will be

frozen and you can just pour off the

organic

organic layer evaporate it and

reconstitute it in reconstitution

solvent

now this proceeds procedure consists of

of many steps

its quite labor

intensive so if youre having large

number of samples

then and this may not be the preferred

method for you

in that case solid phase extraction may

be better

because its easier to automate this

this process

so solid phase extraction

uses a difference of affinity between an

analyte and interference for a solid

phase

also called a

sorbent here you see example of solid

phase columns and theyre also available

in the 9 well plate format

so it all starts with conditioning the

column with the conditioning solvent

and

then you will load your sample

the analyte in your sample

will absorb

to the

to the stationary phase

while the interference components will

just flow through to the waste

after several washing steps

you can then elude

your analyte from the column

by an element

and

there is a possibility to evaporate

this solution and reconstitute it

now there are several types of

absorbents available depending on the

characteristics of your analyte

or polar analyte also the sorbent should

be polar

for example a cdcard column can then be

used

the nonpolar c

sorbents

are

applicable for a nonpolar analyte and

for charged molecules ion exchange spe

columns are available

now before

doing the sample preparation steps it is

important to add an internal standard if

youre doing a liquid chromatography

um essays because um you need to control

for variability that can occur during

sample preparation

hplc injection and ionization

an internal standard is added at a fixed

concentration to calibration standards

quality control samples

and you study samples

before you do your

sample preparation

the preferred internal standard is a

stable isotope labeled compound such as

a

or a deteriorated version of your

analytes

if these are not available or

too expensive then

a structurally similar analog can be

used

you want to have an internal standard

thats structurally very similar to your

analyte because it should exhibit

a

similar behavior in times of in terms of

similar extraction recovery

similar chromatographic retention time

and also a similar ionization response

as the analyte

you will use the peak area of the

internal standard to

normalize the response of your analyte

so

the area ratio is used as the outcome

of your

of your chromatographic essay as shown

over here

so for example if there is any loss of

your analyte during

during extraction then also

your

peak area of the internal standard will

decrease you know to a similar extent

and your area ratio wouldnt change

and this will greatly improve the

precision of your method

heres an example of an internal

standard that we used when we developed

an essay

for the quantitation of seraphinity

human plasma

we use a carbon and a deuterated

version of seraphinip

so this part of the molecule is the only

difference with

the with the analyte the rest is similar

and we can see

here in the chromatograms that the

chromatographic behavior is very very

similar

the retention time is almost the same so

this is the type of

interior standard that you would prefer

for your essay

extra sample preparation you need to

separate the analyte from

any other components that are still

there in your matrix

very often

uh sample or analyze separation is

performed by liquid chromatography

here you see a schematic overview of

an lc system coupled with a mass

spectrometer as the detector

so your sample is injected over here

using an auto sampler

and after injection

it gets mixed with the mobile phase

consisting of solvent a and solvent b

for refirst phase methods ill

shortly discuss what it is

solvent a is typically

an aqueous

solvent while solving b is an organic

water miscible solvent such as methanol

or acetonitrile

so the separation principle of liquid

chromatography is based on the

distribution of the analyte between the

mobile phase and the stationary phase

which is the picking material

of the lc column

each component in sample

interacts differently with the adsorbent

material of the stationary phase

so just to give you an example

this is a nonpolar

stationary phase that is used to

retain nonpolar analytes

so we see here that the polar

molecules in your sample

they will be retained

less strongly by your stationary face

and they will travel through the color

more quickly

so they will

elude earlier in time

whereas the analyte

will have more interaction with the

stationary phase

and will move slower slower through the

column and will be detected at a later

point in time

so these differences in binding affinity

to the stationary phase leads to

separation of the analyte

now in the next slide

over here you see that there are

different types of liquid chromatography

methods

there is high performance for high

pressure liquid chromatography hplc

and the other subtype is ultra high

performance or pressure and liquid

chromatography uh plc

differences between these methods are

shown over here

uhblc

columns

have a smaller column particle size less

than two micrometers

so

these columns can be run at a much

higher pressure

just result in a shorter runtime

and also resolution is higher meaning

that the peaks are narrower compared to

hvlc methods

disadvantages of new hplc methods

are that its more costly

the instrument is more expensive

only the highest rate of solvents can

can be used

and maintenance should also be carried

out more frequently

and hplc methods are also more robust

than uh plc methods

so each of these methods

has their advantages but also there are

disadvantages

now there are different types of hplc

and uhfc columns on the market because

there are several ways that an analyte

can be retained in the column

so first

there is a separation based on polarity

um so for nonpolar analytes also the

stationary face of the column needs to

be nonpolar

this is the case for reverse phase

columns

in reverse phase columns

large alkyl chains are bonded to the

stationary phase particles as shown here

for

polar analytes also a polar stationary

phase should be used

and so you would then have a silica

column as shown over here

for charge analytes um

you can

carry out ion exchange chromatography

so

in this example the

analyte is a negatively charged

molecule and its

bonded by positively charged beads in

the stationary phase

so

any positively charged molecules in

uh in your sample will just go through

directly to the waste because they wont

interact with the with the stationary

phase

and by applying a salt gradient or

increasing the ph

you will then be able to elude the

negatively charged ions

and

process the sample further for for

detection

another separation technique is

size exclusion chromatography

here the stationary face

or the column is packed with fine power

speeds

and

these beads have different sizes and

only the smallest molecules will be

captured

by these beads and the larger molecules

will

flow through

and be you know eluded sooner in time

compared to the smaller molecules

carbon columns

[Music]

are also out there when your

analytes contains several enantiomers

so chiral cone in parallel columns

a um

enantiomer of a chiral compound is

bonded to the stationary phase

and the

in your sample will have different

uh binding affinities

for this in engineer

so in this example you see this this

upper enantiomer will bind more strongly

and will be retained longer compared to

this nature mirror

now lets move on to the mobile phase

because here there are also a couple of

variations possible because you can

change the composition

of mobile phase a and b

if the composition remains constant

throughout your method and then this is

called high spread evolution as shown

over here um this

graph shows you

a chromatogram

for uh

an lcu method to detect the albumin

dio and caffeine

and we see that caffeine the peak of

caffeine

occurs later in time because uh caffeine

is more nonpolar

than the other two compounds

this is a reversed phase method by the

way

you can however reduce a runtime by

gradually increasing the percentage of b

meaning that your mobile phase will

become

more and more nonpolar over time

thereby forcing nonpolar compounds such

as caffeine earlier from from the column

so also caffeine will elute early in

time

making

the runtime shorter than an isocratic

idolation method

so gradient allusion is a good method

when a sample contains components of a

wide range of polarities

well after having discussed sample

preparation and analyze separation lets

move on to analyte detection

nowadays

mass spectrometry is the most widely

used detection methods

so ill primarily focus on that method

however still many labs use uv or

fluorescent detection so ill also

shortly discuss that technique

there are

several uv detectors available such as

variable wavelength detector or a diode

array detector

in the next slide i will show you how a

diode array detector works

here a deuterium lamp

emits polychromatic light in the uv

phase region

and

it will flow through the flow cell and

the flow cell is connected directly to

the output of the hplc column

so in the flow cell

the analyte will absorb

light and any transmitted light will

heat the grading which disperses the

light

onto a diode array element

which

measures the intensity of light at each

wavelength

so you would then be able to generate a

uv

spectrum while also

generating a chromatogram at the same

time

uv detectors are

very reliable and easy to use

disadvantages are that they only work

for analytes with chromophoric activity

meaning that the analyte should have

conjugated double bounds

as you can see here for betacarotene

and benzoic acid

also if compounds have the same

chromophores then it can be difficult to

distinguish these because theyll

generate say uv spectrum

so specificity and also sensitivity

is less compared with mass spectrometry

methods

and therefore

ms detectors are nowadays the detectors

of choice

so lets move on to ms

an ms machine consists of

three components

the ion source to ionize sample

mass analyzer

which will separate the ions

and finally a detector

and here you see a selection

of widely used ion source mass analyzers

so lets first discuss electrospray

ionization

here the sample is ionized in the liquid

phase

so

here

the sample will be introduced from the

lc column

and a high voltage is applied

and

here

in spraying nozzle charge droplets are

being formed

ionization can be carried out in a

positive ion mode which

generates

protonated

molecules whereas also negative ion mode

can be carried out in which your analyte

will become deprotonated

now

over here

in this process charged droplets are

being formed

and

eventually the solvent in the droplets

will evaporate so droplets will become

smaller and smaller in size

due to repulsive forces between the

charges

the droplets will become even more

smaller

until a droplet only contains one single

analyte

so the smallest

droplets will then be

separated in the next stage

atmospheric pressure chemical ionization

um

is more effective in iron in ionizing

less powder and smaller analytes than

electrospray ionization

here ionization occurs in the gas phase

so hplc effluent

flows through the nebulizer probe which

is also heated to temperatures up to 00

degrees celsius

the effluent is evaporated completely

and then it enters the ionization region

where a corona discharge needle

ionizes the samples and this can be also

carried out in positive or negative ion

modes

and this entire process is done

under atmospheric pressure

and

the

ion separation processes later on will

be carried out

under high vacuum conditions

when youre dealing with

macromolecules such as peptides or

proteins theyre that are nonvolatile

and fragile then maldi

matrix assisted laser absorption

ionization

is an appropriate ionization method

maldi is a softer technique than

electrospray ionization or apci

so that causes less fragmentation of the

analyte in the ion source

so for maldi

the sample is first mixed with the uv

absorbing matrix

on a metal plate

here you see a commonly used multi

matrices

and what happens next is that a laser

will be

directed onto the sample

this will irradiate and desorb the

sample

um containing analytes and also your

multi matrix

your analyte will then become ionized

and

an electrostatic field will be applied

which will accelerate the ions

into the mass analyzer and typically

the detector is

thats commonly used with maldi is the

type of flight detector as shown over

here

its a tube of approximately one meter

in length

with a detector here

at the top

and the time of light is the measure of

time that

ions need to cross the tube and make it

to the detector

and here low mass ions for which the

detector faster than high mass ions

so

this will lead to separation of the ions

now another mass analyzer and perhaps

most uh wellknown and widely used in

bioanalysis is the triple particle

instrument

a quadrupole is a set of four metal rods

and a triple protocol is three sets of

them in a row so this is q

q

and q

so if we take a closer look at these

rods

they are arranged parallel

to one another and opposite rod pairs

are connected electrically

a radio frequency voltage with the

direct current offset voltage is applied

between one pair and another

and this will create positive and

negative charges that are alternating

and this all leads to an oscillating

electric field

in which only ions of a certain master

charge ratio will be able to travel

through the rods

these are the socalled wrestling ions

other

ions will have unstable trajectories

such as the blue one over here and it

will collide with one of the rods and

will not make it through

the next stage which is the collision

cell

now the resonant ions

that will be that will enter the

collision cell will be bombarded by

neutral gas molecules such as argon or

nitrogen and here fragments will be

created

and these fragments will then be

selected in the next mesh analyzer at q

and only the wrestling fragment ions

will make it to the iron detector

so heres an example of a molecule with

a massive charge ratio of

after fragmentation only this fragment

will be selected and detected

there are several types of scan methods

that can be carried out with a

with an msms

machine

so without fragmentation you can do a

full scan

in which compounds of every mass are

plotted against time

a more

specific way of

you know selecting ions is to do a

selected ion monitoring scan in which

the ms is said to scan over a very small

mass range

for quantitation purposes

multiple reaction monitoring is most

often carried out

here you specifically monitor a

transition from

a

parent ion to a specific fragment ion

and normally you would just see one big

ear chromatogram so this is a very

specific and also very sensitive

scan method

now the last

type of nas analyzer ill discuss

is the iron trap

as the name suggests this instrument is

used to trap ions inside the mesh

analyzer by using electric or magnetic

fields

here you see the

different types of iron traps that are

out there

for the sake of time i will only

highlight the linear iron trap

which is shown over here

so here uh the linear iron trap is

a set of three quadrupler rods

um

so there are three sections the front

and center and the back section

and ions are trapped by an electric

field

they are confined radially by a

twodimensional radio frequency field

thats created by an alternating current

and this current is applied to all of

these three segments

in the actual direction

a

direct current potential

is applied

and

this will create a socalled potential

well

and this also makes sure that the ions

cannot escape the iron trap

heres an example of

how a chap i would look like

now you can

use the iron trap as a

selection device by capturing only the

ions of interest and ejecting the

ions that are not important for your

analysis

you can eject ions by gradually

increasing the radio frequency voltage

which makes the ions

unstable so then theyll be able to to

leave the trap through one of the

ejection slits

the trap ions can be

characterized further by by fermentation

you can do this by introducing

ventilation gears such as helium

and well this process of ejection and

fermentation can be can be repeated

several times and this is called

multistage fragmentation

so heres an example of a multistage

fragmentation experiment

um here the drug byzantine is being

fragmented it has apparent massive

charge ratio of

um after fragmentation um

four

fragments are observed in this product

ion scan

and each of these four fragments can

then again be isolated and fragmented

so you would then be able to obtain

these ms spectra which are shown over

here for the three major fragments

most commercial insurance can repeat

this

process several times sometimes to the

0th or the th level and this is a

very useful

instrument for qualitative analysis for

example if you need to identify

metabolites of the drug

so we just discussed

several mass analyzer

it is possible to combine these

analyzers to increase the functional

capabilities of your method

just to give you an idea this figure

shows you

commonly used hybrid configurations in

bioanalysis

so the simplest configuration

is tandem mass spectrometry

with the three particles

in some machines the third particle can

also function as a linear iron trap so

then you have a portable

linear ion trap

and here i just some other examples of

other combinations

now

i just want to make sure that everyone

understands difference between a

chromatogram and a mass spectra so ill

therefore show you this graph

a chromatogram

shows the retention time on the

horizontal axis

and peak in density on the yaxis

so it shows you

the chromatographic separation of your

penalize over time

at each moment in time

you can also

look at the different master charge

ratio that are present

and then youll have a mass spectrum

where you have the massive charge ratios

on the horizontal axis and still on the

vertical axis peak intensity

so a mass spectrum shows you which ions

are

most abundant at a certain point in time

and this spectrum can be used to

elucidate the chemical identity or

structure of your compounds

so we just discussed chromatographic

assays for large molecules such as

proteins and peptides ligand binding

assays can also be used for quantitation

in biological matrices

so in these essays quantitation is based

on the analyze affinity for ligands such

as an antibody or an antigen

there are differences with

chromatographic assays

for example very often its hard to

isolate these large molecules from the

matrix

therefore ligand binding assays are

often run

without

separating the analyte from the matrix

also the analyte is measured indirectly

by

monitoring the binding reaction with sa

reagents

and the dynamic range of assays of these

ligand binding assays are narrower

than chromatographic assays

here we see the different

ligand binding as essay platforms

the

widely used and

wellknown elisa is the most commonly

used platform

and within the elisa platform there are

several subtypes such as direct

competitive sandridge and the bridge

eliza

in the next slide i will highlight how a

sandwich eliza works

so here

the wells of the elisa plate are

precoated

with a capturing antibody

then

when the sample is is added

the analytes will be captured by this

antibody

and the next step is to add a second

conjugated antibody

shown over here and this second antibody

will bind

to the analyte

at a different binding site

so

you will create basically a sandwich

the conjugated enzyme is very often

horseradish peroxidase

and after adding its substrate tmb

blue color formation will be observed

this reaction will be stopped by an acid

stop solution and the blue color will

turn into yellow and the intensity of

the yellow color can be measured on a

plate reader at 0 nanometers

so for each experiment a calibration

curve will be

generated

and well after measuring your steady

samples

you can calculate the absorbance

to the concentration and

you would have your results

a sandwich elisa is very specific

because

the capturing and the detection steps

require very unique epitope recognition

to generate an essay signal

all right

so

after completing method development you

need to assess the performance of your

essay and this will be done during

validation

i will already apologize because this is

a very boring topic but its also very

important and therefore i think you

should know about this

so bioanalytical method validation

is based on procedures and criteria

outlined

in the fda guidance

last version of the guidance was

published in 0

and its required for pivotal studies

that are submitted in drug applications

that need

regulatory decision making

during method validation several

questions are addressed such as

uh will the mesh will the method measure

the intended analytes

so specificity and selectivity are for

daily parameters

whats the variability

with the measurements and also how

accurate are the are the measurements

whats the range

of your essay that provides

reliable data

and how stable

is the analyte during sample collection

sample handling and storage so several

stability experiments will be done

here

you see

several validation parameters

and there are also

some post validation runs that i will

cover in the next section

so first

i will explain to you the difference

between calibrators and quality control

samples qcs

cad operators

its basically biological matrix to

which a known amount of analytes

has been added

and these samples are used to construct

calibration curves

as shown over here which runs from the

llc to the uloq the upper limit of

quantitation

calibrators need to be prepared freshly

before each run

and then the quality control samples

these are also

biological matrix samples to which a

known amount of analyte has been spiked

and these samples are used to monitor

the accuracy and precision of the method

and also determine instability of the

samples

quality control samples

they span the

calibration curves

so

qc samples need to prepare at the very

at the lowest level the llq

then there are qcs at the lower range of

the curve midrange

and at

the higher range

and

these qc samples need to be prepared at

least

freshly for for one run

then it is acceptable acceptable to

freeze a batch of qc samples and thaw

as subsets of these samples for

subsequent validation runs

calibrators and qcs are prepared from

separate stock solutions

that are prepared by doing separate

ratings

and they must go through the exact same

sample preparation procedure as the

study samples

here here you see a list of the fde fda

criteria for the calibration curve

for both chromatographic essays and

ligand binding essays

for the sake of time i will

primarily focus

uh on the chromatographic criteria

uh if you are interested in the ligand

binding criteria please feel free to

review these tables at your own pace for

read the fda guidance

but for now let me just discuss the

chromatographic

criteria

so

cataracts that need to be included

are blank samples that contain no

analyte and no internal standards

its a zero calibrator which only

contains internal standards

and then at least six nonzero

categories

need to be run

ranging from the lower limit of

quantization up to the copper limit

calibration curves for chromatographic

edges are typically linear

and you should use the simplest

regression method as possible

a nonrated progression

doesnt work then it is possible to use

a weighted regression effect as shown

over here

of calibrators and a minimum of six

nonzero calibrators need to be within

of the nominal concentration

the lower limit of

quantitation

sample can

deviate

and dimensionals of 0

calibration curves for ligand binding

assays are typically nonlinear so a

four or five parameter logistic metal

model is is used

and well the

other criteria for calibrators and

decombining essays are shown over here

so lets move on to accuracy and

precision

precision

is a measure of how closely the

measurement results are to each other

so in this example

precision is very good but accuracy is

poor because accuracy shows the

proximity of the results to the true

value

so you want to have a method thats both

precise and accurate

so you want to have this

this this this

result accuracy and precision

need to be validated in at least

three independent runs for

chromatographic essays

in each round four qc levels need to be

included

and there need to be at least five

replicas

at each level

the concentrations that are measured

need to be within or 0 percent of

the nominal values

and also precision need to be within

or 0 percent

selectivity and specificity

will assess how well the analyte can be

detected and measured in the presence of

other matrix components that can be both

endogenous and exogenous such as other

drugs that a patient may be using

so when validating seductivity

at least

matrix from six individual sources needs

to be spiked with the analyte at the llq

and

the values the concentrations need to be

within 0

of the nominal value

also there shouldnt be any interference

at the retention time of the analyte and

internal standards

and the response of the internal

standard need to be

pretty consistent so within five percent

of the average is response in

calibrators and qcs

in ligand binding essays at least 0

individual sources

need to be tested and these should also

include lipamic or hemolyzed samples

and an additional qc levels is also

validated for ligandbinding assays

so specificity then it focuses on the

interference of crossreacting molecules

other drugs that may be present or

metabolize in your samples

here

you will spike samples that contain any

of these expected interference

components and you spike these samples

with your analyte at the llq

and results should again be within 0 of

the nominal values

and well the interference and the is

response criteria are

similar as what i showed you in the

previous slide on set activity

carryover can be

a big problem

in liquid chromatography method

it happens when there are leftovers of

the analyte

somewhere in the chromatics

somewhere in the chromatographic system

which can be the column or the sampling

needle in the auto sampler

and you will observe carry over when you

see a peak

in a blank sample directly after

injection of a high concentration sample

and this has negative impact on the

accuracy of your measurements

therefore you should

minimize carryover during method

development

so the fda criterion on karyov is that

a peak in a blank sample

after injection of a high concentration

sample

should not exceed 0

of the response of an llq sample

so

this in this example

you see that theres a very small peak

in

the blank sample which is well below the

twenty percent response of of an ll qp

so this is an acceptable acceptable

result with regard to carryover

now recovery describes the efficiency of

your extraction method

how much loss is observed

and it is calculated by dividing the

response of extracted samples

by both extraction blanks that are

spiked with the analyte so this number

represents 00 percent

recovery every coffee doesnt need to be

very high or it doesnt need to be 00

percent more importantly is that it is

consistent and reproducible and it needs

to be validated at at least three qc

levels

then

there are several stability experiments

that need to be carried out

benched up stability which assesses

stability in an unprocessed sample

then process sample stability

stability after three free start cycles

stock solution stability and long term

stability experiments will need to be

carried out

and

qc

concentrations at the low and the high

level

should be within of the nominal

concentrations

well after successful essay validation

you can go ahead and run your study

samples

you typically run the samples in the

following order

first calibrators

need to be injected followed by study

samples by default in chronological

order

and then lastly qcs

will be

injected

at least

and a minimum of six nonzero

calibrators need to pass

meaning that nonzero calibrators need

to be within

of the nominal values and loq

qcs need to be within 0 percent

for the qcs

at least percent need to be within

the 0

range and per qc level

more than half of the qcs

also need to be within

you will also look at potential

interference because there shouldnt be

any interfering peaks at the retention

time of the analyte and internal

standard

if results are below the llq these

should be reported as pql

if

constraints are

higher than your calibration curve then

you need to dilute and reanalyze these

samples

another option is to extend and

revalidate the standard curve

to verify the reliability of reported

analyte concentrations in study samples

a portion of the study samples are

reanalyzed and this is called incurred

sample reanalysis or isr

and this is expected for new drug

applications

so for isr

0

of the first 000 samples need to be

reanalyzed

and percent will be reanalyzed of any

remaining samples

and samples will be selected around the

maximum concentration and also in the

elimination phase

so you will then do the following

calculation

you will subtract the original

concentration from the repeated

measurement and divide this by the mean

value of

above both

values and

multiply this by 00

this will

give you the difference percentage of

difference from the mean values

so for chromatographic essays percent

of the samples need to be within 0 of

the mean

and

for ligand binding essays

um

or

of the samples they should be within 0

of this mean value

there are different types of validation

if you do all

validation experiments then youll do a

full validation also partial validation

is possible

which can vary between

just doing one accuracy and precision

run up to a nearly full validation

partial validation needs to be done when

a method is being transferred between

labs

or when there are any changes in for

example

analytical detector

a change in sample appraised procedures

or if sample volume or matrix change

slightly

another type of validation is

crossvalidation which is done when two

different analytical methods are used to

generate data for the same study

or you can also have this situation in

which

two or more labs

use the same method to generate results

for one single study so then you need to

assess interlaboratory reliability

crossvalidation is carried out by

analyzing a shared set of qc samples

and its also deposited to its also

possible to analyze

nonpooled or pulled subject samples

and

the difference between

the test method and the reference method

should be very close to to each other

the best requirements for

crossvalidation

it is important to note that its not

always necessary or feasible to conduct

a full fda foundation

most importantly is that the level of

validation needs to be appropriate for

the intended purpose of the study

so if youre doing an exploratory study

then

you can have less stringent validation

requirements

so you could get away with a reduced

number of analytical ones or replicas or

also

includes or reduced numbers of

validation parameters

now then about

ligand binding essays because these are

often available as commercial kits and

these kits they were typically designed

to diagnose a condition in patients and

later on they were repurposed to

measure analyte concentration

concentration from the kinetic and

pharmacodynamic studies

sometimes these kits are fine and dont

require additional validation

but sometimes you need to do additional

validation runs for example if the

calibration curve only consists of one

or two calibration points

or if the actual qc concentrations are

not known

because concentration ranges are not

acceptable

additional validation is also needed if

categories and qcs

are prepared in a matrix gets different

from your study samples

or if the reference standard is

different from the analyte in your

samples

so then you need to do additional

validation experiments

so let me

end with an example of how we developed

an essay at roswell park for encircamite

and human plasma

and salutamide

is an oral drug

used in the treatment of castration

resistance prostate cancer

it competitively inhibits androgen

binding to the energy

receptor and we developed this assay in

support of a phase d study

in which ensolutamide

was given with or without giraffe in

patients with hepatocellular carcinoma

at the time of essay development

there were already three published

essays

and so these were pretty helpful for us

in the essay development process

here you see the metabolism of

enzolitamide its metabolized by

cytochrome p0 enzymes in the liver

uh to

the m metabolite which then is being

metabolized into n dash material and

solidamide or m

and this is the major active metabolite

therefore

we included also this metabolite in our

assay

as we normally do for drug assays and

plasma we use one of our triple particle

mass spectrometers

and protein precipitation was used here

as the sample preparation method

so 0 microliters of plasma was mixed

with 00 microliters of

verbal solution that contained the

internal standards

we used

the structurally analog

epilidamide as an internal standard

because

radio labeled insulitamide was not

wasnt stable in our lab

so after vortex mixing and centrifuging

supernatant was transferred to a 9

plate

then samples were diluted

vortex mix centrifuge

and finally one migrator was injected

into the lcms system

and salutamide is a nonpolar compound

therefore we carried out the first phase

chromatography using a c column

here we see the composition of the

mobile phases

and we applied gradient dilution

total runtime was 9 minutes

so ionization of

the sample was done

using electrospray ionization

in the positive ion mode

and here you see the

transitions that were monitored

and just to give you an example the

fragmentation pattern of insulinite is

shown over here and we monitor the 09

fragments

so here you see the chromatograms at the

lower limit of quantitation 0 nanograms

per mils and solution might elude it at

0 minutes

and destination and ludamite is slightly

more polar therefore

alludes a little bit earlier at 9

minutes

and the internal standard had a

redemption time that was pretty close to

that of enceladumite minutes

here you see an output of the massback

software

showing you the calibration curve of

insulator mites running from 0 up to 0

000 nanograms per mils

and here are the validation results so

for accuracy and position

four qc levels were tested

and we see for ensolutamide

that the precision

represented by c percent was

within

0 and within

and also deviations from nominal

concentrations were also well within the

or the 0 requirements

and similar results were observed for

for the metabolite

so accuracy and position were validated

selectivity was

assessed by

spiking blank plasma samples from six

individual sources

and

we see at the llq that deviations from

nominal concentrations were well between

the 0 requirements

and also we didnt see interferences in

the blank samples at the redemption time

of enzalutamide and the metabolite and

internal standards

in recovery

recovery for both analytes was

consistent

and was very high greater than 9

percent

at all qc levels

and

this was also true for new metabolites

and we can also see that the variability

between samples was also very very low

so recovery was consistent and

reproducible

and finally

disability results we see that

after doing benchtop

stability process sample stability and

stability after prestart cycles

we see that for the low and the high qc

levels the deviations from normal

concentrations were all within the

requirements

and also for the metabolites

deviated phenomenal were all within

percent

so

in conclusion this essay was validated

successfully and was used to measure

samples of the interaction study

i would like to conclude

with these figures showing you what you

can actually do with a validated essay

because the

concentrations that will be

measured can be used to generate these

concentration time curves as shown here

for enzoludamide

and the metabolite

and these concentrated curves

are very handy to obtain important

pharmacokinetic parameters such as the

area under the curve

which

gives you

a measurement or an indication of the

systemic exposure of the truck

you can read off the

maximum plasma concentration

and

the terminal slope can also be used to

calculate elimination halflife

so

this concludes my talk

i hope you now have a better

understanding of the importance of

bioanalysis

the different

types of essays and the process of essay

development and validation

please direct any questions you have on

this lecture to the course directors

and thank you very much for your

intention