hello uh my name is bob parise and today

a quick a quick introduction to

lc mass spectrometry

uh explain a little bit about the the

equipment

uh

run some compounds through here uh also

were going to talk about

sample preparation

and then at the end

well go through some data and do some

data analysis

so what we have here

is an hplc mass spectrum

mass spectrometer and this part here is

a

hplc

and

over here

is the mass spectrometer

so the

role of the hplc

in this process is to delete two things

is one to deliver solvents

and

compounds

uh

to the

mass spectrometer uh

also

using a column in the hplc we are able

to separate compounds

the hplc consists of three r each blc

consists of three parts

uh

a pump which is located on the bottom

an auto sampler which injects samples

and

lastly a comb compartment

that has a column inside which is used

to

have some retention and also

separate compounds

so

the way it works is the pump on the

bottom pumps mobile phase

which is located on top and today well

be using acetonitrile

and water that has formic acid that

helps ionize the compound

thats drawn into the pump

and

then

goes into the pump and then comes out

this uh the mobile phase will be flowing

through here at a flow rate of 0 mils

per minute

into the auto sampler

uh the job of the auto sampler is to

inject the samples

we keep our samples in either

these

bile

plates

or we also have

can use

9 or well plates

theres a needle here uh when you set

the machine to run it will

pull the sample tray out the needle will

come over pick it itll pick up and then

make an injection once that happens

uh this the mobile phase and the sample

will travel through to the column

compartment

where you can we have the option of

heating

the mobile phase if we have to

and it will

then go into the column and based on the

chemistry of the compound

and the column

it will uh slow down and elute

slower than the mobile phase coming

going through this enables us to

uh

separate if we have multiple compounds

were able to separate them based on the

chemistry of the

compound

the type of mobile phase were using and

also the stationary phase inside the

column so once it goes through the

column

it will the mobile phase will travel

into the mass spectrometer

uh and the master drummer has

uh four

different uh

main pieces to it one is the source

where the mobile phase

which enters is a liquid is ionized

heated and turned into a gas

its the

the area where our compound will be

ionized for detection

once it goes into the source

it will

be drawn into the mass spectrometer

which would has

two quadrupoles separated by collision

cell

so what were going to do today

is were going to do what they call

multiple reaction monitoring

uh where we will select

uh a mass

that would be the mass of the

our precursor or our compound

and well use the quadrupole number one

to

lock in on that mass and well only

allow that mass to come through

and then that will enter the collision

cell and then in the collision cell

theres energy and gas and we will

adjust those parameters

to fragment the compound into

uh product ions

and then we will after that everything

is optimized we will be able to

set up our hplc mass spec

method to detect only

uh

compounds coming through

that have that specific

precursor

um

product

transition that gives us a very specific

way of detecting things

so the first thing were going to do is

to tune in our compound and the compound

ive selected is

lmp which is an nci compound

and

uh well tune on that

both that compound and its deuterated

internal standard which is essentially

the same compound where

but its three mass units heavier

heavier

the three of the hydrogens were are

replaced by deuterium so chemically it

behaves exactly the same way

in the mass spectrometer except for

being three mass units heavier

so we have

i prepared a syringe

that has

one microgram per ml of both and its

deuterated

uh internal standard i will turn that on

and well go through this

this line here is a liquid

at seven microliters per minute and at

this point im only going to use the

hplc for solvent delivery the only thing

im gonna do with the hplc is set the

flow at 0 mils per minute

and that will come through here

and it will meet up with our

two compounds we have in here

with this t

and that will give us a continuous flow

of our compound and that will allow us

to

change the parameters of the mass

spectrometer to uh optimize the largest

uh m plus h ion

so ive just turned the

mass spectrometer on on tuning mode

and

the mass of our compound is of lnp four

uh seven four is

and its internal standard duty rated

internal standard is

in this case were ionizing it with

a proton

so

we end up with a massive charge of

and

and what i do is i change the different

parameters

in the source region of the mass

spectrometer to optimize to get the

largest uh

uh

precursor or compound scans here so we

have a number of things like change

theres curtain gas theres an iron

spray voltage

a temperature to

turn the mobile phase from a liquid to a

gas

and theres also two other gases that i

apply

and then theres also the clustering

potential

that helps to

free our compound from any uh

clusters that may form with uh the

solvent

thats thats in there

so i have uh optimized this already with

this and you can see

i have

two nice peaks here uh so thats our

precursor

uh thats our m plus h ions the next

step i want to do is to

uh

have these uh

fragment these compounds in the

collision cell and look for fragments of

these compounds

so what i will do here is i will stop

this

requiring i will change it from a q

scan

and then i will

use a product ion scan and i will take

the mass i have here and entered here

this will let our first quadrupole will

lock in on that mass

and then the compound will be fragmented

in the collision cell and i will set the

second quadrupole to scan for any

fragments

so we can see here this is our precursor

scan this is lmp

plus a hydrogen which is

and by adjusting the collision voltage

in the

collision cell

by raising it

we can see that i get one major uh

product ion scan

so this is a what we call

a precursor product scan mass transition

once i have this and i have all these

numbers here

i go ahead and i put this into our data

acquisition

and then when we do an injection where

we inject samples

uh it will look for the mass

spectrometer will look for

uh

species that only have this particular

characteristic that means it has to have

a

a precursor

mass of and then a product line of

9

i so this is for the

our our compound and i can change it for

our internal standard

which is three mass units heavier

yes

its four mass units heavier i just saw

that there

sorry for that mistake its actually

but the same uh product eye of 9

and one once i had like i said i want to

have this information i will go ahead

and put this

into

our data acquisition

program okay so once that we have the

tuning parameters established

uh

the second

part to develop the method would be to

actually do the chromatography

you want to develop a method thats

thats reproducible

uh

thats

quick it doesnt have a long run time

but also accurate

so

we do the chromatography part of this

now is so now the tuning is done

and were going to actually

you know you have to inject samples and

be able to see them

so

i set up a method here too that will use

acetonitrile and water

pumped like i said earlier through

through the column it picks up the

sample and then i can vary the

percentages of both the water and the

acetonitrile over time

to move my

peak where i want it to come out

and

ive done this

earlier

and heres an injection that we already

did

and

in so the xaxis is time

and

the sample is injected at time zero

and our compounds come out

at

minutes

with using this method

uh

and

its monitoring both compounds here

so i can separate them and look at them

and you can notice that the

uh

the internal standard comes out

the internal standard on the bottom

comes out exactly the same time

as our

compound of interest

this is crucial in mass spectrometry

because

whatever happens things in the source

region where its ionized

can change

uh fluctuations in power and the

building or

what have you in there uh having

something that loots at the same time

uh

allows compensation for

differences in ionizations

so we have a method here

where

uh our compound comes out at

so once we have our

method where actually

hplc method were actually able to then

start to

process samples and analyze samples

okay so once once we have our lcms

method and were able to detect the

compound

the next step is able to we have to be

able to take our compound out of plasma

or any other biological

matrix that you might want to examine

but today im going to

uh

do plasma

so

theres different types of extraction

techniques that they they are techniques

to try to get your compound out of

plasma

uh

so you

that makes it cleaner for the mass

spectrometer

uh so we have four uh different methods

that we use here in this laboratory

uh

three of them are using liquid and then

over here

is a solid phase extraction apparatus

so i usually generally with the new

compound come to study i will

i will try different techniques to see

what our best extraction efficiency is

and today were going to use

acetonitrile

uh

terpedo terpenomethyl ether

dichloromethane

and im going to show you just quickly

this

three different solvents

test im putting 00 microliters of

plasma in each one

and i will follow that with

different types of

solvents the first is a c nitrile

and you can use a c nitro for one to two

methods

one it would be a dilute and shoot and

thats the easiest method you would

simply add it to your sample

centrifuge

your sample take off the supernatant and

inject that into the mass spectrometer

or you could uh blow it down

take the supernatant off blow it down

and then resuspend

the second one is tbme terbutal methyl

ether

the third one is dichloromethane

i want to show you the difference with

these

fum the plasma

so with the asean nitrile

uh

that acts to precipitate the proteins

where they come down to the bottom after

center after vortexing and centrifuging

it it turns out like this

you can see that

and you simply

can take the supernatant its one one

face

here now like i said you can either

inject that into the mass spectrometer

with some methods i do that or if i have

to concentrate the sample what i can do

is blow it down

uh in an end of app which ill show you

a little later

and then resuspend it makes a more

concentrated sample

the ether sample

forms two layers

you can see our plasma is on the on the

bottom

plasmas head heavier than ether

and the

ethers on top

so

depending on the chemistry of the

compound if it likes the ether

the goal is here is to try to get as

much of the compound out of the plasma

into the ether phase

that that can be blown down later

and then lastly i have dichloromethane

and this is a messier thing i do not use

this as much anymore

as much anymore with mass spectrometry

as i did when i used to do just hplc

but you can see our plasma is on top

and now to get to our

i know that what we want here is to have

the compounds come out of the plasma

into the dichloromethane

you can see this is a little more

difficult because you actually to get it

out you actually have to put a pipette

through the plasma

to the bottom layer blow out some of the

plasma and then aspirate

the

chloromethane off

lastly what i have is a solid phase

extraction apparatus which is here

and

we can also do dilute and shoot or

do protein cleanup

with this

you can see the solid phase extraction

on the bottom this will do 9 samples at

once

and i can collect it by vacuum

into a 9 well plate

for further processing or direct

injection into the mass spectrometer

okay for todays example were using

seven like i said lmp and this

was developed this asset was developed

about eight years ago when we had an

older mass spectrometer that didnt have

the sensitivity of the mass spectrometer

i showed you today

uh if i would do this method over i

would do it probably at a c a nitrile

diluting shoot where i would add a c

nitride like this to the plasma

vortex it

spin it down

and inject it straight in the mass

spectrometer but

our older mass spectrometer didnt have

that so we had to concentrate our drug

that we would use

uh 00 microliters of plasma

and then wed use tert butyl ether

and

extract the compound that way and then

blow it down

uh and then resuspended 0 microliters

so youre making it four times more

concentrated

uh

that we needed that with the older mass

spectrometer it didnt have the

sensitivity of the like i said the one i

showed you today

i showed different extraction technique

and like i said today were going to use

the

mtbe methyl therapy

butyl ether extraction technique and

once uh

we

add the plasma and the ether the next

step would be the

vortex

the sample

i usually do that for one minute

and then when thats

when thats done i will

centrifuge using a centrifuge put the

upper dwarf tubes into the centrifuge

for five minutes at 000

times g

and then whats nice about the ether you

said there was two different layers

theres the plasma layer thats on the

bottom

and the ether layer on top

well what i do then is

i can freeze that sample and it only

takes a few minutes and minus 0

and once thats done

we have here

is a

plasma thats frozen on the bottom but

our ethers is remains

uh liquid

and i can decant that

into a glass tube

or pipette

and then i place the tube

into an apparatus thats called an

enevap

and what the

purpose of this instrument or this piece

of equipment

is to

dry your sample down

by turning it on i will

blow nitrogen through these

needles and that will blow

or that cause our ether to evaporate

okay so we

weve

glued down our sample as we evaporated

the

uh ether from the sample in the end evap

and heres our

tube and what we need to do is resuspend

it

and so i will

take some acetyl nitrile water in this

case 0 percent of c nitrile 0 water

00 microliters of that

add it to our tube

and then vortex it

because vortex

and now we have to

transfer

00 microliters

to an hplc vial

so now we have our sample

thats been extracted from plasma

its now an hplc vial

and its ready for injection

okay

so

now i have my hplc valve

and i will place that into the

auto sampler rack

and now the sample is ready to be

analyzed

i then turn my hplc on

set up my sequence table

and inject my sample

so im developing a method

once i have the

particular extraction technique thats

suitable that is reproducible

and

it gets the greatest extraction

efficiency

im ready to start to validate the

method

and

then

i can

do that first by preparing a standard

curve

and

in this case

we have

our standard curve will be from one

nanogram to a thousand nanograms and i

also make quality control samples

uh

and i believe

ours are two nanograms 0 nanograms and

00 nanograms

per mil

i prepare my standards and then i

pipette just as i did before 00

microliters of plasma in all my tubes

the different standard concentrations

and the qc concentrations

and then this time i will i add

internal standard which i will do that

first here

now i add internal standard to every

sample except the blank

and like i said before the internal

standard is used

sort of type to normalize

uh the data so whatever happens to our

compound happens to internal standard

for instance

if i were to spill some of my

sample during the preparation

i will spill

a proportion of internal standard the

same amount if i lose 0 microliters i

will lose internal standard and i will

lose

my compound

but in the end when we quantify the data

that will normalize each other

also accounts for

difference in ionization like i said

earlier if theres a problem with the

ionization are not a problem actually

but if fluctuation in building power or

when that when it first

enters the source whatever happens to

the compound will happen to the internal

standard

i added the internal standard so yes

when we do the standard curves qcs and

patient plasma

uh unknowns and internal standard each

one add our plasma in this case we add

our

uh t uh

tbme or ether

and process them like i showed earlier

uh by vortexing the centrifuges and

blowing them down and putting them in

hplc valves

uh

we set up our run

and in this case we i ran one standard

curve and qcs

and what were looking at here is the

one the one on the left

is our actual compound this is our

and this one here is our internal

standard

and if you can see this here we ran one

through a thousand nanograms per mil and

then i ran two qcs at each level

and what were looking for here

what the program will do was is actually

it will take these areas

and it will

takes the area of our

uh standard in this case this ones 0

gram per mil and divides it

uh with the area of the internal

standard

and that gives you an area ratio so as

you go up

uh in concentration the area ratio goes

up

and the program will do a linear

regression

in this case its one through a thousand

and what we want to do here

were looking for here is actually the

accuracy of the back calculated

concentrations

we want it to be between

and

and we can see in this case they are

theyre all in that area so that that

shows that this

this uh assay is

is linear for this drug from one

nanogram per ml

to

a thousand nanogram per ml and when you

have an unknown sample

it will take an area of that unknown

sample of the

compound in this case seven for four and

it will

do the same thing as it does for

standard curve in that it will divide

the area of the unknown

against the internal standard

and that will give you a number

on the yaxis

and that will calculate into our

standard curve and it will give us a

concentration

well thank you for your attention and i

hope this brief demonstration uh

help expand a little bit of your

knowledge on

lcms as far as

analyzing patient samples thank you