Advance Analytical Course

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Lecture No. # 09

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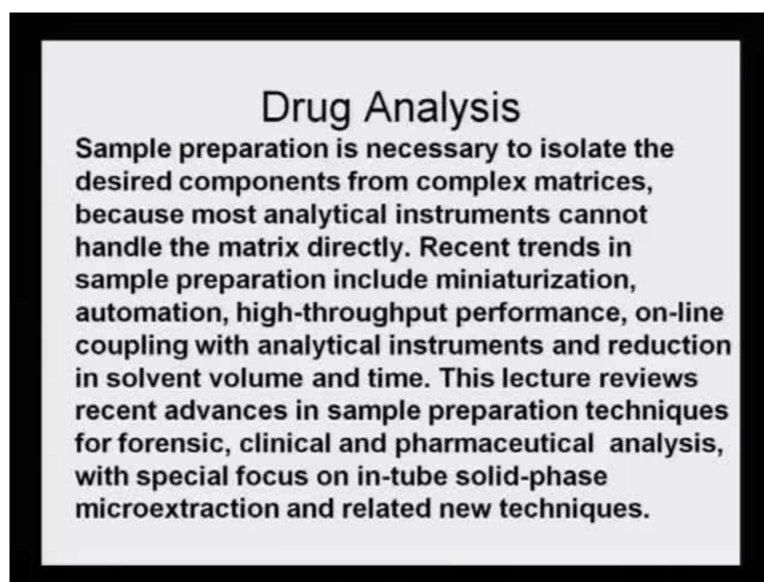

This is again a very important field, where analysis plays a very vital role. Drug analysis, for example, the sample preparation is necessary to isolate the desired components from complex matrices, because most analytical instruments cannot handle the matrix directly. So, it is important to take the drug out of the matrix and then analyze it. Recent trends in sample preparation include miniaturization, automation, high-throughput performance, on-line coupling with analytical instruments, and reduction in solvent volume and time. Because it is again a problem if too much of solvent is used in sample preparation, that solvent has to be removed at some stage.

So, the latest trend in drug analysis has been that it should have automation; it should have a high-throughput performance. That means, the analytical data derived from the analysis should be of high quality, and if possible, if an on-line coupling with any in analytical instrument can be done, it would reduce the time. This lecture reviews recent advances in sample preparation techniques for forensic, clinical and pharmaceutical

analysis, with special focus on in-tube solid-phase microextraction and related new techniques.

Now, you will notice that as we are proceeding from one lecture to another lecture, new techniques are being introduced to you. It is because the necessity or the requirement of that particular substrate gave rise to the generation of this new technique. What is the necessity?

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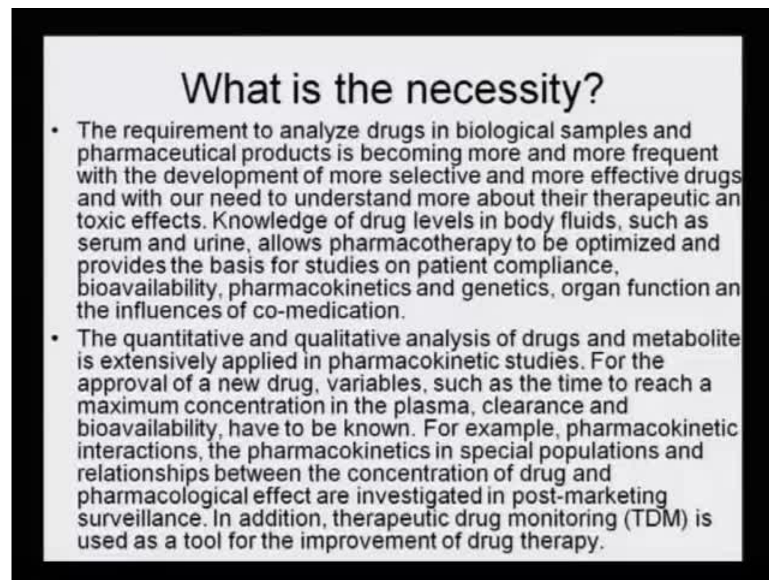

Now, why a new technique should be at all used? That is a very basic question. Because if there is no necessity, there would not be any research; we all know that the mother of research is the necessity. If necessity does not exist, research will not take place. So, the same way, the requirement to analyze drugs in biological samples and pharmaceutical products is becoming more and more frequent with the development of more selective and more effective drugs, and with our need to understand more about their therapeutic and toxic effects. So, knowledge of drug levels in body fluids, such as serum and urine, allows pharmacotherapy to be optimized and provides the basis for studies on patient compliance, bioavailability, pharmacokinetics and genetics, organ function and the influences of co-medication.

Now, it is important, because if too many drugs are being given to a patient, how these drugs are reacting on its body, what is the metabolic pathway, how they are interacting among themselves, is also very important and what should be the dosage for a person?

Now, you know for sure that when small children have to be given medicine, they are given in much milder dose as compared to adult, because their metabolism does not allow to be able to handle a very high dose, and it could even reach a toxic level for them. So, that is why it is important to understand the necessity to be able to analyze these drugs.

The quantitative and qualitative analysis of drugs and metabolite is extensively applied in pharmacokinetic studies. For the approval of a new drug, variables, such as the time to reach a maximum concentration in the plasma, clearance and bioavailability, have to be known and understood. For example, the pharmacokinetic interactions, the pharmacokinetic in special populations and relationships between the concentration of drug and pharmacological effect, are investigated in post marketing surveillance. In addition, the therapeutic drug monitoring, that is, the TDM, is used as a tool for improvement of drug therapy. So, there is a whole lot of new science that needs to be understood when we are analyzing drugs.

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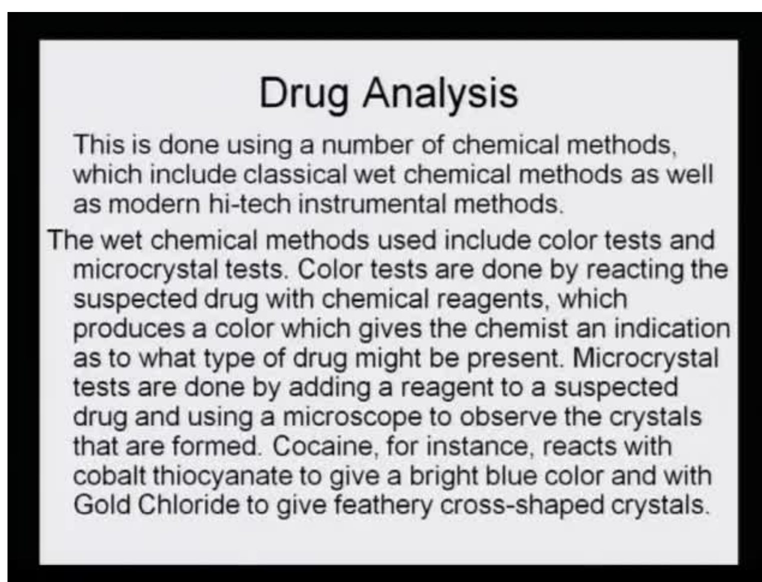

Drug analysis – this is done usually by using a number of chemical methods. No analysis can actually be carried out - chemical analysis - without using any instrument. So, that is of course, very well understood and has been clear from the very first lecture. And these include classical wet chemical methods as well as modern hi-tech instrument methods. So, either we do it by chemical analysis, or we do it by using chromatographic

techniques or spectroscopic techniques. Some instrument has to be used in order to be able to analyze the drugs.

The wet chemical methods used include color test and microcrystal tests. Color tests are done by reacting the suspected drug with chemical reagents, which produces a color, which gives the chemist an indication as to what type of drug might be present.

Microcrystal tests are done by adding a reagent to a suspended drug and using a micro scope to observe the crystals that are formed. Cocaine, for instance, reacts with cobalt thiocyanate to give a bright blue color and with gold chloride to give feathery cross-shaped crystals. So, you see that there are certain reagents, which can give color test with the required drug that needs to be analyzed. And one example that has been given to you or shown to you is the example of cocaine. Now, if cocaine is present in a particular sample, if cobalt thiocyanate is added, it will give the bright blue color, which ordinarily will not be present if cocaine is absent. So, that means that the formation of blue color itself is an indication that cocaine is present. So, this is simple wet chemical method; one of them to be able to explain to you what wet chemical methods mean. It is simply that in the matrix or in the sample, a reagent is added, and it should give a required color as what has been prescribed to show the significance that the particular compound is present.

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Main Instruments

The instrumental tests used include gas chromatography, mass spectrometry, and infrared spectrophotometry. The data from these instruments is handled by computers, which not only organize and print hard copies of the data, but also have databases that can be searched and compared to spectra of unknowns in order to aid the chemist in identifying unknown substances

Main instruments that are usually used in drug analysis are the gas chromatography, mass spectrometry and infrared spectrophotometry. The data from these instruments is handled by computers, which not only organize and print hard copies of the data, but also have databases that can be searched and compared to spectra of unknowns in order to aid the chemist in identifying unknown substances. When I was covering the lecture GC and GC/MS, I told you that there are softwares available, which give the different libraries of compound, like one of them is a NIST library for GC. And it is a software that needs to be bought and that is loaded on the computer, which is connected to the GC machine. When an unknown sample is run, it is compared with the known GC or chromatogram; or if it is a GC/MS, the spectra and the spectra matching or the chromatogram matching helps us to identify the unknown compound.

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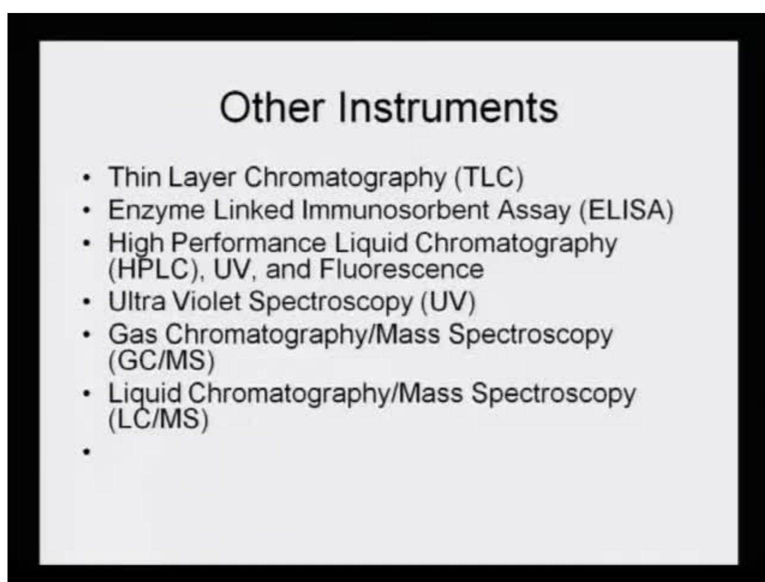

Other instruments that are used for drug analysis are Thin Layer Chromatography (TLC); Enzyme Linked Immunosorbent Assay (ELISA) test - you must have heard about this; High Performance Liquid Chromatography (HPLC) with either UV or fluorescence detector; Ultra Violet Spectroscopy; Gas Chromatography with Mass Spectroscopy, that is, GC/MS; and Liquid Chromatography with Mass Spectroscopy, that is, LC/MS.

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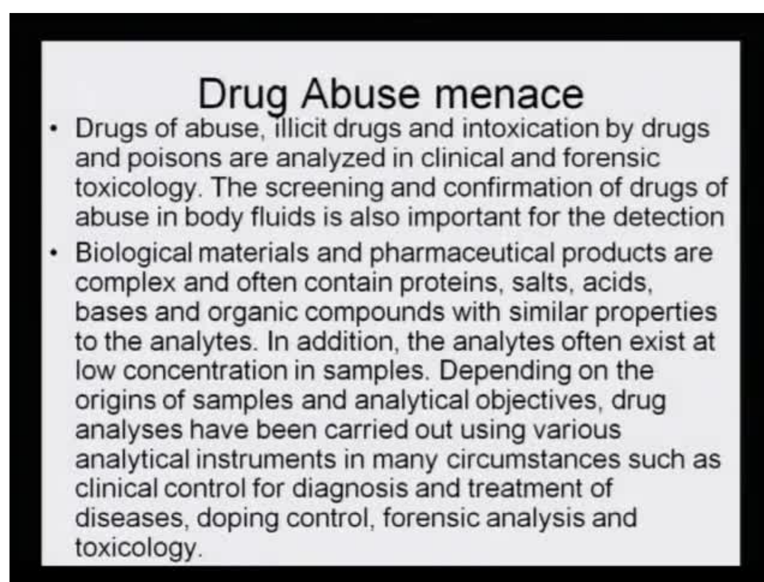

So, you have already known about LC/MS, GC/MS, HPLC, TLC. The only new test that is not known to you is the ELISA test. We will elaborate a little bit about that. But, before we do that, drug abuse menace - drugs of abuse, illicit drugs intoxication by drugs and poisons are analyzed in clinical and forensic toxicological labs. The screening and conformation of drugs of abuse in body fluids is also important for the detection, because how do we find out that a taxi driver or a car driver was driving with alcohol intake? So, that is one type of test, which gives the police an idea that the driver was under some kind of intoxication.

Biological materials and pharmaceutical products are complex and often contain proteins, slats, acids, bases and organic compounds with similar properties to the analytes. Now, always, biological matrices are very complicated. They have many other compounds and some of them would be structurally similar to the compounds that are already present in the matrix. In addition, the analytes often exist at low concentration in samples. Depending on the origins of samples and analytical objectives, drug analyses have been carried out using various analytical instruments in many circumstances, such as clinical control for diagnosis and treatment of diseases, doping control, forensic analysis and toxicology. So, these are the various fields where drug abuse menace can be checked, and can be tested and analyzed.

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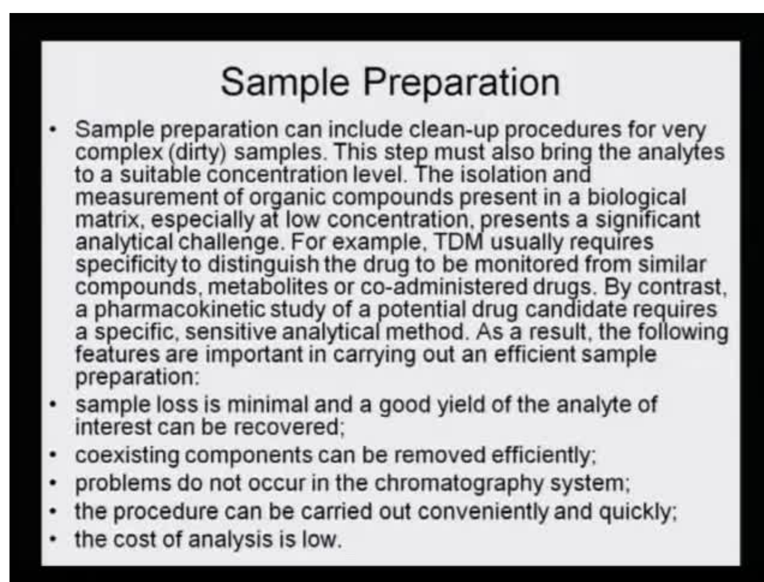

Sample preparation - as I have repeatedly told you that sample preparation is the most crucial step of any analysis; be it a GC analysis or a LC/MS analysis or a GC/MS analysis or a UV-based analysis, everywhere sample preparation plays a very vital role, because if the sample has not been properly extracted and specifically extracted, the analytical data will go haywire. Sample preparation includes clean-up procedures for very complex or dirty samples. This step must also bring the analytes to a suitable concentration level. The isolation and instrument of measurement for organic compounds present in a biological matrix, especially at low concentration, presents a significant analytical challenge.

Now, you have to remember, that from the matrix, the compound must be extracted properly, and to a fairly good amount. Even if it is in low concentration, it has to be extracted properly. For example, TDM usually requires specificity to distinguish the drug to be monitored from similar compounds, metabolites or co-administered drugs. By contrast, a pharmacokinetic study of a potential drug candidate requires a specific, sensitive analytical method. As a result, the following features are important in carrying out an efficient sample preparation. So, what are the following methods that one needs to keep in mind when one is being an analyst?

Sample loss should be minimal and a good yield of the analyte of the interest can be recovered from the matrix; coexisting components can be removed efficiently; problems

do not occur in the chromatographic system that it should not clog the column, it should have a throughput, which is acceptable; the procedure can be carried out conveniently and quickly; and last but not the least, the cost of analysis should be low, because if the large number of samples have to be analyzed and if each test cost is very high, then it will not be a very feasible method.

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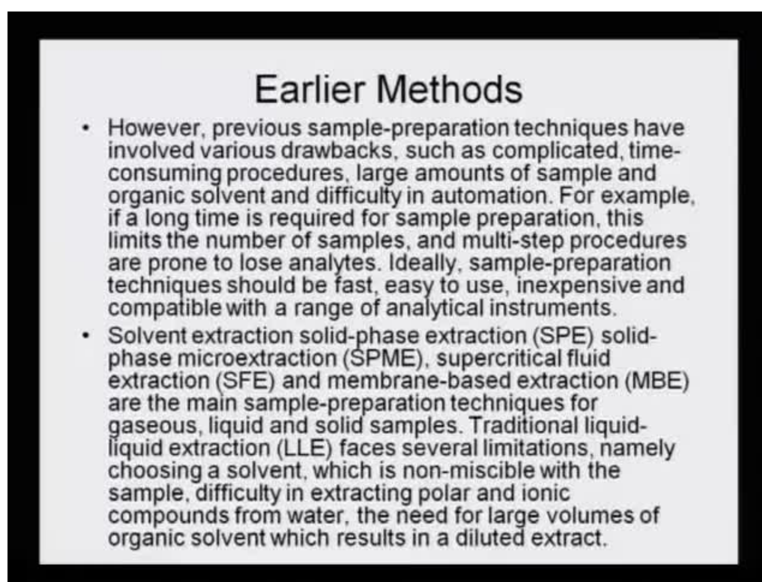

Earlier methods that were practiced - however, previous sample-preparation techniques have involved various drawbacks; and that is the reason why new methods were devised, such as complicated, time-consuming procedures, large amounts of sample and organic solvent and difficulty in automation. For example, if a long time is required for sample preparation, this limits the number of samples, and multistep procedures are prone to lose analytes. Ideally, sample-preparation techniques should be fast, easy-to-use, inexpensive and compatible with a range of analytical instruments. Unless and until these last qualities are to be fitting in the analytical method, it will be of no use for the analyst, because very long drawn extraction processes can always reduce the number of the samples that can be done throughout the day. If too much of solvent is used during the analysis process or the sample-preparation process, at some stage of the other, the solvent has to be removed. So, all these drawbacks were tried to overcome by using some newer techniques.

Solvent extraction - solid-phase extraction, that is, the SPE, solid-phase microextraction (SPME), supercritical fluid extraction (SFE) and membrane-based extraction, that is, MBE are the main sample-preparation techniques for gaseous, liquid and solid samples of drugs. Traditional liquid-liquid extraction faces several limitations, namely, choosing a solvent, which is non-miscible with the sample, difficulty in extracting polar and ionic compounds from water, the need for large volumes of organic solvent, which results in diluted extract. So, you see in order to overcome all these difficulties, which were faced by analysts, who were using liquid-liquid extraction, the more modern methods that came up for their rescue were the solid-phase extraction, the solid-phase microextraction, supercritical fluid extraction and membrane-based extraction.

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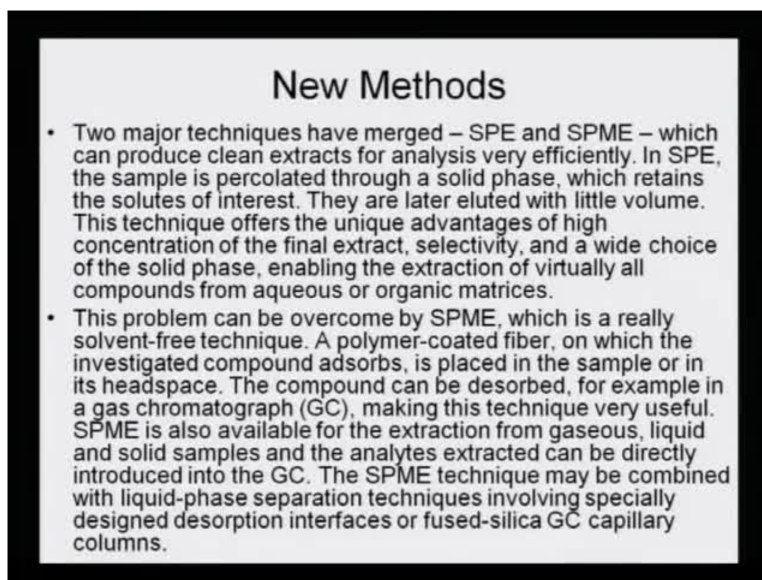

New methods - two major techniques have merged - the SPE and the SPME. As what I mentioned a little while ago, which can produce clean extracts for analysis very efficiently. In SPE, that is, solid-phase extraction, the sample is percolated through a solid phase, which retains the solutes of interest. They are later eluted with little volume of another solvent. This technique offers the unique advantages of high concentration of the final extract, selectivity, and a wide choice of the solid phase, enabling the extraction of virtually all compounds from aqueous or organic matrices. Now, you see that it has so much of versatility. First thing is that the extract or the matrix is directly contacted with the solid phase extractor, and then it retains the solute, which needs to be analyzed and allows the other material to run off. Now, from these adsorbed solutes, more solvent and

a different solvent in which the solubility of this analyte is more is just added optimally, so that the final extract that is obtained, is of the right concentration. So, there is no wastage of solvent; it is much faster and there are different types of solid phase extractors for a variety of compounds.

This problem can also be overcome by solid-phase microextraction (SPME), which is a really solvent-free technique. Now there in SPE, there was still some solvent being used, but SPME is totally solvent free. A polymer-coated fiber, on which the investigated compound adsorbs, is placed in the sample or in its headspace. The compound can be desorbed, for example, in a gas chromatograph or GC directly, making this technique very useful. So, what is done? It is absorbed on the SPME polymeric-coated fiber; and this fiber is then connected as a headspace to the GC, making this technique a very useful technique. SPME is also available for the extraction from gaseous, liquid and solid samples, and the analytes extracted can be directly introduced into the GC as what I mentioned. The SPME technique may be combined with liquid-phase separation techniques involving specially designed desorption interfaces or fused-silica GC capillary columns. So, they can be directly desorbed. Desorbed means one time it is absorbed and then from this polymeric fiber, it is desorbed into the GC column directly. So, there is no possibility of any kind of contamination and also, there is no solvent that has been used for such transferring process. So, the analyte is just directly being injected on the GC.

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Final analyte

The liquid-phase microextraction (LPME) technique which uses a porous polypropylene hollow fiber as an extraction device, is used in drug analysis of biological matrices.

- The final aim of the sample preparation must be to isolate and to purify the analyte and to introduce it into GC, a high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) in a manner that is compatible with each instrument.
- In this way, preparation of biological samples for drug analysis is usually carried out in conjunction with the techniques mentioned above. Furthermore, automated sample-preparation systems are used in conjunction with one or a combination of these techniques.

Final analyte - the liquid-phase microextraction or the LPME technique, which uses a porous polypropylene hollow fiber as an extraction device, is used in drug analysis of biological matrices. The final aim of the sample preparation must be to isolate to purify the analyte and to introduce it into GC, a high-performance liquid chromatography, that is, HPLC or capillary electrophoresis, that is, CE in a manner that is compatible with each instrument. So, now, you see there is another new method, which is specifically for liquid samples and it is called liquid-phase microextraction (LPME). It has a porous polypropylene hollow fiber and through that the drug within the matrix is passed through; and with the small solvent, it can be directly incorporated in the GC machine or HPLC machine or capillary electrophoresis machine. In this way, preparation of biological samples for drug analysis is usually carried out in conjunction with the techniques mentioned above.

Furthermore, automated sample-preparation systems are used in conjunction with one or more combination of these techniques. So, sometimes, only one method suffices the need and sometimes, a combination of methods for extraction is also applied, so that the sample can be extracted properly, and it can be transferred to the analytical machine carefully.

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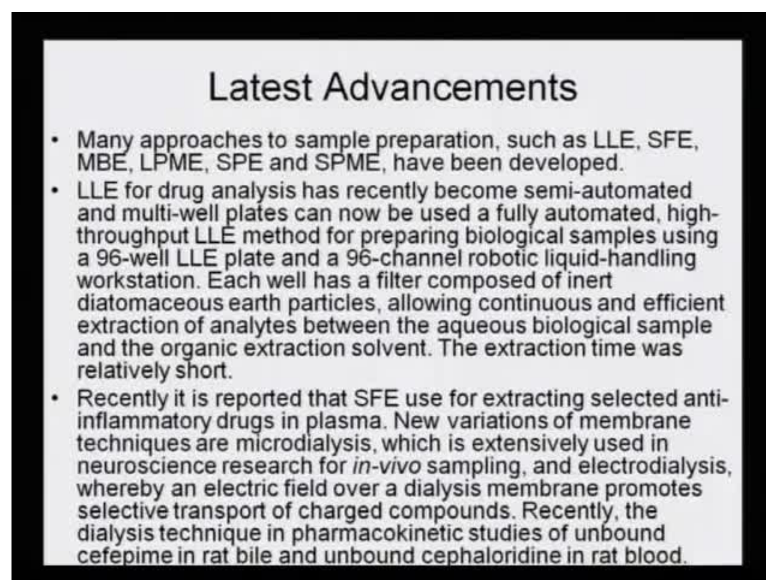

Latest advancements – many approaches to sample preparations have been, such as liquid-liquid extraction, solid-phase extraction, membrane extraction, liquid-phase

microextraction, solid-phase microextraction and so on; and of course, supercritical fluid extraction. The LLE for drug analysis has recently becomes semi-automated and multi-well plates can now be used in a fully automated, high-throughput LLE method for preparing biological samples using a 96-well LLE plate and a 96-channel robotic liquid handling workstation. So, now, there are liquid-liquid extractors, which have 96 such small units combined to each other, so that from one it goes into another one; and whatever extraction has taken place in the first step, will be enhanced in the second place and in the third place, and so on. And in the final, the most enhanced and most concentrated analyte will be obtained through this LLE and semi-automated LLE extraction method. The extraction time also thus relatively reduces.

Recently, it is reported that supercritical fluid extraction used for extracting selected anti-inflammatory drugs in plasma. New variations of membrane techniques are microdialysis, which is extensively used in neuroscience research for in-vivo sampling, and electrodialysis, whereby an electrical field over a dialysis membrane promotes selective transport of charge compounds. Recently, the dialysis technique in pharmacokinetic studies of unbound cefepime in rat bile and unbound cephaloridine in rat blood was carried out.

I am just giving you some of examples in order to show the specificity where all we need to make variations as an analyst. Since this is an advanced analytical course, I want to give you a very holistic picture of what can be done and how they can be done.

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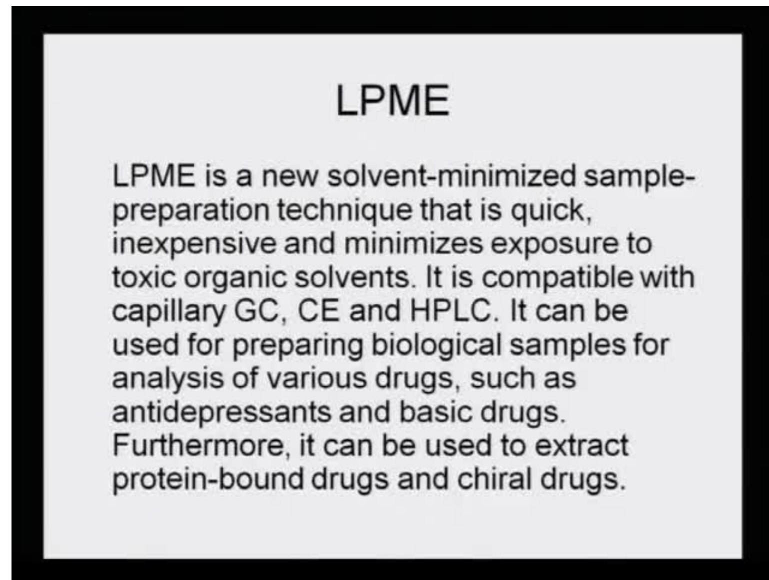

LPME - we will slightly elaborate on this method. Liquid-phase microextraction is a new solvent-minimized sample preparation technique that is quick, inexpensive and minimizes exposure to toxic organic solvents. It is compatible with capillary GC, with capillary electrophoresis and with HPLC. It can be used for preparing biological samples for analysis of various drugs, such as antidepressants and basic drugs. Furthermore, it can be used to extract protein-bound drugs and chiral drugs also. So, you see that LPME is very useful.

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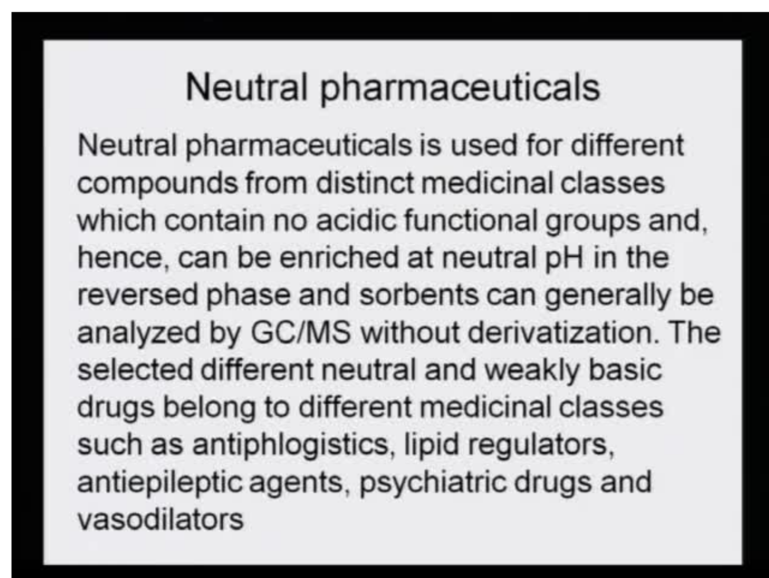

How do we analyze neutral pharmaceuticals? There are specialized dedicated techniques for analyzing neutral compounds. Neutral pharmaceuticals is used for different compounds from distinct medicinal classes, which contain no acidic functional group, and hence, can be enriched at neutral pH in the reversed phase and sorbents can be generally used and analyzed on GC/MS without making any derivative. I had given an example in the last lecture, that derivatization is sometimes possible and is also necessary, because the compound per se may not be having a very good response on the GC/MS. The selected different neutral and weakly basic drugs belong to different medicinal classes, such as antiphlogistics, lipid regulators, antiepileptic agents, psychiatric drugs and vasodilators. So, these are some very typical classes of neutrals pharmaceuticals, which need to be analyzed in a very particular manner.

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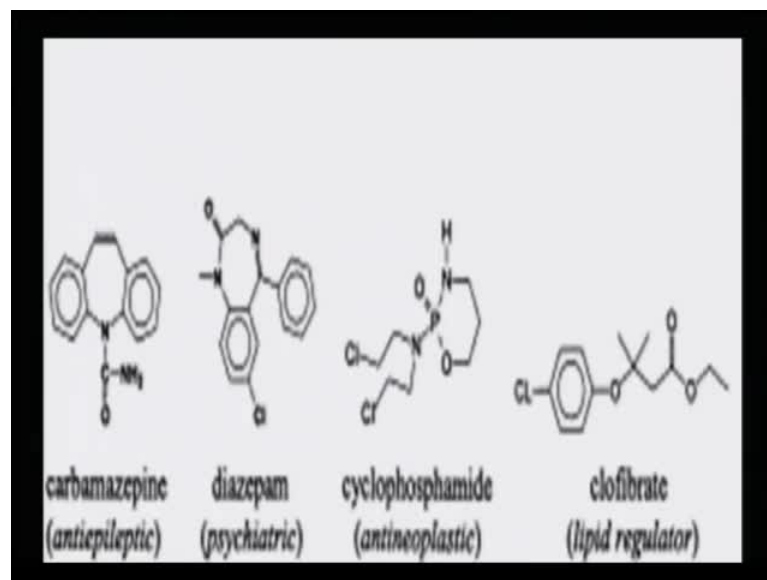

Drugs - I have just shown some diagrams, that is, carbamazepine, diazepam. And all these drugs have very typical structure and very typical functional groups.

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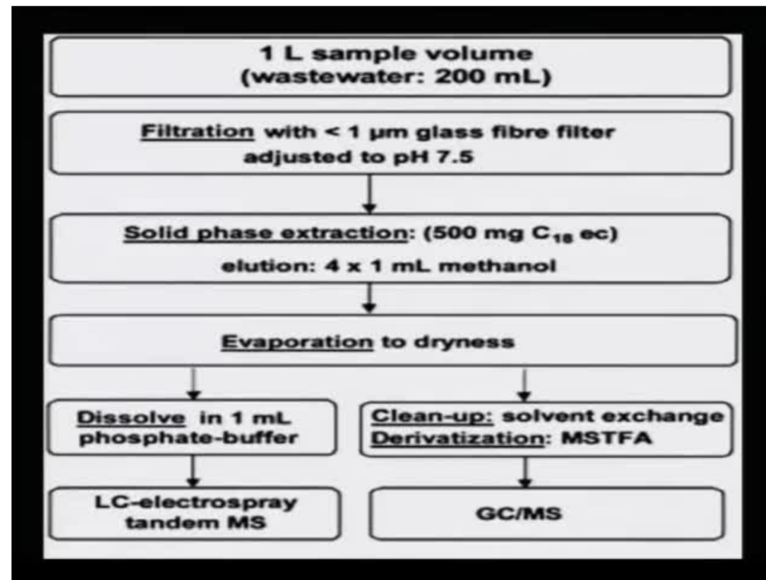

This is a layout of how the sample should be handled. Suppose if it is a waste water sample and a drug, whether it has run into the waste water needs to be analyzed; then, it has to go through filtration, solid-phase extraction, that is, SPE and then evaporation to dryness; and then finally, putting into the phosphate buffer or either carrying out an LC/MS or a GC/MS of the derivatized product.

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Analysis of Antibiotics

For the determination of antibiotics in water down to the lower ng/l range a multi-analytical method as shown in next slide. The analytes belong to different groups of antibiotics such as penicillins, tetracyclines, sulfonamides and macrolide antibiotics.

Analysis was performed by LC–ES/MS/MS, except for chloramphenicol in the positive mode. Chromatography requires the use of C8- and C18-bonded silica columns and water/acetonitrile mixtures containing ammonium acetate.

Analysis of antibiotics - for the determination of antibiotics in water down to the lower level of nano gram per liter range, a multi-analytical method has been shown. The

analytes belong to different groups of antibiotics, such as penicillins, tetracyclines, sulfonamides and macrolide antibiotics. Analysis was performed by LC-ES/MS/MS, except for chloramphenicol in the positive mode. Chromatography requires the use of C8- or C18-bonded silica columns and water and acetonitrile mixtures containing ammonium acetate buffer, was used as a solvent or the mobile phase. Now, these are certain designed techniques for the special analysis of antibiotics. If I have to give you a layout, the simple methods of extraction and then derivatization or putting a phosphate buffer, and then doing in LC/MS, is the usual flowchart of the analysis.

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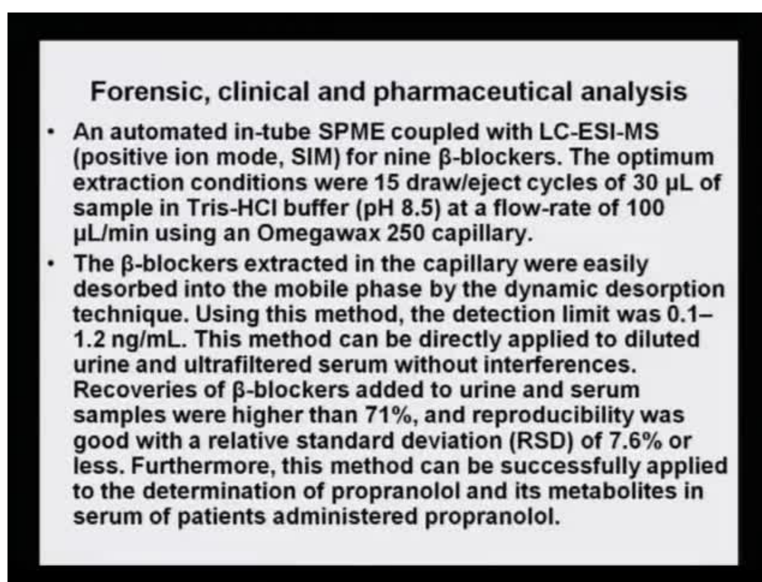

Forensic and clinical and pharmaceutical analysis - how they are carried out? An automated in-tube SPME coupled with LC/MS having ESI-MS positive mode SIM for 9 beta blockers was carried out. The optimum extraction conditions were 15 draw/eject cycles of 30 micro litters of sample in Tris-HCl buffer at a flow rate of 100 micro liter per minute using an omegawax capillary column was used.

The beta blockers extracted in the capillary were easily desorbed onto the mobile phase by the dynamic desorption technique. Using this method, the detection limit was almost 0.1 to 1.2 nano gram per milliliter. This method can be directly applied to diluted urine and ultrafiltered serum without any interferences. You see that even nano gram in 1 mL of the sample can be analyzed, and which is a big achievement, because these quantities are always in very small and trace quantities. These beta blockers will never be in gram

scale. Recoveries of beta blockers added to urine serum samples were higher than 71 percent and reproducibility was good with a relative standard deviation of 7.6 percent or even less. Furthermore, this method can be successfully applied to the determination of propranolol and its metabolites in serum of patients administered with propranolol.

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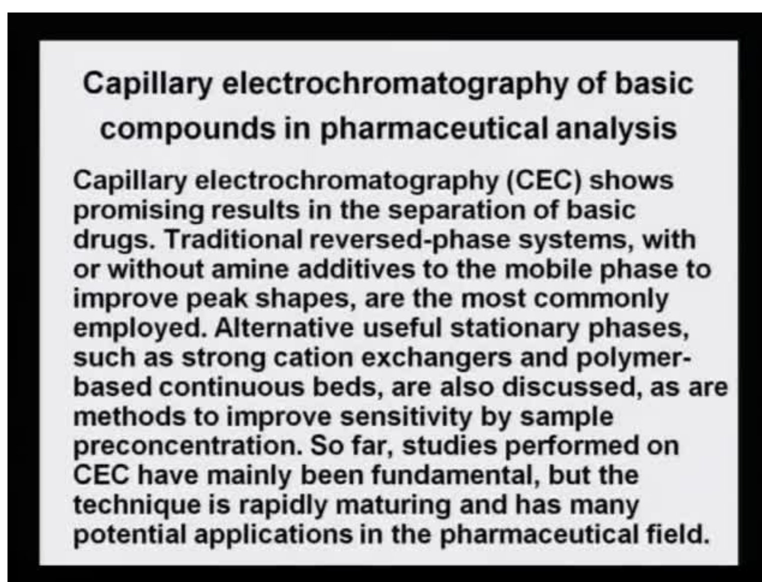

Capillary electrochromatography of basic compounds in pharmaceutical analysis - now, another technique or another chromatographic technique is being introduced for your knowledge, and that is called capillary electrochromatography. It shows promising results in the separation of basic drugs. Traditional reversed-phase systems with or without amine additives to the mobile phase to improve peak shapes, are the most commonly employed system in today's analytical methods. Alternatively, useful stationary phases, such as strong cation exchangers and polymer-based continuous beds, are also discussed, as are methods to improve the sensitivity by sample preconcentration. So far, studies performed on CEC have mainly been fundamental, but the technique is rapidly maturing and has many potential applications in the pharmaceutical field and its analysis.

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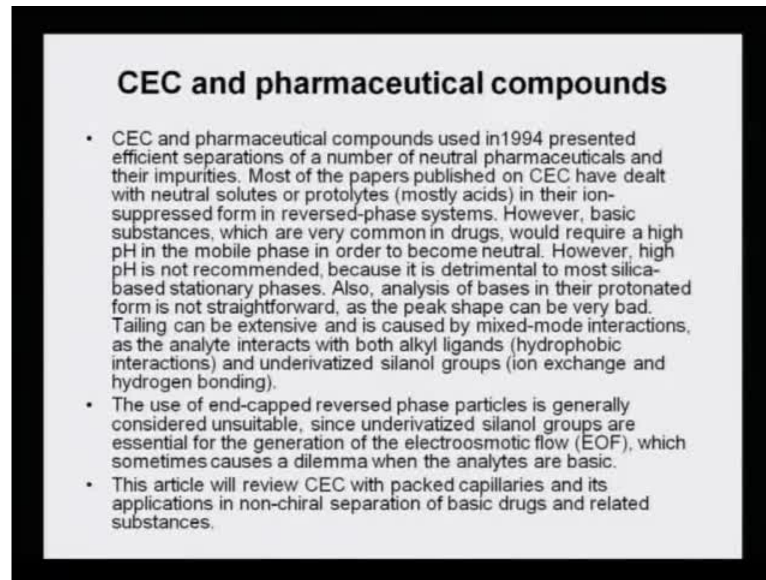

CEC and pharmaceutical compounds - it is a very recent advancement, but still I am introducing you to this capillary method, chromatographic method, because it is of very great importance in the analysis of certain drugs and pharmaceuticals. CEC and pharmaceutical compounds used in 1994, presented efficient separations of a number of neutral pharmaceuticals and their impurities. Most of the papers that were published on CEC have dealt with neutral solutes and prototypes, and some of them were acidic in nature in their ionic-suppressed form in reversed-phase systems. However, basic substances, which are very common in drugs, would require a high pH in the mobile phase in order to become neutral. However, high pH is not recommended, because it is detrimental to most silica-based stationary phases. So, it will harm the stationary phase. Also, analysis of bases in their protonated form is not so straightforward, as the peak shaped can be very bad. Tailing can be extensive and is caused by mixed-mode interactions, as the analyte interacts with both alkyl ligands, that is, the hydrophobic interaction and the underivatized silanol groups (ion exchange and hydrogen bonding).

The use of end-capped reversed phase particle is generally considered unsuitable, since underivatized silanol groups are essential for the generation of electroosmotic flow, which sometimes causes a dilemma when the analytes are basic. This information I have taken from an article just to make you aware that so much of research is being carried out and newer and newer techniques are being introduced for the analyst.

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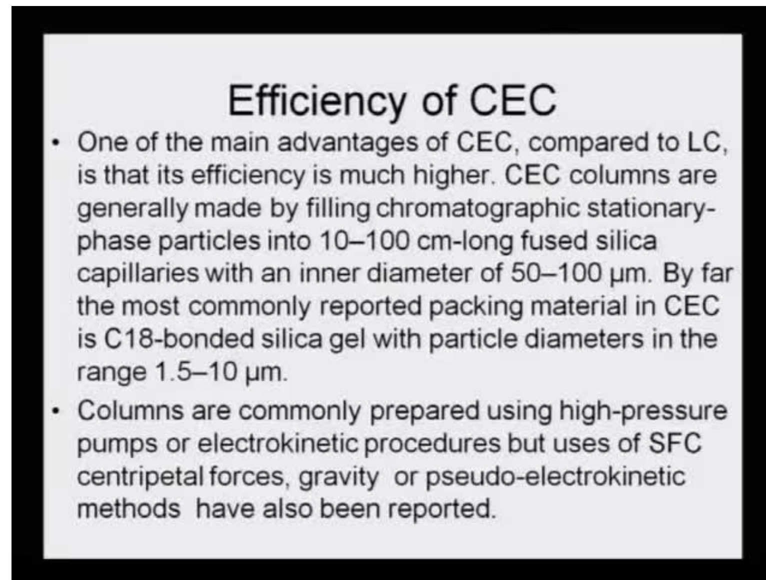

Efficiency of CEC - one of the main advantages of CEC, compared to LC, is that its efficiency is much higher. CEC columns are generally made by filling chromatographic stationary-phase particles into 10 or 100 centimeter long fused silica capillaries with an inner diameter of 50 to 100 micro meters. By far, the most commonly reported packing material in CEC is C 18-bonded silica gel with particle diameters in the range 1.5 to 10 micro meters. Columns are commonly prepared using high-pressure pumps or electrokinetic procedures, but uses of CFC centripetal forces, gravity or pseudo-electrokinetic methods have also been reported in the preparation of these CEC columns.

So, with this, I have been able to introduce you to the idea of using analytical techniques for pharmaceutical drugs.

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Now, I will switch over to another very important topic and this is recent developments in assessing the bioavailability of persistent organic pollutants in the environment. You will understand that I am switching from one type of analysis to another type of analysis only to make you aware that these are very important areas of analysis and they need to be covered.