REVIEW ON LIQUID CHROMATOGRAPHY

SUBMITTED TO:

NAVOJT MAM

SUBMITTED BY:

VIVEK

18MFS8002

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LIQUID CHROMATOGRAPHY

Abstract:

Recent years have seen the development of powerful technologies that have provided forensic scientists with new analytical capabilities, unimaginable only a few years ago. With liquid chromatography–mass spectrometry (LC–MS) in particular, there has been an explosion in the range of new products available for solving many analytical problems, especially for those applications in which non-volatile, labile and/or high molecular weight compounds are being analyzed. The aim of this article is to present an overview of some of the most recent applications of LC–MS (/MS) to forensic analysis. To this end, our survey encompasses the focuses on trace analysis (explosives and dyes), the use of alternative specimens for monitoring drugs of abuse, systematic toxicological analysis and high-throughput analysis. It is not the intention to provide an exhaustive review of the literature but rather to provide the reader with a 'flavor' of the versatility and utility of the technique within the forensic sciences.

Introduction:

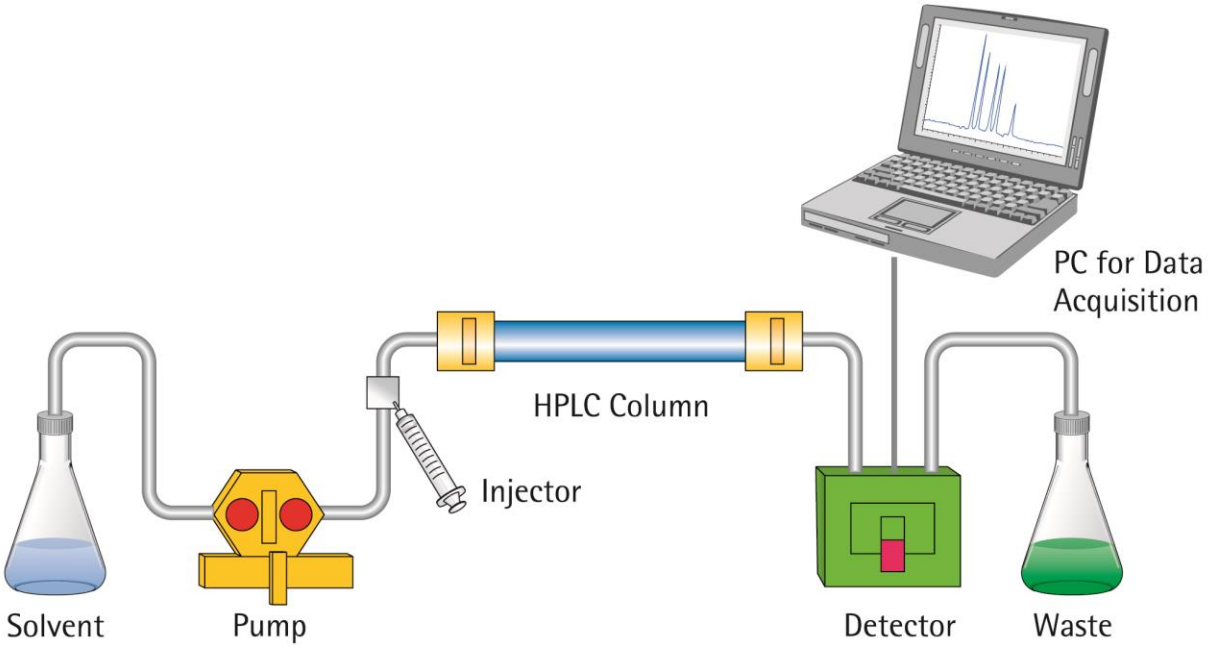
High-performance liquid chromatography (or High pressure liquid chromatography, HPLC) is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds[1]. HPLC mainly utilizes a column that holds packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used[2]. The sample to be analyzed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase. The amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase. The time at which a specific analyte elutes (comes out of the column) is called retention time. Common solvents used include any miscible combinations of water or organic liquids (the most common are methanol and acetonitrile).[3] Separation has been done to vary the mobile phase composition during the analysis; this is known as gradient elution. The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte.[4]

INSTRUMENTATION:

The HPLC instrumentation involves pump, injector, column, detector, and integrator and display system. In the column the separation occurs. The parts include:

- **Solvent Reservoir:** The contents of mobile phase are present in glass container. In HPLC the mobile phase or solvent is a mixture of polar and non-polar liquid components. Depending on the composition of sample, the polar and non-polar solvents will be varied.
- **Pump:** The pump suctions the mobile phase from solvent reservoir and forces it to column and then passes to detector. 42000 KPa is the operating pressure of the pump. This operating pressure depends on column dimensions, particle size, flow rate and composition of mobile phase.
- **Sample Injector:** The injector can be a solitary infusion or a computerized infusion framework. An injector for a HPLC framework should give infusion of the fluid specimen inside the scope of 0.1 mL to 100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).
- **Columns:** Columns are typically made of cleaned stainless steel, are somewhere around 50 mm and 300 mm long and have an inward distance across of somewhere around 2 and 5 mm. They are generally loaded with a stationary phase with a molecule size of 3 μm to 10 μm. Columns with inner diameters of <2mm are regularly alluded to as micro bore segments. Preferably the temperature of the mobile phase and the column should be kept consistent during investigation.
- **Detector:** The HPLC detector, situated toward the end of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized detectors are UV-spectroscopy, fluorescence, mass spectrometric and electrochemical identifiers.
- **Data Collection Devices or Integrator:** Signals from the detector might be gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic information. The PC coordinates the reaction of the indicator to every part and places it into a chromatograph that is anything but difficult to interpret.

The schematic representation of a HPLC instrument ordinarily incorporates a sampler, pumps, and a locator. The sampler brings the sample into the mobile phase stream which conveys it into the column. The pumps convey the mobile phase through the column. The detector generates a sign relative to the measure of sample component rising up out of the segment, consequently taking into consideration quantitative investigation of the example parts. A computerized microchip and software control the HPLC instrument and give information data. A few models of mechanical pumps in a HPLC instrument can combine numerous solvents in proportions changing in time, producing a sythesis slope in the portable stage. Most HPLC instruments likewise have a column broiler that considers altering the temperature at which the partition is performed.



TYPES OF HPLC

Depending on the substrate used i.e. stationary phase used, the HPLC is divided into following types:

- **Normal Phase HPLC-** In this method the separation is based on polarity. The stationary phase is polar, mostly silica is used and the non-polar phase used is hexane, chloroform and diethyl ether. The polar samples are retained on column.
- **Reverse Phase HPLC-** It is reverse to normal phase HPLC. The mobile phase is polar and the stationary phase is non polar or hydrophobic. The more is the non-polar nature the more it will be retained.
- **Size-exclusion HPLC-** The column will be incorporating with precisely controlled substrate molecules. Based on the difference in molecular sizes the separation of constituents will occur.
- **Ion-exchange HPLC-** The stationary phase is having ionically charged surface opposite to the sample charge. The mobile phase used is aqueous buffer which will control pH and ionic strength.

FORENSIC APPLICATIONS:

- **Garpeev.et.al (2003) studied that the explosive material is analyzed in liquid chromatography technique.** The analysis of trace levels of explosives is critical in crime scene forensic investigations, homeland security and environmental analysis. LC–MS is a well-established technique for explosives in associated complex matrices such as post-blast residues and in environmental samples such as soil and plant material extracts. Although these compounds have a low vapor pressure they tend to be heat labile and can degrade at the high temperatures typically used in GC injectors. Thus, LC–MS is particularly well-suited to the analysis of these relatively polar molecules, heat labile

compounds. Many of the methods rely on the formation of cluster or adduct ions for identification the formation of cluster ions of 1, 3, 5-trinitro1, 3, 5-triazacyclohexane (RDX), one of the most commonly used military explosives.

- **Hunag.et.al (2005) analysis the dyes in this technique.** Textile fibers found at a crime scene can be used as chemical evidence in a wide range of crimes; dye identification and comparison can be of particular importance. Recently, Huang et al. have used LC–MS to enable unambiguous differentiation between structurally related textile dyes which were previously indistinguishable by UV–visible absorption profile or by micro spectrophotometry. They concluded that where single stage LC–MS fails to differentiate, analysis should be extended to include LC–MS/MS of the extracted dye mixture.

- **P.kintz (2004) separated the components of any poison present in hair sample.** In addition to the convenience of sample collection, any drugs and metabolites incorporated into hair tend to persist much longer than in conventional specimens. Recently, hair has been used to document drug exposure in a variety of scenarios such as forensic and workplace testing to monitor compliance to drug therapy and particularly for investigating cases of drug-facilitated crimes (DFC). The availability of standard reference materials for drugs of abuse in hair is vital and enables those laboratories performing hair analysis to check the accuracy of their methods. Over the last few years DFC, e.g. sexual assault and robbery have been increasing; these crimes are often difficult to prove due to factors such as the low concentrations of drugs used, or their rapid clearance from the body.

- **Pharmaceutical Applications:** The pharmaceutical applications include controlling of drug stability, dissolution studies and quality control.

- **Environmental Applications:** Monitoring of pollutants and detecting components of drinking water.

- **Food and Flavour Applications:** Sugar analysis in fruit juices, detecting polycyclic compounds in Vegetables, analysis of preservatives.

- **Clinical Applications:** Detecting endogenous neuropeptides, analysis of biological samples like blood and urine.

- **Cannabinoids:** HPLC methods for determination of cannabinoids were reviewed by Raharjo and Verpoorte. Using UV detection, lower limits of quantification of THC from serum of 5 ng/ml were described [106]. However, electrochemical detection at +0.9 V vs. Ag/AgCl is preferred due to the oxidable phenolic structure of the compounds. Limits of detection of 1 mg/ml in serum and 5 mg/ml in urine for THC and THC-COOH were determined and the methods proved to be suitable for routine confirmation of immunoassay results.

- **Opioids:** The analysis of opiates from serum or urine was performed using DAD, variable wavelength UV, electrochemical and fluorescence detection. The determination

of morphine, 6-acetylmorphine, codeine, methadone, its metabolite EDDP as well as cocaine, benzoylecgonine and coca ethylene from urine and plasma by SPE on Bonduel Certify cartridges and HPLC-DAD using an RP8 column and a phosphate buffer pH 6.53/acetonitrile gradient mobile phase was described by Ferna´ndez et al. The method with a limit of detection of 100 mg/ml for all substances in urine and between 10 and 50 ng/ml in plasma was applied in 23 drug fatalities. Similarly, morphine, morphine-3-glucuronide and morphine-6-glucuronide were determined from serum with limits of detection of 10, 60 and 90 ng/ml by UV detection. A clearly higher sensitivity for these three substances was achieved by fluorescence detection (LOD 5 and 3 ng/mg, respectively, and electrochemical detection (LOD 0.102, 0.135 and 0.135 mm). In addition to that, the combined use of fluorescence and ECD enabled an improved specificity. HPLC with UV detection was regularly used for analysis of methadone and its metabolite EDDP. Since L-methadone is about 50 times more effective than Methadone and both the L-enantiomere and the race mate are prescribed, the enantioselective analysis is frequently performed. A good separation was achieved on cyclodextrin bonded phases. The low therapeutic range of buprenorphine (0.5–5 ng/ml) could sufficiently sensitively be measured by HPLC with ECD.

- **Benzodiazepines:** The analysis of benzodiazepines from biological fluids using HPLC was reviewed by Berrueta et al. Because of their characteristic and intense UV absorption, benzodiazepines including the low-dose drugs such as alprazolam or lorazepam can be analyzed with high sensitivity and selectivity using a diode array detector. Liquid–liquid extraction with 1-chlorobutane or n-hexane/ethyl acetate as well as SPE with C18 extraction columns or online SPE proved to be suitable for this task. The detection limits were generally between 2 and 10 ng/ml and were sufficient for confirmation of positive immunoassay results in daily routine. A sensitive determination of benzodiazepines with detection limits of 2–14 ng/ml was also possible using reductive electrochemical detection at a hanging mercury drop in combination with UV detection.
- **Amphetamines and designer drugs:** HPLC methods with UV or diode array detection for determination of amphetamine, methamphetamine, methylenedioxymethamphetamine (MDMA), methylene dioxyethamphetamine (MDE), 4-methylthioamphetamine (MTA), mescaline or 4-bromo-3, 5-dimethoxy-phenethylamine and some of their metabolites in urine were described. The detection limits after usual SPE were between 20 and 80 ng/ml. However, a tremendous increase in sensitivity to 0.5 mg/ml was possible by specific sample preparation using single-drop liquid–liquid–liquid extraction. The basic drugs were extracted from the alkaline sample into the hexane layer and from there simultaneously or successively re-extracted into a 5 ml drop of 0.02 H3PO4 with an enrichment factor of about 500. A high sensitivity could also be obtained in detection of these compounds from plasma, urine oral fluid or hair using HPLC with fluorescence detection. Whereas the methylenedioxy-compounds MDMA, MDE and MDA display a sufficiently intense native fluorescence, amphetamine and methamphetamine were derivative, e.g. with 4-[4, 5-diphenyl-imidazolyl-(1)]-benzoylchloride. Electrochemical detection was enabled by pre-column derivatization with 3,4- dihydroxybenzaldehyde. Enantioselective analysis of amphetamines by HPLC were reviewed by Herraez-Hernandez et al. and Liu et al. Fluor metric detection was preferred also for this purpose.

- **Cyanide, thiocyanate and azide:** The determination of cyanide in body fluids by HPLC with FLD after derivatization with 2, 3-napthalenedialdehyde and taurine was described by several authors. The reaction leads to a highly fluorescent benzoisoindole derivative. Fluorescence detection has the advantage in comparison to UV/VIS absorption that the reagents which are added in large excess do not fluoresce. Felscher and Wulfmeyer isolated HCN from blood by micro distillation before derivatization and described a detection limit of 2 mg/ml. In a simpler and faster procedure, Mateus et al. added 0.5 ml water and 2 ml methanol to 100 ml blood, vortexed and centrifugated the mixture and performed the derivatization directly in the supernatant. For analysis, 10 ml of the obtained solution were injected and detected at excitation and emission wavelengths of 418 and 460 nm, respectively. The detection limit was below 25 mg/ml. In a similar way, thiocyanate was determined with high sensitivity (detection limit 0.16 nmol/l) from saliva and plasma by HPLC with FLD after derivatization with 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one. However, in case of the plasma samples, SCN had to be separated before derivatization using anion-exchange cartridges. Azide was determined in body fluids and tissues of fatalities by HPLC-DAD after derivatization with 3,5-dinitrobenzoyl chloride or benzoyl chloride. In the first case, the samples were treated with acetonitrile and 0.02 N K2CO3 for protein precipitation and the supernatant adjusted to pH 5 with diluted HCl. After 10 min reaction with 3, 5-dinitrobenzoyl chloride, 50 ml of the solution were directly injected into HPLC. The detection limit was 0.08 mg/ml. The procedure with benzoyl chloride was very similar with a detection limit of 0.2 mg/ml. In the chromatograms, the derivatives were well separated from the excessive reagents and no disturbing peaks from other sample constituents were seen.

CONCLUSION:

The HPLC is mostly used analytical technique. It is having several advantages. With the use of HPLC one can produce extremely pure compounds. It can be used in both laboratory and clinical science. With the use of HPLC the accuracy, precision and specificity can be increased. The only disadvantage of HPLC is high cost.

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