CHAPTER
1

Analysis of Drugs in Biological Matrices

URINE AND BLOOD

- Urine is the sample of choice for the screening and identification of unknown drugs due to high concentration of drugs in urine.
- Improvements in sample preparation, chromatography and in detector techniques have made blood accessible as a screening matrix.
- Another great advantage is that drugs can be detected just after intake prior to metabolism and/or filtration.

Sample Pretreatment

- Conventional sample pretreatment techniques for drugs of abuse analysis in urine samples are:
  - Liquid–liquid extraction (LLE)
  - Solid phase extraction (SPE)
- However, they are rather laborious, time consuming and using large amounts of toxic solvents. Therefore, solvent less sample preparation techniques such as:
  - Liquid-phase micro extraction (LPME)
  - Supercritical fluid extraction (SFE)
  - Solid phase micro extraction, had already been proposed.
- In case of urine screening, immunoassays (IA) are used to differentiate between negative and presumably positive samples.
- Enzyme-linked immunosorbent assay (ELISA) technique is utilized on whole blood samples for cannabinoids, amphetamines and opiates.
- Capillary electrophoresis (CE) has been proven to have great utility in the analysis and detection of drugs of abuse. But CE is not sensitive enough to be applied to trace analysis due to low injection volumes and limited detection path lengths.

ORAL FLUID SAMPLE (SALIVA)

- Detection time of drugs in oral fluid (5–48 hrs.) is similar to that in blood (1–2 days) whereas the detection times in urine can be much longer.

- Due to short detection times, oral fluid is a feasible matrix for confirmation analysis of driving under the influence of drugs (DUID) cases, where indications of recent drug use is required.
- Samples can be collected with the Stat Sure Saliva Sampler TM device.

Sample Pretreatment

Liquid–liquid-extraction (LLE)

Solid-phase-extraction (SPE)

- Samples were analyzed using gas chromatography–mass spectrometry (GC–MS) with the mass selective detector (MSD) operating in either electron ionization (EI) or negative-ion chemical ionization (NICI) mode.
- The compounds analyzed included cannabis, cocaine, amphetamines, opioids, benzodiazepines and other psychoactive medicines.

HAIR SAMPLE

- Drugs are fixed inside the hair matrix, therefore a digestion procedure is necessary before the extraction of drug from the matrix.
- An automatic solid-phase extraction method is used.
- The method is used along with analysis by gas-chromatography-mass spectrometry (GC/MS) in selected ion monitoring mode (SIM), for the following drugs: codeine, 6-monoacetylmorphine (6-MAM), morphine, cocaine, methadone, ecstasy (MDMA) and Eve (MDE).

NAILS

- Nail samples are usually obtained by cutting the excess overhang of the nail plate using cosmetic nail clippers
- The four key steps in preparing nail samples are:
  1. Decontamination
  2. Cutting into small segments
  3. Digestion/Hydrolysis (alkaline, acidic or methanolic)
  4. Extraction (usually LLE)
- Analyzed by GC.
- A variety of licit drugs (β-blockers, sedatives, anticoagulant agents, antidepressants and antipsychotics) illicit drugs (cocaine, cannabinoids, morphine and AM related compounds, including their metabolites) has been detected and determined in nails.

TEARS

- Sampling tears is the chief problem to producing precise, reproducible analytical results.
- Two main procedures for collecting tears are:
  1. Direct sampling and
  2. Indirect sampling

Direct sampling comprises collecting tears with capillary tubes and requires previous stimulation.

Indirect sampling uses absorbing supports that are very similar to Schrimer strips (classically used to diagnose dry-eye syndrome).

PROPERTIES OF THE BIOLOGICAL MEDIA

- Blood
- Plasma and Serum
- Urine
- Milk
- Cerebrospinal Fluid
- Bile
- Saliva
- Blister Fluid
- Synovial Fluid
- Aqueous Humor

Biological Samples Listed in Descending Order of Fluidity Corresponding to Degree of Difficulty of Analysis

• Liquid	• Tears • Sweat • Saliva • Urine • Bile • Cerebrospinal fluid
• Mixed	• Plasma • Serum • Blood • Feces
• Solids	• Brain, fleari, kidney, liver lung, muscle bone

Blood

- Blood is the most complex of the biological fluids mentioned. As collected from a subject or animal, the blood consists of a buffered clear fluid containing solubilized proteins, dissolved fats and solids, and suspended cells.
- The major constituent, the red blood cells, or erythrocytes, can be separated from the clear fluid, or plasma, by simple centrifugation. However, if the blood is not treated carefully, the cells can burst and separation of undesirable components becomes more difficult.
- For example, ferric ions released from erythrocytes may chelate with some analytes causing poor extraction from the aqueous phase.
- The cells can be caused to burst by heating or by freezing, or by mechanical means such as stirring, but the most common cause is by changing the ionic

strength of the surrounding fluid by the addition of water; the resulting osmosis causes the cells to swell and rupture.
- It should extract in isotonic saline.
- If blood is allowed to stand without the addition of anticoagulating agents, then the red cells will eventually clot and the resultant fluid (serum) can be decanted.
- Serum is, in most respects, similar to plasma except that it does not contain the soluble factors that lead to the clotting phenomenon. On the other hand, if anticoagulants are added and plasma subsequently prepared, then these factors remain in the plasma and may give rise to subtle differences when serum and plasma are analyzed.
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- Protein can also be denatured using proteolytic enzymes, a procedure that should avoid the possibility of damage to the analytic using chemical-type denaturation. Such procedures are generally found in the preparation of tissue for drug analysis, but the enzyme subtilisin has been successfully used for the digestion of plasma proteins.
- Protein binding is a phenomenon that is important for the transport of drugs in the blood, and sometimes for the solubility of the drug also.
- Acidic drugs such as barbiturates are generally considered to be strongly bound, whereas basic drugs tend not to be.
- It is important to recognize that some changes in plasma constituents can have an impact on the actual levels of drug, as distinct from factors which interfere with the analysis. An example of this is the potential for some drugs to displace others from their protein-binding sites. In such cases, the physiological effect of the drug may depend on only the unbound, or free, concentration, yet because there are fewer binding sites available, the total drug measured will appear inappropriately low for the observed effect.

Urine
- Urine is generally free of protein and lipids and therefore can usually be extracted directly with an organic solvent. Urine does, however, have a wide variation in

its composition, this most obviously being seen in the dark amber of an overnight specimen compared with the pale urine collected during the day.

- The normal type of compound found in urine is water soluble, whereas most drugs are lipid soluble and can be extracted with an appropriate solvent.
- One of the greatest difficulties that arises, however, is the volume of urine that may be produced over fixed time intervals. if the urine volume is large, then the sample is dilute and the method may be operating near its limit of detection,
- Urine is the most commonly used biological matrix for testing of drugs of abuse. It is easier to collect than blood, easier to analyze, and a urine sample will be positive for a drug for a significantly longer time period than blood. After a single dose of most types of drugs, the parent drug or a metabolite may be found in urine for a few days.

Milk

- Milk is not a very usual fluid for the analysis of drugs, but it is occasionally of interest when trying to establish whether drugs may be transferred from mother to infant by this route.
- Most authors seem to be able to adapt existing methods for urine' or serum' without much difficulty. The main problem would seem to be the presence of fats in milk (approximately 4,5% in mature human breast milk), and a method for phyllo Quinone uses lipase to hydrolyze fats in the sample prior to HPLC.
- Alternatively, the defatting step that is recommended above for processing plasma samples may be sufficient for the analysis of tenoxicam in human breast milk. In this procedure, fats were removed by washing 0.5 ml breast milk, buffered to pH 3 to 4, with 10 ml /7-hexane

Cerebrospinal Fluid

Cerebrospinal fluid is another biological fluid which is thought to be closer to the site of action of drugs and natural biological agents than the usually assayed plasma or serum. Not surprisingly, attempts have been made to analyze drugs with pain- killing activity in this fluid. Samples are, however, not so easily obtained, although when they are available, techniques that have been developed for plasma or serum seem to be readily applicable.

Bile

- Bile is a complex and variable fluid which is not frequently used for the assay of drugs, partly because of the difficulty of collection and also because any systems of removing bile will interfere with the process being investigated. Nevertheless, liver perfusion studies in vivo will often involve such analyses, particularly where conjugating enzymes are being investigated. Because of its character, methods developed for fluids with well-established consistency are not immediately adapted for bile.
- For example, blood, urine, vitreous humor, and cerebrospinal fluid could be analyzed directly for morphine using commercial RIA kits, but bile needs to be

made alkaline and extracted with dichloromethane: propanol and the residue reconstituted in serum.

Saliva

- Saliva is a colorless, transparent or translucent, somewhat viscid material of low viscosity.
- Its attraction for bioanalysis is that it is relatively free of interfering substances, is easily extracted by organic solvents, and is thought to reflect the levels of nonprotein bound drug in the blood. For nonionized drugs such as steroids, the drug should readily cross from plasma to saliva and this would represent a noninvasive method of monitoring.
- Normal subjects may produce up to 2 L of saliva in 24 h, with a more or less continuous flow of 15 ml between meals. Collection of a reasonable sample in a reasonable time is tedious for normal subjects but larger samples can be readily obtained by stimulation of the glands by chewing an inert material such as parafilm or by using citric acid.
- The advance device consisting of sucrose granules enclosed in polyurethane-bonded cellulose semipermeable membranes, which, when placed in the mouth, collect an ultrafiltrate of saliva. This ingenious device bypasses several of the problems of collection and handling of the fluid.
- Stimulated saliva is also reported to be more consistent in its pH value (pH 7.0 to the composition of saliva compares well with those of blood, plasma, and serum.
- The analytical methods for determining drugs in saliva do not seem to have experienced any particular problem in adapting existing methods for other fluids such as plasma or serum; minor problems have been noted with absorption of phenytoin to the mucoid proteins of saliva and occasional adsorption of drugs to the stimulation material.
- The principal problem with analysis of drugs in saliva is not a technological problem but is to establish the relevance of results of such analyses. Assuming the transfer of drug from blood to saliva is by passive diffusion, then the relationship between saliva and blood concentrations can be expressed from theoretical considerations.
- Researchers can then decide whether the levels to be expected are within their analytical capabilities and once analytical values have been obtained, they can make the appropriate extrapolations to determine blood concentrations. By applying the usual Henderson-Hasselbach equation, the ratio of drug concentrations for a weak acid (pKa) in saliva and plasma can be expressed as:

$$\text{Ratio} = \frac{1 + 10^{\text{pH of saliva-pKa}}}{1 + 10^{\text{pH of plasma-pKa}}} \times \frac{\text{fraction unbound in plasma}}{\text{fraction unbound in saliva}}$$

with a corresponding equation for weak bases of

$$\text{Ratio} = \frac{1 + 10^{\text{pKa-pH of saliva}}}{1 + 10^{\text{pKa-pH of plasma}}} \times \frac{\text{fraction unbound in plasma}}{\text{fraction unbound in saliva}}$$

Blister Fluid

Blister fluid obtained from suction-induced skin blisters is similar to interstitial fluid and exudate in a mild inflammatory reaction and contains proteins and lipids. It has been used for studying the pharmacokinetics of ibuprofen.

SMALL ORGANIC MOLECULES

- The vast majority of chemical compounds used as drugs are small molecules of molecular weight 200 to 300 Da. Most of these compounds are weak acids or weak bases and the drugs themselves are usually in the form of salts, the particular salt being chosen for its physicochemical properties in the formulation stages rather than its pharmacology; once in the body, buffering in the blood and other fluids makes the original salt form mainly irrelevant to its subsequent biological activity.
- A few of these small molecular weight drugs may be uncharged, relatively lipophilic molecules, such as the steroids. Other uncharged drugs include esters, which may be active or may act as prodrugs releasing either an acid drug or an alcohol, and quaternary amines.

Examples of Organic Molecules Used as Drugs

- Uncharged Steroids
- Acetylsalicylates
- Phenacetin

The neutral character of steroid drugs, esters, and ethers means that the partition of such compounds into organic solvents is independent of the pH. This property gives good scope for partial purification of such compounds by simply removing the acids and bases from an organic extract by successive washes with dilute acid and dilute alkalis; final washing with distilled water and drying by simple filtration through sodium sulfate or cellulose gives a relatively clean extract for urine samples, but the presence of lipids in plasma or serum is more problematic and requires careful choice of organic solvent for extraction. On the other hand, aqueous samples containing weak bases and weak acids as analytes can be readily defatted with a hexane wash. Thus, the charged nature of the small-molecule drug is an important consideration in the analytical determination.

Conjugates

- Few drugs are administered as conjugates, but many drugs form conjugates *in vivo*. It was originally thought that formation of conjugates was a final step in making xenobiotic water soluble for elimination in the urine and thus little attention was given to the analysis of the conjugates. However, the presence of enzymes responsible for xenobiotic metabolism, including the formation of conjugates, is now recognized as an important parameter in determining the individual's genetic control of his own metabolism.
- The classical procedure in determining conjugates was to assay the sample for unchanged drug and then after a suitable hydrolytic procedure determine the

"total" drug; the difference between the two procedures would be considered the amount of conjugated drug. By using selective hydrolysis, either by carefully controlled pH conditions, or by using enzymes considered specific for particular conjugates, such as p-glucuronidase for morphine glucouronide and sulfatase attempts could be made to determine the individual conjugates.

- However, chemical hydrolysis could also degrade the drug itself, and enzyme hydrolyses were less specific than hoped, or else would not always be complete.
- There have been many successful approaches to direct profiling of drugs and drug metabolites in biological fluids. The ability of liquid chromatography to work with a wide variety of structurally diverse compounds has made it very useful in this area and the powerful structural tools of physical chemistry are now also being used to assess the content of untreated biological fluids, sometimes with no chromatographic purification.

Chirality

- The fact that almost all synthesized drugs which had an asymmetric carbon were marketed as racemates. The whole basis of organic chemical structure is based on the tetrahedral carbon atom and the concept of mirror-image isomers is an inevitable consequence. Many of the synthetic drugs initially produced by the pharmaceutical industry were semisynthetic and based on naturally occurring precursors such as steroids and β-lactams. Fortuitously, the marketed compounds were optically pure, and any consideration of enantiomeric forms of the drug did not normally arise.
- Propranolol and ibuprofen probably represent, respectively, cases where one isomer is pharmacologically inactive at the doses used, or the inactive enantiomer is converted *in vivo* to the active isomer. As such, any clinical implications may not be expected, and there would be no harm in using the cheaper produced version of the drug. However, ignoring the presence of the second isomer may lead the unsuspecting pharmacokinetics or clinical pharmacologist to assume that the presence of enantiomers can also be ignored. If indeed the enantiomers are handled by metabolizing enzymes and transport proteins in an identical manner, there would be no problem, but considering such processes can be highly stereo chemically selective.
- Because of the unavailability of chirally selective analytical methods, and perhaps more importantly because of the general unawareness that a problem may exist, the separate pharmacokinetics of enantiomers were not reported for many drugs in the literature until about 10 years ago.
- A recent paper on aspects of chiral high-performance liquid chromatography in pharmaceutical analysis includes the current European regulatory viewpoint on racemates vs. single isomers. Chiral problems are not limited to the parent drug; in some cases a nonchiral center can be introduced by metabolic processes,
- The success of the bioanalyst in devising chiral separations for following separate enantiomers following dosing of racemates has undoubtedly influenced the trend

toward single-isomer products. This success has, paradoxically, diminished the very reason for the development of such methods; if the drug dosed is a pure enantiomer, then there will be no need to follow the isomer that was not dosed!

PROBLEMS WITH ANALYSIS OF BIOLOGICAL MATRICES

Factors affecting the stability of drugs and drug metabolites in biological matrices:

- External factors
- Light temperature
- pH
- Oxidation
- Enzymes

Factors influencing degradation External factors	**Examples of affected drugs and metabolites**	**Approaches to avoid effect of factor on sample stability**
Light	Nifedipine Nisoldipine Vitamin D Flunitrazepam Lysergic acid diethyl amide Lomefloxacin	Cover samples Use sodium lights or ultraviolet-filtered lights
Temperature	Simvastatin Aspirin Temazepam Testosterone Benzodiazepines Methylenedioxyamphetamine Norepinephrine	Use reduced temperatures during sample collect storage and processing
pH	Gemcitabine triphosphate Clopidogrelat Camptothecin Cisplatin	Identify/adjust pH as needed for sample storage handling; buffer samples appropriately
Oxidation	Ascorbic acid Mitoxantrone Tiopronin Psilocybin Levodopa	Add reducing agents Collect samples in ethylenediaminetetraacetic
Enzymes	Glucagon-like peptide-1 Parathyroid hormone Enfuvirtide	Add enzyme inhibitors Acidify samples

- Structural factors
  i. Ester pro-drug
  ii. Tautomerization

iii. Acyl glucuronide metabolites
iv. Chiral interconversion
v. H/D (hydrogen-deuterium) exchange effect

Factors influencing degradation	Examples of affected drugs and metabolltes	Approaches to avoid effect of factor on sample stability
Ester pro-drug	Cefetamet pivoxyl Nafamostat Phenyl carbamates *O*-lmidomethyl 17β-estradiol	Add esterase inhibitors Use rapid processing of samples Use low temperatures for sample storage and handling
Lactone/acid conversion and tautomerization	Camptothecin FK 506	Use low temperatures for sample storage and handling Use pH control
Acyl glucuronide metabolites	Telmisartan Ibuprofen Clopidogrel Mycophenolic acid SCH X	Use low temperatures for sample storage and handling Use pH control
Chiral interconversion	Thalidomide S-adenosylmethionine BMS-207940 MK-0767	Use low temperatures for sample storage and handling Use pH control
H/D exchange	Rofecoxib (deuterium-labeled internal standard)	Avoid exchangeable hydrogen labels

Photochemical Stability

- It is a common source of analyte instability in a biological matrix when the sample is exposed to ambient light.
- Most laboratories use fluorescent lighting, which contains a significant amount of UV radiation.
- Many industrial laboratories utilize UV filtered light to minimize stability problems.
- Degradation of vitamin D in blood serum can be minimized by using blood collection tubes that are wrapped in foil and placed in light-proof boxes after centrifugation.
- Nifedipine and nisoldipine are examples of drugs that are sensitive to photochemical degradation.
- In whole blood, after 1 hour, nifedipine degrades by 14% versus samples stored in the dark.
- This indicates the importance of protecting nifedipine from light throughout the storage and processing of samples for its analysis.

Thermal Stability

- Temperature is a critical factor to consider and control in addressing issues of sample stability.
- Simvastatin is a common cholesterol lowering drug that is known to be temperature sensitive.
- The lactone ring of simvastatin is rapidly hydrolyzed to its β-hydroxy acid form, simvastatin acid.
- The hydroxylation of simvastatin in the lactone form occurs rapidly in heparinized plasma, which means that thawing such samples and conducting all of their processing at 4°C within 3 hours is necessary to maintain good stability
- Thermal stability is of particular importance in forensic applications.
- Blood chemistry changes after death. Thus, the stability of an analyte in fresh whole blood cannot be used directly to determine the stability of the same compound in postmortem blood.
- A postmortem sample may be kept at either ambient or subambient temperatures for much longer periods of time than are typically observed with more typical clinical samples.
- Temazepam is common injectable street drug.
- 77% of temazepam remains after storage over 6 months at 5 and 25°C. At -20°C, the stability is increased and 95% of this drug is recovered.

Effects of pH

- It is critical to properly buffer biological matrices for highly pH sensitive compounds or their metabolites because the pH of the matrix itself can change over time.
- The pH of plasma, bile and urine will increase over time from pH of 7.2–7.4 to as high as 8.8 upon storage at room temperature.
- Cisplatin is an example of drug that is highly susceptible to sample pH.
- Cisplatin and its monohydrated complex degrade rapidly at pH 7.4 and 37°C in the presence of samples such as ultrafiltrates, plasma and blood.

Effects of Oxidation

- Oxidation in samples is inhibited by placing in the matrix an antioxidant such as ascorbic acid or sodium metabisulfite.
- Mitoxantrone is one example of a drug that undergoes oxidation reactions.
- This drug is relatively unstable in plasma, with a half-life of approximately 70 hours at room temperature.
- Therefore, addition of 0.5% w/v solution of ascorbic acid to plasma samples is necessary during sample storage and processing.

Enzymatic Degradation

- Many drugs, such as those that contain an ester group, are susceptible to enzymatic degradtion.

- Approaches to inhibit enzyme activity include the use of a protein denaturant such as sodium fluoride or the use of an organophosphate enzyme poison.
- Cefamet pivoxil (i.e. the orally absorbed ester form of cefamet) is highly unstable in plasma.
- After just 1 hour, upto 70% degradation of this drug can occur in untreated plasma.

Chiral Interconversion

- Under the right conditions, all chiral compounds are susceptible to interconversion.
- This may occur at a physiological pH and temperature or it may require an extreme pH and elevated temperatures.
- [R]- and [S]-thalidomide are two forms that have quite different pharmacokinetic and pharmacodynamic properties, requiring the use of adequate methods for chiral separation and analysis of thalidomide.

Stability of Large Molecules

- In many cases, large biomolecules are even more reactive than small compounds to enzyme degradation.
- Their stability is also affected by temperature and by fluctuations in temperature that occur during sample freezing and thawing.
- The analysis of large biomolecules in clinical samples is often accomplished by collecting samples in EDTA plasma, storing these samples at –70°C and processing them as rapidly as possible to avoid degradation.
- Parathyroid hormone (PTH or parathyrin) is an example of one large biomolecule that is used as a drug.
- PTH is an 84-amino acid peptide that is susceptible to hydrolysis by proteolytic enzymes in biological samples.
- PTH is significantly more stable in EDTA plasma than in serum. EDTA inhibits serine proteases that were leading to part of the greater instability of PTH in serum.

Effects due to H/D (Hydrogren-Deuterium) Exchange

- LC-MS/MS methods commonly utilize internal standards that are labeled with deuterium. The resulting labeled compound will closely mimic the properties of the corresponding analyte during sample storage and processing.
- When utilizing MS detection, an isotopically labeled internal standard can be differentiated from the analyte through the use of their different m/z ratios.
- Deuterium may be exchangeable with the hydrogen that is found in water in aqueous solutions and samples
- This will lead to a change over time in the relative amount of internal standard that remains in the isotopically labeled form, thereby leading to an apparent change in the amount of analyte that is measured vs the internal standard.

ANALYSIS OF PEPTIDES AND PROTEINS

Liquid Chromatography of Peptides and Proteins

Separation Mechanism

- Size exclusion chromatography (SEC)–diameter of the molecule
- Ion–exchange chromatography (IEC)–electric charge of the molecule
- Reversed phase chromatography (RPC)–presence of hydrophobic domains
- Hydrophobic interaction chromatography (HIC)–presence of hydrophobic groups
- Affinity chromatography (AC)–biospecific interaction

Detection of Peptides and Proteins in LC and Capillary Electrophoresis

UV = 280 nm, < 230 nm

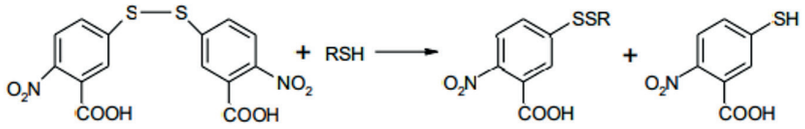
Derivatization and UV/VIS detection

Ninhydrin A570

Dabsylchloride A430

DTNB–selective detection of thiol groups

Absorption maxima at 412 and 357 nm

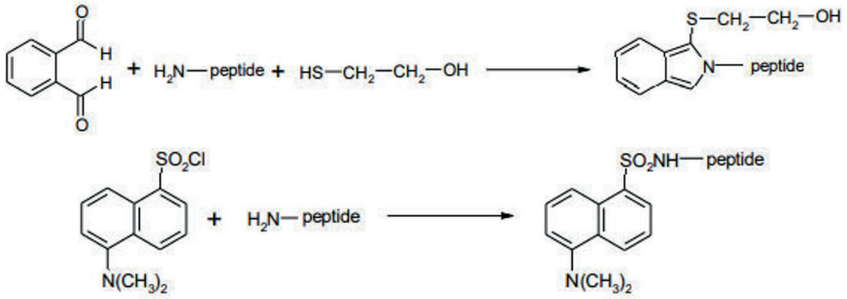


FLD–fluorescence of proteins (caused by Trp)

Derivatization and FLD

Reaction with o-phtalaldehyde and mercaptoethanol

Reaction with dansylchloride



Mass spectrometric detection

SEC of Peptides and Proteins

Gels

Modified dextran (Sephadex, Superdex), agarose gels…

Size of columns

4.6 × 250 mm, 10 × 300 mm, 16 × 600 mm…

Mobile phases

Conc. solutions of urea, guanidine aqueous solution of ana electrolyte (0.2–0.5 M) + buffer (e.g. 0.02 M phosphate) or simple buffer (Tris-HCl) sometimes an organic solvent is added (10–30%, MeOH or ACN)

Separation mechanisms

Low separation efficiency

IEC of Proteins

Stationary phases

Cation exchangers–groups -SO3-, -COO-

– anion exchangers–groups -CH2CH2-N+ Et3, -CH2CH2-NH+ Et2

Gradient elution

acidic buffer (cation exchangers)

– alkaline buffer (anion exchangers)

+ increasing concentration of an electrolyte

Separation

According to charge of the molecules at the actual pH

RPC of Peptides and Proteins

- Hydrophobic interactions between stationary phase and non-polar amino-acids (Phe, Val, Leu, Ile) bound in the separated peptides
- Expected retention time can be calculated as a sum of retention factors of the amino-acids plus a correction of the molecular arrangement

Stationary phases

SiC18, SiC8, SiC4, SiPh…

Porous graphitic carbon

Mobile phases

1. Water (or aqueous buffer solution) + acetonitrile (ACN) or methanol (MeOH) or tetrahydrofurane (THF)
2. (1) + ion-pairing agent most often cca 0.1 % trifluoracetic acid CF3COOH (TFA) or hexafluorobutane acid (HFBA) gradient elution (increasing portion of ACN or MeOH, constant conc. of TFA)

HIC of Peptides and Proteins

- Hydrophobic stationary phase
- **Mobile phase:** Quite concentrated aqueous solution of an electrolyte (several mol/l)
- **Gradient elution:** Decrease of the electrolyte concentration the hydrophobic interaction weakens
- Suitable for separation of native proteins

Electrophoretic Methods of Peptides and Proteins Analysis

- Gel electrophoresis in tubes or planar gel electrophoresis
- In agarose gels
- In polyacrylamide gels (PAGE)
- Capillary electrophoresis (CE)
- Capillary zone electrophoresis (CZE)
- Micelar electrokinetic (capillary) chromatography (MEKC, MECC)
- Capillary isoelectric focusation (CIEF)
- Capillary gel electrophoresis (CGE)
- Capillary isotachophoresis (CITP, ITP)

Isoelectric focusation, IEF

- In a gel with pH-gradient the particles of peptides or proteins migrate to get to the place of its isoelectric point
- Very high resolving power: the substances differing in pI by only 0.002 can be resolved

SDS-PAGE

- Electrophoresis in polyacrylamide gel in the presence of sodium dodecylsulphate (SDS)
- SDS is bound to proteins so that the molecules received the same electric charge
- Proteins are separated according molecular weight–larger molecules are less mobile

Two-dimensional Gel Electrophoresis (2D GE)

Detection of proteins in a gel

- Organic dyes, e.g. Coomassie blue
- Reaction with fluorescamine to form fluorescent derivatives
- Reduction of silver ions to metallic silver
- Blotting and application of immunochemical techniques

Capillary Electrophoresis (CE)

Sampling

- Hydrostatic method
- Hydrodynamic method
- Electrokinetic method

Electroosmotic flow

The inner surface of silica capillary is negatively charged; electric double layer is formed EOF

Capillary Zone Electrophoresis (CZE) of Peptides and Proteins

- The quality of separation depends on pI values of the compounds and the kind of buffer

- Interaction of molecules with the capillary surface peak brroadening, slow analysis
- Interaction suppression is achieved by increasing of ionic strength of the buffer
- Effect of pH of the buffer:
  - Too alkaline buffer: negative charge of both surface and protein molecules
- EOF is accelerated, migration is suppressed
  - Too acidic buffer: negative charge of the surface disappears, positive charge of proteins
- EOF is suppressed (pH 3)
- Micellar electro kinetic chromatography (MEKC) of peptides and proteins
- A surfactant is added to the buffer
- The separation is affected by the formation of micels of the surfactant and the analyte
- Non-ionic surfactants or anionic surfactants (SDS, alkanesulphonic acids)–the surface of the capillary remains negatively charged
- Cationic surfactants (dodecyltrimethylammonium bromide, cetyltrimethylammonium bromide, tetracecyltrimethylammonium chloride)–the surface turns positive
- The direction of EOF is reversed

Multi-dimensional Separation of Peptides and Proteins

- Liquid-chromatographic fractionation and sequential analysis of the fractions by the second separation technique:
  - SEC RPC
  - SEC IEC RPC
  - SEC CE
- Comprehensive two-dimensional liquid chromatography (LC LC)

Mass Spectrometry of Peptides and Proteins

- Fast atom bombardment (FAB-MS)
- Electrospray ionisation (ESI-MS)
- Matrix-assisted laser desorption ionisation (MALDI-MS) usually the instruments use a time of flight (TOF) analyzer
- Application in proteomics

Determination of Peptides in Food

Carnosin, anserin, balenin

- Occur in muscle tissue
- Allows identification of the kind of meat on the basis of carnosin/anserin ratio
- Markers of addition of animal protein

HPLC determination of carnosin and anserin

- Extraction and deproteinization by diluted HClO4

- IEC separation: Spherisorb SCX (4.6 250 mm)
- Mobile phase
  - A: 20 % ACN in 6 mM HCl
  - B: A+0.8 M NaCl
- Post-column derivatization oPA + ME
- Fluorimetric detection 340/445 n