Review of Literature
Heroin: history and chemistry

Opium is the latex produced within the seed pods of the opium poppy, *Papaver somniferum*. The name opium came from the Greek *opion*, or poppy juice. The plant has evolved from a wild strain, *Papaver setigerum*, which grew in coastal areas of the Mediterranean Sea. The species *somniferum* evolved through centuries of cultivation and breeding for opium. Today, *P. somniferum* is the only species of *Papaver* used to produce opium (Fig. 1). The major constituents of opium are morphine, codeine, noscapine, papaverine, and thebaine. Morphine is 3, 6-diacetyl derivative of heroin (hence it is named as diacetylmorphine) and was synthesized from it by acetylation. The white crystalline form is mainly the hydrochloride salt, known as diacetylmorphine hydrochloride. Fig. 2 shows the structures of all the opiate drugs. It works as painkiller but is also a source of other analgesic drugs such as morphine and heroin.

![Figure 1](image1.png)

*Figure 1:* (A) The poppy plant, *Papaver somniferum*; (B) seed pod, showing the exudates coming out from the top of seed pod.

Though heroin is known from ancient times, it was first synthesized in 1874 by C. R. Alder Wright, a British chemist working at St. Mary’s Hospital Medical School, London. Felix Hoffmann, of Bayer in Elberfeld, Germany used heroin as a medicine and registered it as trademark. From 1898 to 1910, it was marketed as a non-addictive
morphine substitute and cough medicine for children. All opiates get metabolized by liver into identical molecule with varying degrees of concentration in the blood stream.

Figure 2: Structures of Opiate drugs.

**Current Methods for Narcotic drug detection**

Opiates and their derivatives are very potent analgesics and have an important place in medicine (Dams et al., 2002). The illegal trafficking and abuse of heroin (diacetyl derivative of morphine) has become a widespread problem (O’Niel et al., 1984). Therefore, there is a need to accurately determine opiate alkaloids and their derivatives in a diverse range of samples (Hindson et al., 2007). Clinical and forensic testing for these compounds generally involves screening (by immunoassay) and confirmation (by chromatographic techniques), usually on biological fluids like saliva, blood and urine. Due to complex nature of these matrices the conventional methods for detection and analysis of opiate drugs include physico-chemical techniques like gas chromatography (GC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE),
mass spectrometry (MS) and their combinations such as GC-MS, LC-MS, GC-MS-MS etc (Bosch et al., 2007; Janicka et al., 2010).

Chromatographic Techniques

Thin layer chromatography (TLC) and high-performance thin layer chromatography (HPTLC)

Numerous TLC/HPTLC methods have been reported which can separate heroin from morphine (Steele et al., 1965), heroin or morphine from monoacetyl morphine (Mule et al., 1971) and heroin from acetyl codeine (Kaistha et al., 1972). While, lesser-abused opiate alkaloids, namely thebaine, papavarine and noscapine have not been studied extensively. There are three types of opiate alkaloids. First, the poppy alkaloids: morphine, codeine, thebaine, noscapine and papaverine; then, the semi-synthetic and synthetic derivatives used in therapy as antitussives and analgesics, such as pholcodine, ethylmorphine and dextromethorphan. Last narcotic compounds are diacetylmorphine (heroin) and opiates employed as substitutes in the treatment of addiction: buprenorphine and methadone. Common contaminants such as barbital, caffeine and strychnine can be distinguished by TLC from the compounds of interest namely codeine, heroin and morphine. For classical thin-layer chromatography (TLC) of opium alkaloids, it is necessary to use complex eluents with strong alkaline substances to obtain a clean separation between morphinan and isoquinoline compounds. Based on TLC a sensitive method was developed for the quantitative estimation of 16 opioids where further addition of AgNO₃ and KMnO₄ after iodoplatinate, increased its sensitivity (Gorodetzky et al., 1972).

A simple and rapid method for the detection of heroin and its metabolites in blood and urine employs the alkaline hydrolysis in an autoclave for 15 min followed by two-dimensional TLC using ethyl acetate:methanol:ammonia (85:10:5) and ethanol: dioxane: benzene: ammonia (5:40:50:5) as developing solvent systems (Win et al., 1983). For the detection of opiates in opium samples, as well as opiates and adulterants in illicit heroin samples, TLC system was developed, using the chloroform: hexane: triethylamine (9:9:4), by the National Drug Research Centre, Penang, Malaysia, and was most suitable on both laboratory coated and pre-coated plates. In addition, the following two systems,
one on laboratory coated plates chloroform: hexane: diethylamine (30:50:7) and the other on the precoated plates benzene: dioxane: ethanol: ammonia (50:40:5:5) were also amongst the most suitable TLC systems for the analysis of opiate samples (Rajnanda et al., 1985).

A good separation of the components of various chemical classes of alkaloids can be achieved by over-pressured thin-layer chromatography (OPTLC) on aluminium oxide plates with ethyl acetate alone as the mobile phase. A clinical study on urine samples of drug addicts for opioids (morphine, codeine, buprenorphine, dextropropoxyphene, pentazocine) and benzodiazepines (diazepam, nitrazepam) (Jain et al., 2000; Margoob et al., 2004) was done.

Automated multiple development (AMD) TLC gradient method could successfully separate these opiate alkaloids (Pothier et al., 2005). HPTLC has been used for the quantitative detection and screening of the drug ketobemidone in human urine samples with a cut off value at 0.2 μg/ml, which is to level of the one obtained from LC-MS of these samples (Woods et al., 2008).

TLC techniques is mainly used as a basic or primary screening technique to detect opiate and non-opiate compounds in illicit samples which are based on liquid-liquid extractions at controlled pH, solvent concentration and finally identification and semiquantitation by TLC (Reinhardt et al., 2009).

As quantitative procedures, TLC/HPTLC is inferior to GLC or HPLC in terms of rapidity, ease of operation, convenience, precision and sensitivity. However, TLC/HPTLC techniques plays an invaluable role when used as preceding steps to more detailed analysis and can be carried out simply, cheaply and rapidly.

Gas Chromatography (GC) and GC-MS:

Gas chromatography (GC) is superior to TLC/HPTLC as it offers greater selectivity for the separation of various compounds. Numerous GC methods have been published (Kaistha et al., 1972; Gudzinowicz and Gudzinowicz, 1978) for the quantification of heroin and other opiate drugs in illicit traffic on a routine basis. The thermal stability of heroin does not appear to pose any problems.
Baker et al., (1981) devised an identification system using three solvent column systems (both normal and reverse phase). The relative retentions and the ratio of absorption at 254 nm and 280 nm using these three chromatographic systems are used to identify compounds viz. morphine, codeine, heroin, papaverine and noscapine (narcotine). It was reported that 95% of the drugs of forensic interest are characterized using these procedures. Reverse phase chromatography is the most popular mode of liquid chromatography at present. Ion-exchange chromatography generally gives poor reproducibility and causes problems of reduced column life. The use of paired-ion chromatographic reagents with reverse phase systems is a recent innovation. The drugs of interest, however, are satisfactorily eluted by adjusting the pH of mobile phase using appropriate buffers (Love and Pannell, 1980).

An integrated (GC/MS) system combines the unexcelled identification of mass spectrometry with gas chromatography’s powerful separation capabilities. In view of the high running and instrument costs, as well as the complexity of operations, it is difficult to envisage GC/MS as a routine method for opiate identification in the near future. It will, nevertheless, play an essential role in confirmative and definitive analyses of drugs. GC-MS is one of the recommended analytical techniques for the identification and confirmation of opiates in urine (Solans et al., 1990; Maurer et al., 1992).

Owing to the selectivity and sensitivity of the GC/MS/MS analysis, an extremely simple and rapid method was developed which fulfilled the requirements of both clinical and forensic diagnosis of heroin use by direct treatment of methanol washed hair with silylating solution for the extraction of heroin, 6-monoacetylmorphine, morphine, acetylcodine, and codeine (Polettini et al., 1993).

Recently a very sensitive GC technique, surface ionization detection (SID), which is very specific to tertiary amino compounds, was developed, that may allow the trace determination of drugs present in small samples, such as blood stains and hair, extending its applicability in forensic toxicology (Hattori et al., 1994). 6-monoacetylmorphine (6-MAM) is a good indicator for the intake of heroin and was detected in blood, urine and hair of heroin users by GC-MS (Moeller et al., 1995).

The identification and quantification of residues of illegal drugs in human hair is still an important task for the forensic toxicologist. Utilizing its advantages, GC/MS/MS has
been used for the testing of human hair, for most common drugs of abuse like heroin and other opioids, cocaine, cannabis and amphetamine derivatives (Uhl et al., 1997). More recently gas chromatography–fourier transform infrared spectroscopy (GC–FTIR) became the most powerful technique applied for the identification of opiate drugs. The accuracy, sensitivity and selectivity of the system recommend its use for automating the investigations of illicit drugs for epidemiological, clinical, administrative and forensic purposes (Praisler et al., 2000). A new sample preparation technique, high-temperature headspace solid-phase microextraction (SPME), for the screening and confirmation of illicit drugs in urine and in serum was done by GC-MS (Staerk et al., 2000).

In this continuation, another simple and sensitive method for the determination of pethidine has been developed by GC-MS/MS, which is a narcotic analgesic drug in body fluids (Ishii et al., 2003; Dumasia et al., 2003).

Further, two-step silylation for the derivatization of 51 drugs of abuse, was analyzed by the combination of two different gas chromatographic (GC) separations with both electron capture detection (ECD) and mass spectrometry (MS) operating in a selected ion monitoring (SIM) mode (Gunnar et al., 2004). An extraction and determination method of the most important amphetamine derivative in serum has been developed and detected for various opiate molecules (Amri et al., 2004; Hidvegi et al., 2006).

A rapid analysis of methamphetamine and its metabolites in urine is performed by GC-MS using a short narrow-bore capillary column (NBC) (5 m x 0.1 mm I.D.) by selected ion monitoring (SIM). Linearity is found to be over a range from 25–2500 ng/ml (Fujii et al., 2006).

A comprehensive screening method has been developed for the detection of narcotic agents by GC with selected ion monitoring mode. The screening method developed, reduces the amount of urine required and increases the sample throughput without a loss in sensitivity and selectivity (Thuyne et al., 2008).

Methamphetamine (MA) is the most commonly abused drug. An analytical method has been developed by using GC for the determination of methamphetamine in oral fluid (Choi et al., 2009; Bonadio et al., 2009).
High Performance Liquid Chromatography (HPLC)

Since UV absorption and fluorescence properties are exhibited by so many compounds of diverse chemical structures, the specificity of HPLC system is only as good as the separation achieved. Several HPLC have methods like Ion exchange chromatography (Knox and Jurand, 1973), Normal phase (Verpote and Svendsen, 1974), Paired ion reverse phase chromatography (Soni and Duger, 1979), Isocratic reverse phase chromatography (Love and Pannell, 1980; Nobuhara et al., 1980; Poochikian and Craddock, 1980) have been used for the detection and quantification of heroin and other opiates agents.

The quality of detection, therefore, depends on the capabilities of the detection system used. Conventionally, spectrophotometry (Love and Pannell, 1980) and fluorometry (Nelson et al., 1982) are most often used as detectors in HPLC systems used for the analysis of heroin and opiates, while capillary column techniques have been developed for the analysis of morphine, 6-acetylmorphine and codeine (Edlund et al., 1981). A common method of heroin detection used in various testing laboratories employs the GLC system using a stationary phase of 3 % OV 17 silicone gum (Gough et al., 1981). Another approach was put forward to gain high sensitivity by using a double-beam, diode array detector incorporating a fast, integrated microprocessor. The usefulness of this innovation in the analysis of heroin and other opiates has not been investigated (Black et al., 1986).

An improved method for the determination of codeine and morphine simultaneously by using HPLC with automated on-line extraction coupled with electrochemical detection in plasma and urine samples was developed by Heybrock et al., (1990). A fast method was developed for the screening of eleven β-blockers, two narcotic analgesics and two stimulants in urine by HPLC with column switching (Saarinen et al., 1995). Determination of amphetamine in illicit drug samples by HPLC with UV detector was developed by Sadeghipour et al., (1997).

A recent approach is column switching HPLC electrospray ionization mass spectrometry (ESI-MS) for the identification of heroin metabolites in human urine. Urine samples can be directly introduced to the system, and endogenous urinary constituents are removed by using on line column switching solid phase extraction with a strong cation exchange.
(SCX) cartridge column (2.0 mm I.D x 10 mm). Heroin and its metabolites enriched on the top of the column were then successfully analyzed with excellent separation by use of a SCX semi microcolumn (1.5 mm I.D x 150 mm), accompanied by ESI mass spectral detection. The detection limits were in the range of 0.1-3.0 ng/ml (Katagi et al., 2001). Based on a new type of packing for HPLC columns (monolithic silica), a routine, rapid and simple analysis of heroin samples, containing a mixture of an illicit heroin was developed which could separate well resolved peaks in 7 min (Macchia et al., 2004).

Determination of d-3-methoxy-17-methylmorphinan (Dextromethorphan), which is a structural analogue of morphine and codeine, was done by HPLC equipped with PDA (Kim et al., 2006). Direct detection of glucuronide metabolites in biological fluids by HPLC overcame the critical limitations of approaches that involve enzymatic cleavage procedures or derivatization (Kaushik et al., 2006).

A segmental hair analysis for the retrospective multi-parameter evaluation of drugs of abuse including opioids, cocaine and amphetamines was carried out with the use of liquid chromatography-atmospheric pressure chemical ionization- tandem mass spectrometry (LC–APCI-MS-MS) (Klys et al., 2007).

For qualitative and quantitative analyses of phenethylamine in real products including biological specimens simultaneous determination method, based on ultra-fast liquid chromatography coupled with fluorescence detection (UFLC–FL) was also developed (Min et al., 2008).

Separation of drugs at an elevated pressure with 1.7 µm hybrid C18 stationary phase HPLC column provided a significantly better resolution and faster analysis than conventional HPLC and capillary electrophoresis (CE). Twenty-four solutes of varying drug classes including narcotic analgesics, stimulants, depressants, hallucinogens, and anabolic steroids were fully separated in a 13.5 min gradient (Lurie et al., 2009).

Kwok et al., (2010) developed a rapid liquid chromatography-tandem mass spectrometry (LC–MS–MS) method for the simultaneous screening of 19 drugs of different classes in equine plasma using automated on-line solid- phase extraction (SPE) coupled with a triple quadrupole mass spectrometer. Over 70% of the drugs studied gave detection limits at or below 100 pg/ml, with some detection limits reaching down to 19 pg/ml.
Before set up of any experimentation, it is essential to select the sample type and the instrumental technique applied for the appropriate sampling, extraction and purification of illicit drugs (Janicka et al., 2010).

**Electrophoretic Techniques**

In the early 1990s, CE was introduced in the forensic field by the pioneering work of Weinberger and Lurie (Lurie et al., 1995) who showed the potential of this technique for the separation of 18 illicit drugs, as well as heroin impurities and adulterants.

Recent advances in the technology have found application of electrophoresis in the field of drug monitoring in biological fluids (Altria et al., 1998; Lemos et al., 2001; Piette et al., 2002). Capillary electrophoresis is a narrow-bore fused-silica capillary to separate a complex array of large and small molecules. High electric field strengths are used to separate molecules based on differences in charge, size and hydrophobicity. CE separations can be carried out using capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE) and capillary isotachophoresis (CITP) (Wernly et al., 1993; Tagliaro et al., 1996; Wright et al., 1997; Bjornsdotir et al., 1999; Piette et al., 2002).

A capillary electrophoretic (CE) method was developed for the quantitation of complex seizures of amphetamines and related substances which involves the use of minimal organic solvent and had a resolving power which allows the separation of certain compounds (Trenerry et al., 1995; Tagliaro et al., 1998). Small amount of drug can be detected, where separation is performed in fused silica capillaries with internal diameters of 25-100 μm and they provide very high theoretical plate numbers and along with sensitive detection methods such as UV-absorbance and fluorescence which makes it highly promising technique. This technique is gaining popularity because, various modes, which can be used for separation of compounds (Martinez et al., 2000).

A fast and simple method for separation of 16 seizure drug substances using capillary electrophoresis in a non-aqueous separation medium was developed where by controlling the choice of solvents, electrolytes and viscosity increasing additives it was possible to
separate all 16 amines within 2 min with the limit of detection 1 μg/ml (Macchia et al., 2001).

Amperometric detection, followed by capillary zone electrophoresis was developed for the detection of codeine and its metabolites in human urine samples with the detection limits for codeine and morphine $1.6 \times 10^{-7}$ and $6.8 \times 10^{-8}$ mol/l (Zhou et al., 2002).

Capillary electrophoresis (CE) with Ru(bpy)$_2^{2+}$ electrochemiluminescence (ECL) detection system was established for the determination of contamination of banknotes with controlled drugs and a high efficiency field amplified sample stacking (FASS) technique, with the detection limits of 50 nM for heroin and 60 nM for cocaine, respectively (Xu et al., 2006).

An optimization and validation of a capillary zone electrophoresis method has been done for the determination of methamphetamine, morphine, cocaine and 3, 4-methylenedioxymethamphetamine (MDMA) in hair by field-amplified sample stacking. The limit of detection was found to be 20 mg/ml, 2 ng/ml for MDMA, 8 ng/ml for cocaine and 6 ng/ml for morphine (Iwata et al., 2006; Dahlen et al., 2006). Depending on the types of capillary and electrolytes used, the technology of CE can be segmented into several separation techniques.

**Spectroscopic techniques**

**FTIR-ATR spectroscopy**

Gas chromatography-Fourier transform infrared spectroscopy (GC-FTIR) is becoming the most powerful technique applied for the identification of opiate drugs because of the best selectivity in identifying the structural features associated with the full constellation of pharmacological effects of amphetamines (Praisler et al., 2000).

Schulz et al., (2004) performed analysis of opiates directly from poppies in which they looked at the presence of alkaloids in breeding samples. They used Fourier transform (FT) infra-red spectroscopy (IR) and near infra-red (NIR)-FT–Raman spectroscopy techniques. Numerous examples of utilizing this technique to create a sensor exist in the literature often based on the modification of the surface of ATR plate
with a variety of molecules for chemical and biological sensing (Acha et al., 2000; Roberts et al., 2005; Devouge et al., 2009).

Field analysis of opiates in poppy field when combined with FTIR-ATR, can be used for the quantification by untrained personnel. This technique includes the use of basic chemicals and simple method for analysis which enables the identification of minor compounds. The limits of detection for morphine and thebaine were 0.13 mg/ml and 0.3 mg/ml respectively (Turner et al., 2009). These techniques were applied for the simultaneous identification and quantification of opiate alkaloids (Jalsovszky et al., 2009).

**Raman spectroscopy**

It has advanced rapidly in recent years for commercial field applications (Harvey et al., 2002). Raman, IR and surface-enhanced raman scattering (SERS) is employed for the vibrational characterization of papaverine. The vibrational assignments are performed by means of density functional theory (DFT) calculations.

The FT-SER spectrum of papaverine obtained with the developed experimental setup exhibits the same characteristics as the SER spectrum (Leopold et al., 2004). Raman spectra are dramatically amplified when a molecule is adsorbed onto nano roughened noble metal surfaces such as silver and gold. The degree of enhancement enables single-molecule detection, which offers the potential for the unambiguous identification of low-level narcotic drugs (Ryder et al., 2005).

Fibers are one of the most common forms of evidence associated with forensic investigations. The use of adhesive lifters to recover fibers from crime scene samples has long been established as an effective method to recover such items of evidence. Samples of seized ecstasy, cocaine, ketamine and amphetamine, detected by Raman spectra showed that it was possible to identify drugs of abuse from particles trapped within the fibers without interference from the fiber itself (West et al., 2009).

Technological achievements of Raman scattering redeveloped the Raman spectroscopy to become the most adaptable spectroscopy technique for non-invasive chemical analysis of hazards within non-transparent containers and packaging (Brody et al., 2009; Izake et al., 2010).
However, these conventional methods for opiate detection have disadvantages and suffer from drawbacks. Most are complex and time consuming and require costly, bulky instrumentation. Sample preparation for these techniques is tedious and prolonged, and requires skilled personnel, so they are unsuitable for field studies and in situ monitoring of samples. There has therefore been a great demand for developing inexpensive, reliable assay techniques for effective field monitoring of these opiate molecules. Modern techniques to eliminate some of these difficulties include immunochemical techniques, which are based on the specificity of the antibody–antigen (Ab–Ag) reaction. Immunosensing is becoming attractive because Abs can be produced against opiate molecules with low molecular mass (Gandhi et al., 2008; 2009; Tey et al., 2010). Most immunosensors can obtain quantitative results with similar or greater sensitivity, accuracy and precision than other analytical methods because of the availability of high quality Abs against target analytes. Immunosensor technology is therefore important for analysis because it complements existing analytical methods and it provides low-cost confirmatory platform for many compounds, including pharmaceuticals and opiates.

**Transducer based sensors for opiate analysis**

Immunosensors were designed so that the formation of an immune complex on the transducer surface was directly determined by measuring the physical changes (electrical or optical) induced by the binding interaction. Either Antibody or Antigen was immobilized on the transducer surface to form a sensing device and allowed to react with the complementary Ag or Ab to form an immune complex. This formation altered the physical properties of the surface. Depending upon the transducer technology employed, immunosensors were broadly classified into electrochemical, optical, and micromechanical.

[A] **Piezoelectric Immunosensors**

The main principle underlies is the small change in the mass at the surface of crystal led to a change in resonant frequency due to the formation of antigen-antibody complex (Sauerbrey et al., 1959). A crystal of piezoelectric material is sandwiched between two
electrodes and is based on the measurement of small changes in mass due to the formation of immunocomplex on the crystal surface (Fig. 3). The change in the resonant frequency (ΔF) of at the crystal surface (Δm) due to change in the mass can be explained by Sauerbrey equation (Sauerbrey et al., 1959):

\[ \Delta F = -kF^2 \Delta m/A = -2.26 \times 10^{-6} F^2 \Delta m/A \]

where, ΔF is the change of frequency (Hz) due to coating, k is the proportional constant depending upon density and shear modulus of quartz crystal (for AT-cut quartz, the density is 2.648 g/cm³ and shear modulus is 2.947 \times 10^{11} dynes/cm²), F is the fundamental frequency (MHz) of the quartz crystal, Δm is mass (g) of coating deposited, and A is the coated area of the crystal (cm²).

Various groups have reported piezoelectric sensors for the detection of various infectious agents such as for standardization of antibodies (Skaladal et al., 1994) environmental monitoring (Yokoyama et al., 1995), narcotic drugs as follows: A fiber optic evanescent fluoroimmunosensor was developed for the rapid detection of cocaine in leaf extracts of *Erythroxylum* species. The developed fluoroimmunosensor was based on the simple absorption of light by the layer of substrate/ligand bound to biomolecular layer lead to change in fluorescence, phosphorescence, chemiluminescence and bioluminescence etc. The developed biosensor assay was rapid and could detect up to 40 ng/ml in crude leaf extracts (Toppozada, et al., 1997).
Figure 3: (A) Piezoelectric crystal construction, (B) Piezoelectric crystal bound with receptor molecules for antigen detection, and (C) QCM set-up for analysis.

[B] Electrochemical Immunosensors

Various groups used electrochemical sensors for detection of opiate drugs, based on the measurement of amperometric, potentiometric, conductimetric and capacitive changes associated with the Ag-Ab interactions which lead to change in current, potential (voltage), conductance and capacitance respectively.
The first enzyme based method for the electrochemical detection of heroin and morphine was developed by using an enzyme heroin esterase and morphine dehydrogenase with the detection limit of 6.8 µg/ml for morphine (Fig. 4). Heroin esterase resulted in the conversion of heroin to morphine and further oxidation of morphine to morphinone by morphine dehydrogenase resulted in the reduction of NADP⁺ (Bruce et al., 1990 and 19991; Cameron et al., 1994; Holt et al., 1995).

A series of experiment in this continuation was developed by using the same enzymes heroin esterase and morphine dehydrogenase (Fig. 5). The bioluminescent assay was developed with the detection limits of 89 ng/ml for heroin and 2.0 ng/ml for morphine by using a bacterial luciferase from *Vibrio harveyi* (Holt et al., 1996).
Another enzyme based method for the simultaneous detection and quantification of morphine and codeine was developed by flow injection analysis involving morphine dehydrogenase and laccase. The developed method could discriminate between morphine and codeine in less than 1 min after injection and could detect up to 2-1000 µM for morphine and 5-1000 µM for codeine (Bauer et al., 1999).

The electrochemical detection of morphine was done by using prussian blue modified indium tin oxide which oxidized the phenolic -OH group of morphine through catalytic oxidation at an electrode surface where the density of electron was maximum. The morphine antibodies were immobilized on the surface of electrode which resulted in the generation of specific signal. The sensitivity of the developed assay was found to be 16.8 µA/cm² mM (Ho et al., 2004).

Further, chemiluminescence (ECL) based flow injection method was developed for heroin by using tris (2, 29-bipyridyl) ruthenium (II) (Ru(bpy)_3)²⁺. The oxidation of heroin resulted in transfer of electron in the form of ECL signal. The sensitivity of the developed assay was in the range of 2.0–80 µm/dm³ (Zhuang et al., 2005).

Another electrochemical method for the fast monitoring of morphine was developed by using flow injection system. The detection scheme involved the change in the current on the surface of electrode due to the oxidation and reduction of the adsorbed analyte. The
sensitivity of the developed assay was found to be 95.5 and 285 pg/ml (Norouzi et al., 2008).

**[C] Carbon Nanotube based Immunosensors**

The development of nanotechnology as a vast area of research paved the way for miniaturized sensors or nanosensors, which results in reduced weight, lower power consumption, and low cost. Nevertheless, the discovery of carbon nanotubes (CNTs) has generated keen interest among researchers to develop CNT-based sensors for many applications. Bioapplications of carbon nanotubes (CNTs) have attracted much attention in recent years. Ever since the first demonstration of CNT sensing capability on gas molecules (Kong et al., 2000), numerous works were carried out on a variety of biological and bioactive species such as proteins, peptides, DNA, enzymes, antibodies (Huang et al., 2002; Besteman et al., 2003; Star et al., 2006; Poenitzsch et al., 2007; Tey et al., 2010) and their interaction with CNTs was refined for better attachment.

Carbon nanotubes (CNTs) are used for sensing applications due to several intriguing properties. Carbon materials have been used for electrochemical sensing for over a decade. The meticulous feature is their large length-to-diameter aspect ratio which provide high surface-to-volume. In addition, CNTs have an exceptional capability to mediate fast electron-transfer kinetics for a wide range of electroactive species. In addition, chemical functionalization can be used for the attachment of CNT to nearly any desired chemical species, which leads to enhance the solubility (well-dispersed in an appropriate polymer matrix) and biocompatibility of the tubes (Lu et al., 2009). Among other factors, the development of novel biomaterials such as functional polymers, sol-gel materials has also led to an increased use of CNTs as components in biosensors.

Therefore, CNTs, with their attractive electrical properties are perfect model for electrodes and transducer based biosensors. Individual SWCNTs are extremely sensitive to their surrounding environment. Both chemiresistors and chemically sensitive field effect transistors (FETs) incorporating pristine or functionalized CNTs have been shown to be capable of detecting biomolecules measured (Eugenii et al., 2003; Wang et al., 2004; Gui et al., 2007).
A highly sensitive assay was developed for the detection, using carbon nanotubes immobilized on preheated glassy carbon electrode which was based upon the efficient electrocatalytic oxidation for morphine with relatively high sensitivity, stability and long life. The detection limit for the amperometric response was 0.2 μM (Salimi et al., 2004).

A CNT network or single CNT deposited across two metal contact pads as the semiconducting channel of the transistor which function as the sensing interface (Fig. 6). Similar approach was used for the determination on morphine and noscapine using glassy carbon electrode with modified carbon nanotubes, with the detection limit of 8.0×10⁻⁸ mol/l in blood and pharmaceutical sample (Rezaei et al., 2008; Li et al., 2009).

Figure 6: Different schemes for the detection of opiate drugs using a carbon nanotubes (CNT) liquid gated field effect transistors; [A] Direct detection of antibodies; [B] Indirect detection of antibodies by using
To provide sensing selectivity, a specific recognition group (also called a receptor, ligand or probe) is anchored to the CNT channel interface to allow specific recognition of its target analyte in the subsequent step. Real time detection of MAM molecules using our developed novel flexible CNT-LGFET (Tey et al., 2010) with potential LOD beyond femtograms per milliliter in standard buffer solution was demonstrated.

**[D] Micromechanical Immunosensors**

In recent years, the detection approach of cantilevers has increased attention in immunosensing. The formation of immune complex onto the cantilever surface due to specific biomolecular and chemical interaction between ligands and receptors results in physical bending of the cantilever beam in the range of nanoscale or even lower. This mechanical bending leads to change in the surface stress due to antigen-antibody binding and offers a quantitative measurement.

The selectivity and sensitivity of the assay is governed by the molecular probe (receptor) layer on the cantilever surface and by the degree of cantilever deflection after immunocomplex formation [Fig. 7]. Cantilever based sensors are a promising label-free technique for detecting diverse specific biomolecular and chemical binding events such as DNA hybridization, protein–protein interactions, and many others because of the thermodynamic origin and the amenability to conventional fabrication procedures.

The absolute deflection of the cantilever, $\Delta z$, can be measured using an optical detection system in liquid with constant flow of Ag. The deflection signal resulted in a differential surface stress, $\Delta \sigma$, so, using Stoney’s equation:

$$\Delta \sigma = \frac{1}{2} (t/L)^2 E/(1-\nu) \Delta z,$$

where $L$ is the effective length of the cantilever, $t$ is the thickness, and $E/(1-\nu)$ is the ratio between the Young’s modulus, $E$ (130 GPa), and Poisson ratio $\nu$ of Si (0.28).

A molecularly imprinted polymer in combination with electrochemical sensing was used for the detection of morphine by using a microfluidic system. The sensitivity of the
developed sensor was 171.5 μA/cm² mM with the detection limit for morphine from to 0.2 mM (Weng et al., 2007).

Figure 7: Micromechanical detection of antibody-antigen interactions. Antibodies are immobilized the surface of gold silicon nitride cantilever and after the binding of target antigen to the probe, deflection occurs due to change in the surface stress of cantilever.

[E] Antibody based Immunosensors

Since, opiates molecules are of molecular mass less than 1000 Daltons. Formic immunocomplex (antibody-antigen) on transducer surface with these smaller molecules in direct immunoassay format usually did not produce significant shifts in physical parameters of mass/optical/electro-chemical transducer devices. These molecules therefore coupled with some carrier such as proteins, enzyme or fluorescence tracer then used in immunoassay development for screening and selection of antibody. Antibodies bound to their antigens with very high degree of specificity which can be exploited for the development of immunoassays which could detect opiate drugs in per billion to lower parts per million ranges. As per the label used, immunoassays can be classified into radioimmunoassays (RIAs), chemiluminescent immunoas:
The competitive immunoassay format monitors the competition between labeled and unlabeled antigen for limited available binding sites of antibodies on transducer surface. Free binding sites of the antibody exposed to the antigen are then monitored by binding to the immobilized antigen. This can also be accomplished by immobilizing antigen on the carrier surface. Fig. 8 shows the immunoassay format for the indirect [A] and competitive [B] assay.

Figure 8: Schematic for [A] Indirect and [B] Competitive Immunoassay.
**[a] Polyclonal Antibody based Immunosensor**

For immunoassay based detection of narcotic drugs, it is imperative to generate an antibody that shows high sensitivity and broad specificity towards target molecule and its metabolites. Polyclonal antibodies have greater advantage that where we can have large pool of antibodies and in its native conformation, which is not in case of scFv/Fab antibodies, therefore are more stable as compared to scFv/Fab due to the presence of heavy chain in constant region.

Polyclonal antibodies based method was developed for the detection of morphine-3-glucuronide (M3G), which is a major metabolite of heroin. The developed immunoassay for M3G in urine showed detection of 762 - 24,400 pg/ml (antibody 1) and 976 - 62,500 pg/ml (antibody 2) (Dillon et al., 2003). In another SPR based assay carried out in continuation of the previous work of Dillon where, the limit of detection for morphine-3-glucuronide (M3G) was even to the level of 270-17,500 pg/ml and the chip was used up to total 15 regeneration cycles to make it cost effective (Dillon et al., 2005).

A similar approach with slight modification was developed by using an immunoaffinity column for the detection of morphine and other metabolites in the urine of heroin abusers. The developed assay showed good reproducibility and sensitivity with the detection limit ranging between 91–105% and 10–20 ng/ml (Qi et al., 2005).

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**Figure 9:** Schematic of SPR (surface plasmoc resonance) based immunosensor. (A) The sensor chip consists of a glass slide with a thin layer of gold which provides chemical inertness and good SPR response. This gold layer is in turn covered with a covalently bound carboxymethylated (CM) dextran matrix attached by a hydroxalkyl thiol linker layer. The matrix allows the covalent immobilization of analytes onto the surface of the chip. [B] Typical sensogram obtained after the interaction of light energy with the gold film and this is used to monitor concentrations of analyte on the surface of the chip.
An indirect competitive immunoassay for the detection of papaverine was developed by using a conjugate of mono demethylated papaverine-O-carboxymethyl ether (MDMPAP-O-CME) and bovine serum albumin (BSA) and used as an antigen (PAP-BSA). The assay showed high affinity to papaverine with an affinity constant of $7.3 \times 10^7$ l/mol. (Yan et al., 2005).

The SPR based immunoassays were highly specific for the detection of small molecules with low detection limits for a wide variety of small molecules usually less then 1000 Da (Fig. 9) (Shankaran et al., 2007). A novel detection method was developed by using lipoate derivatives by formation of imprinted self assembled molecular thin films for morphine in an aqueous environment. Morphine was added as a template on gold substrate. The binding studies were done for the association/dissociation of morphine with SPR and the limits were found to be 10 μM for morphine (Tappura et al., 2007).

A fluorescence based assay was developed for the detection of monoacetyl morphine by using a carboxylic acid derivative of monoacetylmorphine (MAM–COOH), synthesized and conjugated with bovine serum albumin (BSA) for the generation of polyclonal antibodies. The relative affinity constant ($K_a$) of the antibody was $3.1 \times 10^7$ l/mol, and the IC$_{50}$ values obtained for heroin, MAM, morphine, and codeine were 0.01, 0.013, 0.012, and 0.014 ng/ml, respectively (Gandhi et al., 2008).

A protein array microchip based label-free detection of heroin, cocaine, ecstasy and amphetamine was developed by SPR with the detection limits of 0.5 pg/ml for heroin, 2.5 pg/ml for cocaine, 5.0 pg/ml for ecstasy and amphetamine (Klenkar et al., 2008).

The use of enzyme and colloidal gold based reagents started an era of rapid diagnostic immunoassays because of low hazard and no requirement of sophisticated instrumentation. The first target molecule for such assays was Human Chorionic Gonadotropin for diagnosis of pregnancy (Santalahti et al., 1998). Various enzyme-based and colloidal gold-based rapid immunoassays were developed for detection of opiate drugs up to 2.5 ng/ml (Gandhi et al., 2009).
**[b] Monoclonal Antibody based Immunosensor**

Hybridoma technology provides an excellent alternative, where monoclonal antibodies (mAbs) can be produced in unlimited quantity with constant characteristics. Besides, mAbs of desired affinities can also be selected (Malaitsev et al., 1993).

A fluorescence based competitive immunoassay was developed for the detection of morphine in urine samples by using monoclonal antibodies. For this, morphine was conjugated with fluorescein molecules and used as a tracer. The dissociation equilibrium constant ($K_d$) of tracer with anti-morphine antibody was 0.23 nM. The sensitivity of the developed assay for morphine-3-glucuronide (M$_3$G) was 0.2 ng/ml with an IC$_{50}$ of 2 ng/ml. Other opiates and heroin metabolites showed 50 % cross reactivity, when present at 1 µg/ml included codeine, morphine-6-glucuronide, and oxycodone (Eldefrawi et al., 2000).

**[c] scFv Antibody based Immunosensor**

Phage display is now used widely in making recombinant antibodies for clinical, therapeutic and diagnostic applications (Fig. 10). The major advantage of phage display technology as compared with standard hybridoma technology is that the specific scFv/Fab fragments to a particular antigen can be generated in a couple of weeks, animal immunization, sacrifice and bleeding can be avoided, isolation and production is cheaper than monoclonal antibodies. The developed recombinant DNA technology offers an added advantage for the production of novel antibody system which can be possible after suitable cloning of antibody genes in phage display vector, thus improving the sensitivity and reproducibility of immunoassay immunoassay (Robinson et al., 1981; Arnold et al., 1990).

The recombinant antibodies were developed against morphine-3-glucuronide (M$_3$G) by immunizing mice with M$_3$G-BSA conjugate. The developed single chain fragment variable (scFv) antibodies were further characterized by using SPR. The inhibition studies were done for M$_3$G and the developed assay showed the sensitivity ranges between 3-195 ng/ml and 3-97 ng/ml in PBS and urine respectively (Dillon et al., 2003).
Figure 10: Schematic for displaying phage antibody library and stepwise selection of binders for selective antigen.
Similar studies were done in saliva for morphine detection by ELISA and SPR based assays. The sensitivities were found in the range of 6.1-5162.5 ng/ml by ELISA and 381.5-781.250 pg/ml by SPR in saliva (Brennan et al., 2003).

Antibodies against 6-acetylmorphine were already reported and were identified by screening with high density colonies of *E. coli* expressing soluble scFv antibody fragments without prior expression on bacteriophage (Moghaddam et al., 2003). The developed antibodies showed the ability to produce large number of recombinant antibodies from a phage display library and also provide altered affinity and specificity to particular antigen. Therefore, the generation of functional antibody fragments has now become a powerful tool in the diagnostics which could be explored for the detection and diagnostic applications.

**[F] Aptamer based Immunosensors**

Aptamers are nucleic acid molecules which possess high affinity and specificity toward their target analyte. These aptameric structures offers an unprecedented advantages such as imitation expediency, chemical stability etc. and have become progressively more important sensing tool for diagnostics applications.

![Aptamer sequence and folding pathway](image)

**Figure 11:** The sequence of the DNA aptamer and a proposed folding pathway. The binding of target with probe triggers the formation of aptamer-target complex.
RNA aptamers bound to codeine was developed by using an in vitro SELEX process (Fig. 11). The binding properties of these aptamers were determined by SPR where two highest binding aptamer sequences, FC5 and FC45 were showed $K_d$ values of 2.50 and 4.00 $\mu$M, respectively (Win et al., 2006).

A novel electrogenerated chemiluminescence (ECL) cocaine binding aptamer was designed for the detection of cocaine and employed ruthenium complex as an ECL label. The integrated ECL intensity versus the concentration of cocaine was linear in the range from $5.0 \times 10^{-9}$ to $3.0 \times 10^{-7}$ M and the detection limit was $1.0 \times 10^{-9}$ M (Li et al., 2007).

Bioassay strategy using gold nanoparticles for the determination of cocaine, potassium, adenosine and engineered DNA aptamers was also developed. An aptamer was synthesized as two random coils of single stranded DNA, which was reassembled further into the intact tertiary structure in the presence of specific target. AuNPs was used to differentiate between these two forms of DNA due to change in the characteristics of SPR. This method could be used for cocaine detection in the low micromolar range (upto 4.0 $\mu$M) within few min (Zhang et al., 2008).

![Figure 12](image)  
**Figure 12**: The sequence of the cocaine DNA aptamer construct used in this study and a proposed folding pathway. The three positions that were independently labeled with nucleoside $C$, in aptamers 1–3 are shown in color. The binding of cocaine (filled black circle) triggers the formation of a three-way junction. The structure of cocaine is shown above the arrows (Cekan et al., 2009).

The aptamer structural and conformational properties changes due to incorporation of cocaine at 3 different positions (Fig. 12). The DNA aptamer binds with a
cocaine molecule at the junction of three helices. Nucleoside C contains a rigid nitroxid spin label and can be studies directly by electron paramagnetic resonance (EPR spectroscopy and fluorescence spectroscopy after reduction of the nitroxide to yielded th fluoroside C which indicates that these DNA based aptamer biosensor recognized th cocaine molecule upto 1 mM (Cekan et al., 2009).

Different approaches were used for the cocaine detection, one of which wa based on single quantum dot (QD) based aptameric sensor (Fig. 13). The QD surface wa functionalized with aptamer for the recognition of cocaine and the limit of detection wa 0.5 µM for cocaine. These aptameric sensors resulted in the simple sample preparation, high sensitivity, and extremely low sample consumption (Zhang et al., 2009).

![Figure 13: Principle of signal-off single QD-based aptameric sensor for cocaine detection. In the absenc of cocaine, Cy5 fluorescence was detected due to FRET between 605QD and Cy5. While the presence c cocaine led to the formation of a complex structure of a cocaine aptamer complex, and the subsequer abolishment of FRET between 605QD and Cy5, the decrease of Cy5 signal signified the presence c cocaine (Zhang et al., 2009).](image)

Another aptamer based sensor was developed by employing fluorescence principle for th detection of cocaine (upto 2 nM) (Fig. 14) with target induced strand displacement (He c al., 2010).
The basic concept and promising feature of an immunosensor is based on the competitive immunoassay of opiate drugs for the identification and quantification in wide areas of drug development and trafficking. As a result, analytical chemists and toxicologists are frequently called upon to analyze the biological specimens and drug seizures for the presence of parent drug and breakdown products. Immunosensor based analytical techniques provide simple, inexpensive, and sensitive detection methods for the monitoring large number of analytes, including low molecular mass opiate molecules. These techniques are now gaining acceptance, and are competing successfully with other established analytical methods for opiate drug determination.
Despite significant laboratory results and reviewed literature there are yet to make a major impact in the commercial market for the substantial improvements in this technology. The major drug developing authorities and organizations prefer small, cost effective, on-site immunoassay kits which enable early detection of narcotics, which demands significant advancement in the current immunosensor design and performance.

Generation of specific antibodies against various opiate molecules, improvements in the electronic transducer system, reducing cross reactivity and background noise, and increasing the detection limit are a few parameters which still need to be explored before such techniques become alternatives to the established analytical techniques such as GC, HPLC, MS etc. for the analysis of opiate in samples.