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Review

Morphine and its metabolites: Analytical methodologies for its determination

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Abstract

The present article reviews the methods of determination published for morphine and its metabolites covering the period from 1980 until at the first part of 2006. The overview includes the most relevant analytical determinations classified in the following two types: (1) non-chromatographic methods and (2) chromatographic methods.
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1. Introduction

Morphine is an opioid analgesic used for the treatment of moderate to severe pain. It is recommended by the World Health Organisation for the relief of moderate cancer-related pain. It is the opioid of choice in palliative and terminal care. Morphine is predominantly cleared from body by metabolism to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). It is metabolised in man primarily through conjugation with uridine diphosphoglucuronic acid in the 3-position. This conjugate is the major metabolite of morphine in several mammals and it accounts for about 54–74 s of the excretion products in man. A minor portion is glucuronidated in the 6-position or demethylated to nor-morphine.

The assay of drugs in biological fluids presents many difficult analytical problems. Frequently, the drugs are strongly bound to proteins and are present in complex matrix. The analytical techniques employed to overcome the above difficulties include immunoassays, enzyme-multiplied immunoassay and chromatographic methods, such as thin-layer chromatography, gas chromatography and high-performance liquid chromatography, coupled with both liquid–liquid and solid–liquid extraction. For reasons of speed and simplicity, initial testing of specimens is often performed by immunoassay. The development of gas chromatography–mass spectrometry assays for drugs of abuse must encompass both pharmacological and chemical factors. Obviously, if only small amounts of drug/metabolite are present in a sample due to the drugs potency, the difficulty

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of the assay will increase. In addition, high rates of metabolism, distribution and excretion diminish analyte concentration, thus demanding greater assay sensitivity. Fortunately, most gas chromatography–mass spectrometry detectors demonstrate a wide dynamic range in response to analyte concentration. At present, gas chromatography with mass spectrometric detection is receiving wider and wider acceptance. Because of its high reliability and versatility, this tandem gas chromatography–mass spectrometry is applied in the analysis of various classes of compounds (combined, if necessary, with derivatization), helping to identify and quantitatively determine organic compounds in a wide range of absolute and relative concentrations.

On the other hand, the most commonly used methods for sample preparation are liquid–liquid extraction and solid-phase extraction. Following extraction, polar analytes generally are derivatized to improve chromatographic properties.

2. Non-chromatographic methods

For the detection of opiates in whole blood, plasma or serum, non-chromatographic methods are rare. In some papers, the use of immunoassay (IA) as a first step has been reported [1–12]. However, positive results must be confirmed by a second independent method that is at least as sensitive as the screening test and that provides the highest level of confidence in the result. On the other hand, it is said that hair analysis studies on abused drugs started from the time when Baumgartner et al. [13] succeeded to detect opiates in the hair of heroin abusers by RIA (Radio Immune Assay) and estimate their opiate abuse histories by sectional hair analysis.

More recent article describes the development and validation of a radioreceptor assay for the determination of morphine and morphine-6-β-glucuronide (M6G) in serum [14]. The assay is based on competitive inhibition of the μ-opioid-selective radiolabeled ligand [^{3}H]-DAMGO by opioid ligands (e.g. M6G) for binding to the striatal opioid receptor. The assay has been validated according to the Washington Conference Report on Analytical Method Validation. The radioreceptor assay can be performed in serum without prior pre-treatment of the sample. Direct addition of the sample results in no significant loss in maximal binding sites, and therefore, no loss in sensitivity. The assay proves to be selective for a multitude of opioid agonists and antagonists (e.g. morphine and M6G. Moreover, morphine-3-glucuronide (M3G) displays a low affinity for the μ-opioid receptor and according to the literature demonstrates no analgesic activity. This makes discrimination, in relation to the analgesic effect, of the two metabolites of morphine possible. The assay is fast (assay time <4 h, analysis 5 min/sample), easy and the sensitivity is such that very potent agonists, like morphine and M6G, can be measured at the desired serum levels. The assay is accurate (<18%), but precision is limited if measured over several days (>35%).

In other way, contrary to that, the pharmaceutical industry demands poppy cultivars presenting high amounts of the pharmacological active substances which are standardized with regard to their individual concentration in the plant. Also today, opium poppy is one of the most important industrial medicinal drugs containing more than 80 different alkaloids. These include the analgesic and narcotic drug morphine, the cough suppressant codeine, the muscle relaxant papaverine, and the antitumoric agent noscapine. Because usual analysis methods to determine these main important alkaloids are very time-consuming and expensive, new ATR-IR- and Raman spectroscopic methods for the simultaneous prediction of morphine, codeine, papaverine, thebaine and noscapine in poppy capsules, poppy milk as well as aqueous-ethanolic extract were developed [15]. During the last decade several applications of these two complementary spectroscopy techniques have been successfully introduced in the agricultural and food section. Fourier transform (FT) infrared spectroscopy using a diamond composite ATR crystal and near infrared-Fourier transform (NIR-FT)-Raman spectroscopy techniques were applied for the simultaneous identification and quantification of the most important alkaloids in poppy capsules. Most of the characteristic Raman signals of the alkaloids can be identified in poppy milk isolated from unripe capsules. But also poppy extracts present specific bands relating clearly to the alkaloid fraction. Raman spectra obtained by excitation with a Nd:YAG laser at 1064 nm show no disturbing fluorescence effects; therefore the plant tissue can be recorded without any special preparation. The used diamond ATR technique allows to measure very small sample amounts (5–10 μl or 2–5 mg) without the necessity to perform time-consuming pre-treatments. When applying cluster analysis a reliable discrimination of "low-alkaloid" and "high-alkaloid" poppy single-plants can be easily achieved. The examples presented in this study provide clear evidence of the benefits of Raman and ATR-IR spectroscopy in efficient quality control, forensic analysis and high-throughput evaluation of poppy breeding material.

A molecular imprinted polymer of morphine was synthesized [16]. Using the imprinted polymer as the recognition material, sodium sulphite as the protective agent, and a potassium permanganate–morphine chemiluminescence system as the detection system, a molecular imprinting-chemiluminescence method for the determination of morphine was established. The linear response range of this method was 5.0×10^{-9} to 1.0×10^{-6} g ml^{-1} ($r = 0.9981$) and the detection limit was 2×10^{-9} g ml^{-1}. The coefficient of variation for 1.0×10^{-7} g ml^{-1} morphine solution was 2.8% ($n = 9$). This method was applied to the determination of morphine in the urine of the heroin abusers with satisfactory results.

3. Chromatographic methods

In general, narcotic analgesics of the opiate type were preferably screened by chromatographic methods.

3.1. High-performance liquid chromatography (HPLC)

HPLC is the method of choice for simultaneous analysis of morphine and its metabolites. Developed HPLC assay have used ultraviolet-visible (UV), diode array detection (DAD), fluorescence, electrochemical and mass spectrometry (MS) detectors or their combinations. Solid phase extraction (SPE) provides good possibilities for samples clean-up and preconcentration of

Table 1
High-performance liquid chromatography methods for morphine determination

Other species	Detection type	Remarks	Applications	Reference
Codeine, ethylmorphine	–	Reversed-phase and ion-pair formation; alkyl sulfonates and sulphates significantly increased the retention times; the carbon chain length of the pairing ions was linearly related to the log of the capacity factors of these amine drugs	Syrups	[18]
Common opiates, heroin metabolites	–	Using a 200 mm ×2 mm i.d., 3 μm silica column with dichloromethane–pentane–diethylamine-methanol mobile phase; using SPE with Bond Elut Certify cartridges and nalorphine as an internal standard	Urine	[19]
–	Electrochemical	Two methods: one procedure was a standard extraction and the other used a commercially available liquid–liquid extraction column	Cerebrospinal fluid and plasma	[20]
Active glucuronide metabolite	Electrochemical	Based in the method of Svensson [3], but uses only one SPE cartridge prior to chromatography and only a 20 μl injection volume	Human plasma	[21]
M3G, M6G	Electrochemical	SPE; using hydromorphone as the internal standard	Human plasma and cerebrospinal fluid; rat plasma	[22]
6-Monoacetylmorphine (6-MAM)	Electrochemical	SPE	Plasma, whole blood and urine	[23]
Codeine, (6-MAM)	Electrochemical	Applicability of various brans of mixed-phase extraction columns	Blood and serum	[24]
–	Electrochemical	It includes a one-step extraction procedure with hexane-isoamyl alcohol (1:1, v/v) at pH 8.9 (adjusted with phosphoric acid) and reverse-phase LC on a μPorasil column	Human and rabbit pharmacokinetic studies	[25]
M3G, M6G	Electrochemical	Improve one-step SPE	Plasma	[26]
Codeine, norcodeine, normorphine	Electrochemical	Extraction in C_{18}	Plasma	[27]
–	Electrochemical	A SPE, Sep-pak collection, extraction	Human plasma	[28]
Hydromorphone	Electrochemical	SPE, Baxter C_{18}; naltrexone as internal standard	Plasma	[29]
Codeine, hydromorphone, metoclopramide	Electrochemical	Reversed-phase LC; amperometrically at a glassy carbon electrode	Human plasma	[30]
Codeine	Electrochemical	Automatic on-line extraction	Plasma and gastric juice	[31]
M3G, M6G, normorphine	UV at 210 nm; electrochemical	Sample purification with Sep-Pak C_{l8} cartridges, ion-pair reversed-phase HPLC	Studies of morphine kinetics in man and animals	[32]
Codeine and seven metabolites	Electrochemical and UV	Reversed-phase ion-pair method; Sep-Pak light C_{18}	Plasma and urine	[33]
M3G, M6G, normorphine	Electrochemical and UV at 210 nm	SPE employing C_{18} Sep-Pak cartridges; reverse phase ion-pair chromatography with a C_{18} bonded column	Blood	[34]
M3G, M6G	Electrochemical and fluorescence	Using noroxymorphone as the internal standard; SPE	Human plasma	[35]
M3G, M6G	Electrochemical and fluorescence	By two-step SPE; on a reversed-phase C_{18} column	Human plasma	[36]
M3G, M6G, normorphine	Coulometric-fluorescence	Using tetrabutylammonium hydrogen sulphate at pH 10 followed by separation on a single C_{18} SPE cartridge	Plasma	[37]
M3G, M6G	Coulometry for morphine and M6G, UV for M3G	On-line clean-up system	Plasma sample in cancer patients	[38]
M3G, M6G	Coulometric and UV	Using a Supelcosil LC-8 DB reversed-phase column	Human plasma	[39]
Codeine, M3G, M6G	Native fluorescence	Using reversed-phase SPE columns	Serum, plasma and cerebrospinal fluid	[40]
M3G, M6G	Fluorescence	SPE	Neonatal plasma	[41]
M3G, M6G	Fluorescence	Rapid and highly automated determination; on-line SPE	Plasma and cerebrospinal fluid	[42]

Table 1 (*Continued*)

Other species	Detection type	Remarks	Applications	Reference
M3G, M6G	Fluorescence	Immunoaffinity extraction	Blood of heroin victims	[43]
Codeine, propranolol, quinidine, quinine	Fluorescence at λ_{exc} = 215 nm and λ_{em} = 300 nm	By using sodium dodecyl sulphate as mobile phase and direct injection	Urine	[44]
M3G, M6G, normorphine	Fluorescence	Using a C_8 SPE column; reverse phase on a C_{18} column	Plasma	[45]
6-MAM	Fluorescence	Using a Bond Elut SPE column; nalorphine as internal standard	Plasma	[46]
M3G, M6G	Fluorescence	Using a Bond Elut C_8 SPE column	Serum	[47]
–	Fluorescence	By means of heat-acid hydrolysis, pre-column dansyl derivatization, straight phase LC	Hair of heroin addicts	[48]
Codeine	Fluorescence and UV	Using liquid-phase extraction; nalorphine as internal standard	Blood and bile	[49]
M3G, normorphine	UV at 210 nm	Using ion-pair formation; a metabolite which is tentatively identified as the M6G is also coanalysed in this method; the chromatographic system may also be used for the analysis of the morphine congeners codeine, ethylmorphine and heroin (diamorphine)	Plasma and urine; studies of morphine kinetics in man and animals	[50]
M3G, M6G	UV	Use of a SPE cartridge; paired-ion reversed phase with a radially compressed column	Stability studies in human plasma	[51]
M3G, M6G, codeine	Multi-wavelength forward optical detection	SPE	Plasma, urine and cerebrospinal fluid	[52]
Codeine	UV at 240 nm	Using SPE	Plasma	[53]
M3G, M6G	DAD and UV	Simple generic SPE assay	Serum	[54]
M3G, M6G	UV at 210 nm	Symmetry shield and Xterra reversed phase columns	Cancer patient samples	[55]
Diacetylmorphine, M3G, M6G	DAD	Reversed-phase with gradient elution; ethylmorphine as internal standard; extracted using C_{18} ODS-2 SPE columns	Human plasma	[56]
Codeine, normorphine M3G, M6G	DAD	Clean screen, SPE; using a diol column; codeine sa internal standard	Plasma	[57]
Codeine, 6-MAM, cocaine and its metabolites	DAD	Different SPE procedures	Urine, blood plasma and serum	[58]
Codeine	UV	Using Altech C_{18}; quinine as internal standard	Plasma	[59]
Codeine, 6-MAM	UV	Using liquid–liquid extraction; nalorphine as internal standard	Blood	[60]
M3G, M6G	Electrospray ionisation (ESI)-MS	Codeine or naltrexone as internal standard; using ethyl SPE columns	Pharmacokinetic studies in serum	[61]
M3G, M6G	ESI-MS	Using Sep-Pak light C_{18} SPE cartridges, separated on an ODS C_{18} analytical column	Human serum	[62]
M3G, M6G	ESI MS	With deuerated analogues as internal standards; using end-capped C_2 SPE cartridges	Body fluids	[63]
M3G, M6G	ESI -MS	Using deuterated morphine as internal standard	Human serum	[64]
Heroin metabolites	ESI-MS	By combining sem-microcolumn HPLC, a column switching technique and ESI-MS	Human urine	[65]
Opiates, amphetamines, cocaine, benzoylecgonine	Quadrupole time-of-flight-MS	By mixed mode phase SPE; reversed-phase is carried out on a narrow bore phenyl type column	Oral fluid	[66]
M3G, M6G	MS	Extraction by using C_2 SPE cartridges	Pharmacokinetic study in male Sprague–Dawley rats	[67]
Several groups of drugs	MS	SPE; routine approach to the application LC-API–MS	Forensic science	[68]
M3G, M6G	Normal-phase LC-tandem-MS	With a silica column and an aqueous organic mobile phase	Human plasma	[69]
M3G, M6G	LC-tandem-MS	With an automatic 96-well solid phase extraction	Human plasma	[70]

Table 1 (*Continued*)

Other species	Detection type	Remarks	Applications	Reference
M3G, M6G, heroin, (6-MAM)	Atmospheric pressure ion-spray (API)-MS	Nalorphine as internal standard; ethyl SPE columns; water–methanol–acetonitrile–formic acid mobile phase	Serum	[71]
M3G, M6G, 6-MAM	API-MS	SPE; in selected ion monitoring mode	Body fluids of heroin victims	[72]
Codeine, codeine-glucuronide, 6-MAM, M3G, M6G	API-MS	Using C_{18} SPE cartridges; separation on an ODS column in acetonitrile	Body fluids	[73]
Heroin, 6-MAM, 6-acetylcodeine, codeine, M3G, M6G	API-MS	Filtration or SPE; using Zorbax TMS column	Biological fluids	[74]
6-MAM, M3G, M6G	API-MS	SPE with Sep-Pack C_{18}	Biological fluids	[75]
M3G	API-MS	SPE with Sep-Pack C_{18}	Biological fluids	[76]

analytes. The separation of biological samples is very important part of these methods. An interesting review is published by Netriova et al. [17] that includes surveys about using HPLC assay for these determinations. In Table 1 are summarised the principal proposed methods of determination of morphine using HPLC. LC procedures are more often used for the determination of opiates than gas chromatography–mass spectrometry (GC–MS) methods. LC methods in combination with electrochemical detection (ED) or fluorescence detection (FLD) are comparable in sensitivity with GC–MS methods, and have the advantage that they additionally cover the glucuronides of morphine. However, the specificity of GC–MS methods cannot be reached.

An extremely sensitive method for the detection of endogenous morphine in plasma (80 pg ml^{-1}) using HPLC and ED was described by Liu et al. [28]. The stability of 6-MAM (6-monoacethylmorphine) in frozen samples and in samples at room temperature was studied, with good recoveries and sensitivity (1 ng ml^{-1}) [24]. A comparative study of different SPE methods for the opiates, cocaine and benzoylecgonine was performed by Theodoridis et al. [58]. They tested nine different SPE cartridges, and found that Alltech Toxiclean gave the best results. In a variety of methods, low sample volume [40,46,47] or a short analysis time [59,71] and good sensitivity are emphasized.

It should be stated that mass spectrometric detection is still more specific than most of the LC detectors used, such as UV, DAD or FLD. The coupling of LC with MS is still expensive and not widely used. Nevertheless, three publications [62,63,71] appeared in the last years, proving that LC–MS is the method of choice, if the glucuronides of morphine are to be covered. In all other cases, GC–MS is preferable.

In this way, if GC–MS is now the reference technique for the determination of morphine, codeine, and codethyline, heroin and its first metabolite 6-mono-acetylmorphine (6-MAM) in biological fluids, there is still a concern over the direct determination of morphine glucuronides. These metabolites can play an important role in the interpretation of toxic deaths involving heroin or morphine, for at least two reasons: first, morphine-6-glucuronide (M6G) is pharmacologically active and has even been advocated to have a slightly different, and maybe more respiratory depressant action than morphine, due to its binding to a different μ-receptor subtype; secondly, the ratio of morphine over its metabolites can help evaluate the time elapsed between morphine (or heroin) intake and death, as well as the severity of the intoxication when death was delayed and most of the morphine metabolised. Several analytical methods for morphine and its glucuro-conjugated metabolites using LC–MS with different types of interfaces have been reported: at least two LC–MS procedures using a thermospray interface, three using an APCI interface and three using an ES interface (Table 1).

Polettini et al. [74] reported a qualitative LC–TS-MS–MS method for the confirmatory analysis of heroin metabolites in biological fluids. After a simple ultrafiltration of urine samples, the limits of detection (LODs) ranged from 10 ng ml^{-1} (6-MAM, morphine and codeine) to 50 ng ml^{-1} [heroin, 6-MAM, 6-acetylcodeine (6AC), M3G and M6G], while after SPE the LODs were lowered to 1 ng ml^{-1} for all except the glucuronides, the extraction recovery of which was low.

The proposed technique of Tatsuno et al. [75], for the simultaneous determination of illicit drugs of various families in human urine, using a thermospray interface and a single-quad MS, included the analysis of 6-MAM, morphine, M3G and M6G. The authors reported LODs between 2 and 40 ng ml^{-1} in the SIM mode, and between 50 and 400 ng ml^{-1} in the scan mode, without further precision.

The first paper concerned with liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS) appeared in 1992 and was also from a Japanese team [76]. It dealt with morphine and M3G, whose LODs were, respectively, 1 and 3 ng ml^{-1} in the SIM mode and 80 and 350 ng ml^{-1} in the full-scan mode; linearity was verified between 30 and 2000 ng ml^{-1} for morphine, 30 and 1000 ng ml^{-1} for its metabolite. This technique was successfully applied to a urine sample from a heroin addict. Bogusz et al. [73] reported in 1997 the determination of 6-MAM, morphine, M3G, M6G, codeine and codeine-6-glucuronide (C6G) in biological fluids, using LC–APCI-MS and deuterated internal standard: the limits of detection were between 0.5 and 2.5 ng ml^{-1} and the estimated limits of quantitation (LOQs) twice as high for all compounds, except C6G (100 and 200 ng ml^{-1} for LOD and LOQ, respectively). The method was validated between 5 and 500 ng ml^{-1} in serum and revealed convenient also for post-

mortem blood, urine, cerebrospinal fluid and vitreous humour samples in routine work.

Zuccaro et al. [71] published the same year an LC–APCI-MS technique for the determination of heroin, 6-MAM, morphine, M3G, M6G and codeine in mouse serum, using nalorphine as internal standard.

LC–MS procedures, which encompassed several groups of drugs of abuse, were relatively scarce. Miller et al. [77] applied liquid chromatography-electrospray ionisation (LC-ESI) for drug screening (cocaine, BE, codeine, morphine and 6-MAM) in methanolic hair extracts. One hundred and fifteen hair samples were taken during autopsy from homicide, suicide or accident victims. Positive results for cocaine and opiates were recorded in 65% of cases. A method for isolation and determination of opiate agonists (morphine, M3G, M6G, codeine, C6G, methadone, dihydrocodeine, dihydromorphine, buprenorphine, tramadol, ibogaine), cocaine and its metabolites (BE, ecgonine methyl ester, EME), and lysergic acid diethylamide (LSD) in body fluids based on SPE and LC-APCI (SIM) was described by Bogusz et al. [78]. The method was applied in routine forensic casework.

Weinmann and Svoboda [79] demonstrated the usefulness of flow injection ESI-MS–MS (without chromatographic separation) for detection and quantification of several opiates, cocaine, BE, EME and methamphetamine.

Hyphenated LC–MS procedures concerning defined groups or some individual drugs of abuse were more often published in last years. Table 1 summarizes the most important data from these studies.

Among the studies devoted to determination of opiate agonists almost exclusively the LC–API-MS methods has been found. One exception is a paper by Polettini et al. [80], who determined heroin, morphine, M3G, M6G, 6-acetylmorphine, codeine and acetylcodeine in blood and urine by LC–TSP-MS–MS. In the first application of LC–APCI-MS for opiate analysis, the urine samples were extracted with Sep Pak C_{18} cartridges and subjected to analysis on M3G and morphine in SIM and full scan mode [75].

Pacifici et al. [61] applied ESI for determination of morphine, M3G and M6G in serum after SPE with C_2 cartridges. Naltrexone and codeine were used as internal standards. In the study of Tyrefors et al. [62] morphine, M3G and M6G were extracted from serum using C_{18} SPE cartridges and subjected to HPLC (gradient elution) with ESI detection. External standardization was applied, which according to the authors assured better accuracy and precision. The effects of mobile phase composition on the signal intensity were studied. Heroin and its potential metabolites: morphine, M3G, M6G, codeine and MAM were determined after experimental administration of heroin in mice. Nalorphine is used as internal standard. Serum samples were extracted with C_2 SPE cartridges and subjected to LC–MS (ESI) examination in SIM mode [71]. Morphine, M3G, M6G, 6-MAM were determined in autopsy blood, urine, cerebrospinbal fluid and vitreous humor taken from 21 heroin victims. Body fluids were extracted with C_{18} SPE cartridges and morphine-d_3 was used as internal standard. APCI-MS (SIM) was applied [72]. In the next paper of this group, morphine, M3G, M6G, codeine, C6G and 6-MAM were determined in body fluids after SPE by APCI-MS (SIM) in flow-rate gradient [73].

Gerostamoulos and Drummer [34] reported a method capable of measuring morphine glucuronides from postmortem blood with quantitation involving both electrochemical and UV detection. Reversed-phase ion-pair chromatography was accomplished on a reversed-phase (C_{18}) column with a mobile phase of 1 mM lauryl sulfate in acetonitrile/20 mM sodium dihydrogen orthophosphate (24:76, v/v) at 1.0 ml min^{-1} under isocratic conditions. Nalorphine was the internal standard. The LOD was reported as 10 ng ml^{-1} and linear range extended to 5000 ng ml^{-1}. The SPE sample cleanup used C_{18} cartridges with 0.5 ml of blood; SPE recoveries were in the range from 70 to 100%. This extraction provided clean chromatograms with postmortem blood, serum and plasma. Furthermore, the LOD in postmortem blood of 10 ng ml^{-1} compared favourably with LOD values obtained in other studies with serum and plasma. Most importantly, this method eliminated the need for lengthy hydrolysis or overnight incubation to liberate morphine from its conjugate.

An HPLC–MS method for analyses and quantification of morphine and its two glucuronide metabolites was used to determine the stability of M6G and M3G in spiked fresh blood and plasma from live individuals and postmortem blood by Skopp et al. [81]. Prior to analysis, the samples were stored for 6 months in glass vials at −20, 4 and 20 °C. The glucuronides were found to be stable in both blood and plasma at 4 °C for the entire period. Analytes in postmortem blood were only stable at −20 °C. In postmortem blood, the glucuronides are hydrolysed to yield free morphine. The stability of M6G in fresh whole blood and plasma was dependent on storage temperature. M3G was stable even at long-term storage at 20 °C. For both glucuronides, storage at 4 °C in the dark resulted in no degradation for at least 181 days.

A method for the determination of M3G and M6G in plasma, using HPLC with fluorescence detection (FD) was presented by Glare et al. in 1991 [45]. The method took advantage of the compounds' native fluorescence, not requiring derivatization. SPE with reversed-phase (C_8) cartridges was used to clean up the samples. Chromatographic separation was achieved on a C_{18} column with a mobile phase consisting of 10 mM sodium dihydrogen phosphate and 1 mM sodium dodecyl sulfate (pH 2.1), using spectrofluorometric detection (excitation = 210 nm and emission = 350 nm). Nalorphine served as the internal standard and linear ranges spanned 50–200 ng ml^{-1} (M3G) and 100–300 ng ml^{-1} (M6G). Within-run and between-run errors were <13% for the glucuronides. The method is simple compared with previous methods that used multiple detectors and extensive extraction techniques.

Hartley et al. [41] also used HPLC–FD to determine M3G and M6G in plasma. The glucuronides were extracted from plasma by C_8 cartridges and separated on a C_{18} column with a mobile phase of 2 mM sodium dodecyl sulfate in 0.05% phosphoric acid:acetonitrile (71.5:28.5, v/v). This approach required 200 μl of plasma for analyses, which is appropriate for application to detection of M3G and M6G in premature neonates. The method was applied to study the disposition of morphine and its metabolites in neonates.

Wright et al. [22] reported a method to quantitate M3G and M6G in human plasma and cerebrospinal fluid using HPLC with electrochemical detection. SPE served as the sample cleanup technique with recoveries of 84 and 87%, respectively. Chromatographic separation was achieved using a reversed-phase (C_8) column with a mobile phase of methanol:acetonitrile:phosphate buffer (10:10:80, v/v/v). Detection limits for M3G and M6G were 10 ng ml^{-1} each. The inter-assay accuracy for low and medium concentrations of M3G and M6G were <17 and <9% for high levels, respectively. The intra-assay precision for low and high levels of M3G and M6G were <23 and <8%, respectively.

In 1995, Huwyler et al. [42] developed a HPLC–FD method with on-line SPE to detect M3G and M6G in plasma and cerebrospinal fluid. The advantage of fluorescence detection is clearly demonstrated by the clean chromatograms of these samples. The mobile phase used to desorb the analytes from the cartridge onto the analytical column was 200 mM potassium phosphate with a gradient of acetonitrile of 4–12%. Recoveries were over 95% with LOD values of 0.8 and 3 ng ml^{-1} for M3G and M6G, respectively. The automation afforded by on-line SPE enabled high volume rapid analysis. This technique only required 400 μl of sample, an advantage when limited sample is available. A simple method was devised by Aderjan et al. [47] in 1995 for the determination of M3G and M6G using reversed-phase HPLC with fluorescence detection. SPE was done using C_8 cartridges requiring 200 μl of serum sample. The LOD was determined to be 5 ng ml^{-1} for each compound. The method was applied to the analysis of 20 heroin addicts in police custody and 10 heroin-related deaths. By looking at the ratios between M6G, M3G and morphine and between M6G and M3G, one can determine the pharmacokinetics of heroin and gain insight into the time elapsed since last heroin or morphine administration.

LC–MS with electrospray ionization for the detection of M3G and M6G was reported by Tyrefors et al. in 1996 [62]. Separation was achieved on a reversed phase column using a gradient from 4 to 70% acetonitrile with formic acid; the flow rate was at 1.0 ml min^{-1}. The eluant was diluted 1:50 prior to mass spectral analysis. Compounds were detected by selected-ion monitoring. The linear range spanned 5–500 ng ml^{-1} (M3G) and 2–100 ng ml^{-1} (M6G). Short analysis times of less than 5 min were the advantage of this technique compared with previous methods involving 45 min run times. In 1997, Bourquin et al. [56] developed a reversed-phase HPLC method with a diode-array detector to detect metabolites of morphine including M3G and M6G in plasma. Ethylmorphine served as the internal standard and sample cleanup involved SPE with C_{18} cartridges yielding recoveries >80%. The LOQ was 25 ng ml^{-1} for each compound.

Bogusz et al. [78] developed a reversed-phase HPLC with MS (LC–APCI-MS) method to determine polar metabolites without prior derivatization. This paper described the determination of M3G, M6G and C6G following a SPE (C_{18}) protocol that required 0.5–1.5 ml sample. Deuterated internal standards were used for determinations in serum, blood, urine. The mobile phase for M3G and M6G was acetonitrile/50 mM ammonium formate buffer (AMF) (10:90, v/v) delivered at a flow rate of 0.3 ml min^{-1}. Retention times were 2.4 and 2.7 min, respectively. In order to separate C6G, the same mobile phase was used at a flow rate 0.6 ml min^{-1} resulting in a retention time of 4.0 min. The LOD values for M3G, M6G and C6G are reported as 2, 2 and 100 ng ml^{-1}. SPE recoveries were 94 ± 7, 97 ± 8 and 90 ± 6% for these analytes. This SPE method appears to be universal and could be used for the simultaneous isolation of common basic drugs of abuse. Preliminary screening is done with immunoassays, HPLC-diode-array detection and GC–MS. The SIM LC–APCI-MS is then applied for confirmation and quantification of certain drugs and metabolites.

The concentrations of M3G and M6G in serum, urine, and cerebrospinal fluid (CSF) of patients or volunteers receiving morphine during a clinical study were determined using HPLC-electrospray-MS by Schanzle et al. [63]. A reversed-phase separation was used with a C_{18} column and a mobile phase of 1% acetonitrile, 1% tetrahydrofuran and 0.1% formic acid in water. Their retention times were 1.7 and 3.2 min. The LOQ was reported as 0.5 ng ml^{-1} for M6G and 2 ng ml^{-1} for M3G. Analytes were extracted from serum and urine with C_2 SPE cartridges, requiring only 100 μl samples. SPE recoveries for M3G and M6G were 65 ± 4.4 and 71 ± 3%, respectively. The LOQ in urine was 25 ng ml^{-1} for M6G and 9 ng ml^{-1} for M3G. The method was useful to measure M3G and M6G in serum up to 36 h after IV morphine administration of 0.4 mg kg^{-1} in 30 min and in CSF after 24 h.

Most of the methods published on detection of morphine glucuronides use reversed-phase HPLC for separation of analytes. However, in 1997 a normal phase HPLC method with detection by atmospheric pressure ionspray–mass spectrometry was developed by Zuccaro et al. for the determination of M3G and M6G in serum [71]. Using a silica column, the mobile phase of water–methanol–acetonitrile–formic acid was delivered at a flow rate of 230 μl min^{-1}. The reported LOQs for M3G and M6G were 1 and 4 ng ml^{-1}, respectively. SPE with ethyl cartridges served as the method of sample preparation. SPE recoveries were 43.8–44.6% for M6G and 77.2–79.6% for M3G.

Another normal-phase LC–MS–MS method with a turbo ionspray interface was developed by Naidong et al. in 1999 [69]. The authors found that mobile phases with high organic solvent composition and acidic pH were needed to produce good spraying conditions and high sensitivity for MS. An extremely fast equilibration time (5–10 min) was achieved with a mobile phase of acetonitrile, water and formic acid, instead of the traditional solvents of normal-phase LC. Retention times for M3G and M6G were 2.4 and 1.9 min, respectively. Separation of M3G and M6G chromatographically was required as they have identical mass/charge and product ions. In addition, they can fragment to morphine in the LC–MS interface and be falsely detected as the parent compound. Linearity was achieved in plasma over the range of 10–1000 ng ml^{-1} for M3G and 1–100 ng ml^{-1} for M6G. The LOD for M6G was 1 ng ml^{-1}. Method ruggedness was shown by reproducible performance from multiple analysts using several instruments to analyze over one thousand samples from clinical trials. The inter-day precision and accuracy

were <9% relative standard deviation (R.S.D.) and <5% relative error (R.E.) for M6G and <3%R.S.D. and <6% R.E. for M3G. SPE with C_{18} cartridges using 1 ml sample served as the sample preparation technique. Extraction recoveries for M3G and M6G were 70 and 93%, respectively. The glucuronides were stable during long-term storage when refrigerated and when samples were subjected to various freeze-thaw cycles on the bench top. This method allows one analyst to process 200 samples per day and over 400 samples can be analyzed on one instrument per day.

A different type of sample preparation was developed by Beike et al. in 1999 [43]. This method involved the use of immunoaffinity-based extraction to isolate M3G and M6G from human blood. Polyclonal antisera were coupled to an activated trisacrylgel and used for extraction prior to analysis with HPLC–FD. Recoveries were reported as 76 and 88% for M3G and M6G, respectively. The LOD was 3 ng g^{-1} blood for all analytes. The method was applied to the analysis of blood from 23 heroin fatalities.

In 1999, Slawson et al. [82] reported an LC-electrospray–MS–MS method with sub-nanogram quantitation limits for M3G and M6G. The dynamic range for M3G and M6G was 0.25–10 ng ml^{-1}. Inter and intra-assay precision and accuracy were <8% for both metabolites at low, medium and high concentrations.

A two-step SPE was developed as a new cleaner extraction method for M3G and M6G by Meng et al. in 2000 [36]. This involved both hydrophobic and charge-based isolation on carbon and ion-exchange resins, respectively. This combination purified the metabolites from human plasma with maximal removal of interfering substances compared with the C_{18} cartridge alone. The SPE recoveries for M3G, M6G and hydromorphone (internal standard) were 82 ± 6.9, 79 ± 6 and $85 \pm 6\%$. For HPLC separation of the analytes, a reversed-phase C_{18} column was used with a mobile phase; 25% acetonitrile in 0.05 M phosphate buffer and 2.5 mM sodium dodecyl sulfate as the ion pairing agent. The LOD was 0.2 ng ml^{-1} for both M3G and M6G with a combination of electrochemical and fluorometric detectors.

In 2001, Ary and Rona [39] developed a reversed-phase HPLC method with UV detection for the simultaneous determination of M3G and M6G. Chromatographic separation was achieved using a Supelcosil LC-8 DB reversed-phase column and 0.1 M aqueous potassium dihydrogen phosphate/acetonitrile/methanol (94/5/1, v/v/v) containing 4 mM pentanesulfonic acid as the mobile phase. The retention times for M3G and M6G were 4.95 and 8.29 min, respectively. Linearity was observed over concentration ranges of 50–2000 ng ml^{-1} (M3G) and 15–1000 ng ml^{-1} (M6G). R.S.D. values were determined to be 5.15 and 2.57%. The LOD was 10 ng ml^{-1} for both compounds. Morphine glucuronides were extracted from human plasma using C_{18} cartridges. The SPE extraction recoveries were >96% for both analytes. The glucuronides were stable in human plasma for 4 weeks at −20 °C. This assay was used in the analysis of more than 1200 human plasma samples. This method was less expensive than LC–MS and LC–MS–MS methods.

In 2003 Projean et al. [83] developed a quick, simple method for the determination of M3G and M6G in rat plasma by HPLC–MS without the need for a solid phase or liquid–liquid extraction. Sample cleanup was done by protein precipitation with two volumes of acetonitrile and reconstitution in 0.1% formic acid in water. Naloxone served as the internal standard. Chromatographic separation was performed using a phenyl–hexyl column with a step-gradient of acetonitrile and formic acid in water at 1.0 ml min^{-1}. Following protein precipitation using 40 μl of sample, aliquots were directly injected into the HPLC–MS system. Analyte recoveries ranged from 70 to 78%. The metabolites were stable at room temperature for 6 h. With this method 96 samples could be analyzed in less than 24 h of injection time. This method was rapid, simple, and highly sensitive.

Whittington and Kharasch [84] developed a method using LC–MS with a 96-well plate SPE for extraction and detection of morphine and its glucuronides in plasma. The analytes were separated using an isocratic mobile phase consisting of methanol, acetonitrile and formic acid. The LOQ was 0.5 and 5 ng ml^{-1} for M6G and M3G, respectively. The SPE recoveries were in the range of 75–90% in low and intermediate concentration quality control samples. The sensitivity of this technique was comparable to LC–MS–MS. It is also advantageous as only 0.5 ml of sample is required for analysis. The high volume afforded by the 96-well plate SPE coupled to LC–MS allows more than 70 samples to be analyzed in a day.

For a pharmacokinetic–pharmacodynamic study in opioid tolerant patients, who were treated with heroin in combination with methadone, a liquid chromatographic assay with tandem mass spectrometry detection (LC–MS/MS) was developed for the simultaneous determination of heroin, methadone, heroin metabolites 6-monoacetylmorphine, morphine, and morphine-6 and 3-glucuronide and methadone metabolite EMDP [85]. To detect any abuse of substances besides the prescribed opioids the assay was extended with the detection of cocaine, its metabolites benzoylecgonine and norcocaine and illicit heroin adulterants acetylcodeine and codeine. Heroin-d6, morphine-d3, morphine-3-glucuronide-d3 and methadone-d9 were used as internal standards. The sample pre-treatment consisted of SPE using mixed mode sorbent columns (MCX Oasis). Chromatographic separation was performed at 25 °C on a reversed phase Zorbax column with a gradient mobile phase consisting of ammonium formate (pH 4.0) and acetonitrile. The run time was 15 min. MS with relatively mild electrospray ionisation under atmospheric pressure was applied. The triple quadrupole MS was operating in the positive ion mode and multiple reaction monitoring (MRM) was used for drug quantification. The method was validated over a concentration range of 5–500 ng ml^{-1} for all analytes.

A sensitive and reproducible method for the determination of morphine and the metabolites (M3G and M6G) was developed by Bengtsson et al. [86]. The method was validated for perfusion fluid used in microdialysis as well as for sheep and human plasma. A C_{18} guard column was used to desalt the samples before analytical separation on a ZIC HILIC (hydrophilic interaction chromatography) column and detection with tandem mass spectrometry (MS/MS). The mobile phases were 0.05% trifluoroacetic acid (TFA) for desalting and acetonitrile/5 mM

ammonium acetate (70:30) for separation. Microdialysis samples (5 μl) were directly injected onto the system. The limits of quantification (LOQ) for morphine, M3G and M6G were 0.5, 0.2 and 0.5 ng ml^{-1}, respectively, and the method was linear from LOQ to 200 ng ml^{-1}. For plasma, a volume of 100 μl was precipitated with acetonitrile containing internal standards (deuterated morphine and metabolites). The supernatant was evaporated and reconstituted in 0.05% TFA before the desalting process. The LOQs for sheep plasma were 2.0 and 3.1 ng ml^{-1} and the ranges were 2.0–2000 and 3.1–3100 ng ml^{-1} for morphine and M3G, respectively. For human plasma, the LOQs were 0.8, 1.5 and 0.5 ng ml^{-1} and the ranges were 0.78–500, 1.49–1000 and 0.53–500 ng ml^{-1} for morphine, M3G and M6G, respectively.

A method, using 0.2 ml of plasma, was designed for the simultaneous determination of morphine, 6-monoacetylmorphine, amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), N-ethyl,3-4-methylenedioxyamphetamine (MDEA), MBDB, benzoylecgonine and cocaine [87]. The drugs were analyzed by LC–MS, after solid phase extraction in the presence of the deuterated analogues. Reversed phase separation on an Atlantis dC_{18} column was achieved in 10 min, under gradient conditions. The method was full validated, including linearity (2–250 ng ml^{-1}, $r^2 > 0.99$), recovery (>50%), within-day and between-day precision and accuracy (CV and bias <15%), limit of detection (0.5 and 1 ng ml^{-1}) and quantitation (2 ng ml^{-1}), relative ion intensities and no matrix effect was observed. The procedure showed to be sensitive and specific, and was applied to 156 real cases from road fatalities.

Finally, HPLC method was developed for the determination of morphine in plasma [88]. Samples were extracted using Zeolite Y column followed by reversed phase HPLC with fluorescence detection. This method was based on an ex-calibration procedure and was linear between 20 and 200 ng ml^{-1} of morphine. The method was reliable for morphine determination in blood even after five half-lives after the last abuse.

Detection of morphine glucuronide metabolites is very important, a recent review published in 2006 describe HPLC methods to directly detect glucuronides [89].

3.2. Gas chromatography (GC)

Codeine is metabolised in humans by conjugation and by demethylation to morphine; however, the reverse pathway leading to the production of codeine from morphine does not occur. Both morphine and codeine are generally found in biological fluids after codeine ingestion. This results in the need to assay both morphine and codeine simultaneously. Co-extraction of morphine and codeine can be problematic due to the amphoteric nature of morphine; development and use of cation exchange SPE columns have greatly improved the efficiency of multiple analyte extraction [90].

The poor chromatographic characteristics of underivatized morphine analogues necessitate production of stable derivatives. Chen et al. [91] evaluated five derivatizing agents for analysis of morphine and codeine by gas chromatography–mass spectrometry (GC–MS) and found that the acetyl derivative exhibited the greatest stability of those examined. The stability of the acetyl derivatives of morphine and codeine also was demonstrated by Paul et al. [92] who developed a simultaneous assay for these analytes in urine. The extraction was performed with methylene chloride–isobutanol (9:1), followed by acid–base extraction and re-extraction with organic solvent. This procedure was subsequently used by Cone et al. [93] to monitor the urinary excretion of free and conjugated morphine and codeine in human subjects following the intramuscular administration of 60 and 120 mg doses of codeine. Urinary data were reported on free and total codeine and morphine levels for periods up to 120 h after codeine administration.

Mule and Casella [94] and Wu Chen et al. [95] also reported simultaneous assays for morphine and codeine by GC–MS selected ion monitoring (SIM). Both procedures utilized liquid–liquid extraction for the isolation of morphine and codeine from urine and other biological fluids.

Donnerer et al. [96] measured endogenous morphine and codeine in arthritic rats' spinal cords. They hydrolysed tissues in 0.01 M HCl and performed a prepurification step by SPE followed by HPLC purification. The extracts were prepared as the trifluoroacetyl derivatives and analyzed by GC-MS in the full-scan mode.

Analysis of morphine and codeine in hair by various GC–MS techniques has been reported. Pelli et al. [97] reported a highly sensitive identification of morphine in hair of heroin addicts by collisional spectroscopy. Hair samples were extracted with 0.1 M HCl, followed by re-extraction with an organic solvent. Analysis was performed by direct introduction of the residue dissolved in methanol into the ion source. Other investigators have used GC–MS for the determination of both codeine and morphine [98,99] in hair and have concluded that it is possible to distinguish between heroin and codeine addicts by GC–MS analysis, but caution against the use of less specific techniques like radioimmunoassay (RIA). Also, the choice of GC–MS for the identification of morphine and 6-MAM in hair started from 1991. Goldberger et al. [100] identified 6-MAM and heroin in hair samples from 20 heroin users by GC–MS. Nakahara et al. [101] showed that hydrolytic extraction of morphine analogous in hair with 10% HCl for 1 h at 100 °C gave a quantitative recovery of morphine. In their experiments, the total morphine level in hair from monkeys administered with heroin was six times higher than from those administered with morphine. From hair of monkeys and humans intoxicated with heroin, they detected 6-MAM using a methanolic extraction at the levels of 0.7–7.2 ng mg^{-1} as the major component in hair together with morphine but without heroin. Mangin and Kintz [102] showed variability of opiates concentrations in human hair according to their anatomical origin: head, auxiliary and pubic regions. Moeller et al. [103] developed a new extraction method using SPE after incubation of powdered hair samples with β-glucuronidase/aryl-sulfatase in phosphate buffer. Welch et al. [104] have demonstrated that extractions with 0.1 M HCl are efficient at removing morphine from hair. Nakahara et al. [105] compared the efficiency of extraction of 6-MAM and morphine from hair between five extraction methods; methanol, 0.1 M HCl, methanol–5 M HCl (20:1), helicase and methanol–trifluoroacetic acid (TFA) (9:1).

Their findings show that methanol–TFA was the best solvent for extracting 6-MAM and morphine with minimum hydrolysis and maximum efficiency of 6-MAM. Cirimele et al. [106] reported supercritical fluid extraction of codeine, morphine and 6-MAM in drug addict hair. Gygi et al. [107] found that after controlled administration, the incorporation of codeine and its metabolites, morphine, into rat hair occurs in a distinct dose-proportional manner. Jurado et al. [108] and Kintz and Mangin [109] reported simultaneous quantification of opiates, cocaine and cannabinoids in hair by GC–MS. Wilkins et al. [110] developed a new method using PCI–MS for the determination of codeine and its metabolite, morphine. Gaillard and Pepin [111] developed a new SPE method on C_{18} cartridges which allows a very simple protocol of manipulation and a single elution of opiates and cocaine homologs from human hair samples. Polettini et al. [112] evaluated the recovery of extraction of opiates from the hair samples of heroin over-dose corpses and the extent of hydrolysis of acetylated opiates (6-acetylmorphine, acetylcodeine), using alkaline hydrolysis, acid hydrolysis and methanol. Hair analyses of polydrug poisonings including opiates have been discussed as case reports. Hold et al. [113] developed a sensitive method for the combined extraction of cocaine, opiates and their metabolites from human head hair using an enzyme-based digestion technique. Tagliaro et al. [114] reported the findings from hair analysis regarding heroin overdose death.

A number of methods have been developed for assay of morphine in biological specimens. Drost et al. [115] reported a GC–MS SIM method with CI, ammonia–methane (1:5), for the determination of free and hydrolysed morphine in serum and cerebrospinal fluid. Specimens were extracted by SPE and prepared as silyl derivatives. The method was used for determination of the pharmacokinetics of morphine after epidural administration to human subjects undergoing abdominal surgery [116].

Spiehler et al. [117] also reported a method for the determination of free and hydrolysed morphine in blood. Postmortem samples were extracted by organic solvent and derivatized with trifluoroacetic anhydride. Jones et al. [118] developed a highly sensitive method for morphine in urine and body organs. Two liquid–liquid extraction methods were used and the extracts were derivatized with pentafluoropropionic anhydride. The authors compared the use of packed columns with fused-silica capillary columns and found the sensitivity of the assay was increased about 10-fold through the use of capillary columns. Fuller et al. [119] also used liquid–liquid extraction for the determination of morphine in specific regions of rat brain. Morphine levels were measured by GC–MS SIM with methane CI.

The use of GC–MS for detection of illicit drugs in urine led to the discovery that poppy seed products contain significant amounts of morphine and codeine. Diverse studies, summarised in Table 2, have shown that individuals who ingest poppy seeds in foodstuffs can produce detectable levels of morphine and codeine in urine and blood. Also, Table 2 highlight detailed data on the proposed methods. As can be seen, in GC–MS analysis, derivatization is required to overcome the poor chromatographic behaviour of morphine. Silylation or fluoroacetylation are the preferred methods. An interesting method for the determination of opiates, in plasma and whole blood, using automated sample preparation, was described by Krogh et al. [138]. They used on-line dialysis as a purification step. The authors determine the substances using gass chromatography–nitrogen-phosphorous detection (GC–NPD) and GC–MS in parallel. Geier et al. [139] compared different SPE phases and the usefulness of precipitation as a pretreatment method for the simultaneous determination of morphine, 6-MAM, codeine and dihydrocodeine (DHC). Lee and Lee [153] used GC–ED after derivatization with heptafluorobutyric anhydride (HFBA), as well as NPD detection after derivatization with BSTFA, for the determination of morphine and codeine in blood and bile. They stated that both methods were equally sensitive. However, MS methods are preferable, due to their higher specificity [109].

In other paper [158], thirty hair samples were collected from male opioid abusers for whom the presence of morphine in their urine samples was confirmed by thin layer chromatography. The hair samples were decontaminated by washing with isopropanol, deionised water, and isopropanol, dried at room temperature, and cut into small pieces. Samples of the latter (30 mg) were digested by incubation in a mixture of methanol–trifluoroacetic acid (9:1) for 18 h at 45 °C and sonicated to improve the extraction process. The methanolic phase was evaporated to dryness under a stream of nitrogen at 50 °C. The sample was derivatized by addition of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide and 1% trimethyliodosilane at 70 °C for 20 min, with sonication. Derivatized samples (1 μl) were injected into a GC–MS system fitted with a capillary column; the Finnigan MS was operated in SIM mode. Naltrexone was used as internal standard. The masses of the ions selected for morphine and naltrexone were 429 and 557, respectively. The limit of quantitation was set at 0.03 ng mg^{-1} hair.

Analysis of morphine in human solid tissues, such as liver and kidney is particularly important when biological fluids are not available due to putrefaction of the body in suspected cases of morphine or heroin poisoning. When time has passed after ingestion, glucuronide and sulfate conjugation of morphine can be significant; hence, it is difficult to estimate the amount of drug ingested based on only the concentrations of unconjugated morphine in human fluids. In such cases, total morphine concentrations obtained by acid hydrolysis have been used for forensic toxicological examinations. Therefore, it is important to develop a simple and reliable method to determine free and total morphine in human solid tissues. Recently, Kudo et al. [159] developed a reliable, simple and sensitive method to determine free and total morphine in human liver and kidney, using GC–MS. Free morphine or total morphine obtained by acid hydrolysis from 0.2 g tissue sample was extracted using an Extrelut® NT column with an internal standard, dihydrocodeine, followed by trimethylsilylation. The derivatized extract was submitted to GC–MS analysis of EI-SIM mode. The calibration curves of morphine in both liver and kidney samples were linear in the concentration range from 0.005 to 5 μg g^{-1}. The lower limits of detection of morphine were 0.005 μg g^{-1}. This method proved successful when we determined free and total morphine in liver and kidney obtained from an autopsied man who was mis-

Table 2
Gas chromatography methods for morphine determination

Other species	Detection type	Remarks	Applications	Reference
–	EI full scan	With silyl as derivative	Urine	[120]
–	SIM-EI	With pentafluoropropionic anhydride (PFPA) as derivative	Urine	[121]
Codeine	SIM-EI	With acetic anhydride as derivative	Urine	[122]
Codeine	SIM-EI	With trifluoroacetic anhydride (TFA) as derivative	Urine	[123]
Codeine	SIM-EI	With TFA as derivative	Urine, blood	[124]
Codeine	SIM-EI	With acetic anhydride as derivative	Urine	[125]
Codeine	SIM-EI	With silyl as derivative	Urine	[126]
Codeine, 6-acetylmorphine	SIM-EI	With acetic anhydride as derivative; extraction into chloroform–isopropanol (9:1)	Urine	[127]
Codeine, 6-acetylmorphine, heroine	SIM-EI	With TFA as derivative; extraction into chloroform–isopropanol–*n*-heptane (50:17:33)	Urine	[128]
6-Acetylmorphine	SIM-EI	With trimethylsilyl as derivative; extraction into chloroform–isopropanol (9:1)	Urine	[129]
Codeine and 10 potential metabolites	SIM	Study of the effect of different concentrations of isopropanol in chloroform upon recovery; under methane CI conditions by means of a Silar-5CP packed column	–	[130]
Codeine, normorphine, norcodeine, noscapine, thebaine, papaverine, oripavine	SIM	Optimal pH for recovery is 9.5 when using methylene chloride–isopropanol as the extraction solvent	–	[131]
Codeine, cocaine, cocaine metabolites, opiate metabolites	SIM	Extraction method utilized SPE cartridges packed with a co-polymeric phase material; drugs standards and deuterated internal standards were added to drug-free control hair in concentrations representing therapeutic levels; the sample was extracted with acid followed by neutralization and extraction by SPE; extract treated with a silyl-derivatizing reagent	Hair	[132]
Codeine, 6-acetylmorphine, other opiate analytes	MS	With liquid–liquid extraction and analysis by fused-silica capillary column	–	[133]
Codeine, 6-acetylmorphine, other opiate analytes	MS-	With liquid–liquid extraction and analysis by fused-silica capillary column	Urine-	[134]
Cocaine and its metabolites	MS	With clean screen; derivatization with PFPA-hexafluoroisopropanol (HFIP)	Whole blood	[135]
Codeine, 6-acetylmorphine and more opiates	MS	With automated sequential trace enrichment of dialysate; nalorphine as internal standard; column HP Ultra 1; derivatization with PFPA	Plasma, whole blood	[136]
Codeine, 6-acetylmorphine, dihydrocodeine	MS	Using Chromabond C_{18}; methaqualone as internal standard; derivatization with propionic acid anhydride (PAA); column DB-1	Plasma, whole blood	[137]
Codeine, 6-acetylmorphine and more opiates; heroin; cocaine and its metabolites	MS	With clean screen; deuterated analogues as internal standard; derivatization with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA)-methylchlorosilane (MCS); column HP-1	Plasma	[138]
6-Acetylmorphine, heroin	MS	Extraction with ZS DAU 020; deuterated analogues as internal standard; derivatization with BSTFA; column RTX 5	Blood, plasma	[139]
Codeine, 6-acetylmorphine, dihydrocodeine	MS	Using Amchro C_{18} ec; deuterated morphine and codeine; derivatization with PFPA-pentafluoro-1-propanol (PFP); column OV-1	Serum, blood	[140]
–	MS	Using Extrelut, Bond Elut; nalorphine as internal standard; derivatization with *N*-methyl-*N*-(tert.-butyldimethylsilyl)-trifluoroacetamide or diethyl tetramethyldisilazane or ethyl dimethylsilylimidazole; column OV-1	Blood	[141]
Codeine and more opiates	MS	Using Bond Elut C 18; deuterated analogues as internal standard; derivatization with PFPA; column HP-1	Blood	[142]
Codeine, 6-acetylmorphine, cocaine and its metabolites	MS, MS–MS	Using SPEC MP3 microcolumn; deuterated analogues as internal standard; derivatization with BSTFA-MSC	Blood	[143]
Codeine	MS	Derivatization with PFPA and acetic anhydride; deuterated internal standard and selected ion monitoring	–	[144]
Codeine, 6-MAM	MS	Extracted at neutral pH by SPE prior to derivatization to their trifluoroacetyl derivatives; in the electron impact mode	Urine	[145]

Table 2 (*Continued*)

Other species	Detection type	Remarks	Applications	Reference
Codeine, hydromorphone, hydrocodone, oxycodone	MS	Hydrolysed with β-glucuronidase and extracted by SPE on Bond Elute Certify cartridges at pH 6.8; nalorphine as internal standard	Urine	[146]
6-MAM	MS	Extraction with enzymatic treatment; derivatization with PFPAA (70 °C, 30 min)	Hair	[147]
6-MAM	MS	Extraction with enzymatic treatment; derivatization with silylation	Hair	[148]
–	MS	Also by radioimmunoassay using an antibody-coated tube kit	Post-mortem blood	[149]
Heroin, 6-MAM	MS	Liquid–liquid extraction at pH 9.5, propionylation at room temperature; using full-scan ion trap	Blood, urine and vitreous humor	[150]
–	MS	Comparison of SPE and SFE	Whole blood	[151]
Codeine, M3G, M6G and more opiates	Negative ion chemical ionization-MS	Deuterated analogues as internal standard; derivatization with heptafluorobutiryc anhydride (HFBA)	Plasma	[152]
Codeine	Electron capture	Using liquid–liquid extraction; nalorphine as internal standard; derivatization with HFBA; column 1.5%OV 17 + 1.95%OV 202 on Chromosorb W-HP	Blood and bile	[153]
Codeine	Nitrogen-phosphorous detection	Using liquid–liquid extraction; nalorphine as internal standard; derivatization with BSTFA; column HP-1	Blood and bile	[153]
Codeine, 6-MAM	Nitrogen specific and/or flame ionization	Routine determination of 6-MAM, the specific metabolite of heroin	Urine	[154]
–	Not reported	Derivative not reported	Urine	[155]
Codeine, 3- and 6-acetylmorphine, heroine, nalorphine, naloxone, ethylmorphine, naltrexone	–	Extracted with chloroform–isopropanol–*n*-heptane (50:17:33) at pH 9.2, followed by back-extraction with dilute acid and re-extraction with chloroform	Plasma	[156]
Codeine, hydromorphone	–	Using a one-step extraction procedure: the samples were extracted with toluene–hexane–isoamyl alcohol (78:20:2) at pH 9.9	Blood	[157]

ingested morphine compound in the hospital, which resulted in the cause of death being morphine intoxication.

Demonstrating the presence or absence of opiate compounds in postmortem fluids and/or tissues derived from fatal civil aviation accidents can have serious legal consequences and may help determine the cause of impairment and/or death. However, the consumption of poppy seed products can result in a positive opiate drug test. Lewis et al. [160] have developed a simple method for the simultaneous determination of eight opiate compounds from one extraction. These compounds are hydrocodone, dihydrocodeine, codeine, oxycodone, hydromorphone, 6-monoacetylmorphine, morphine, and thebaine. The inclusion of thebaine is notable as it is an indicator of poppy seed consumption and may help explain morphine/codeine positives in cases where no opiate use was indicated. This method incorporates a Zymark® RapidTrace™ automated SPE system, and trimethyl silane (TMS) and oxime-TMS derivatives. The limits of detection ranged from 0.8 to 12.5 ng ml^{-1}. The linear dynamic range for most analytes was 6.25–1600 ng ml^{-1}. The extraction efficiencies ranged from 70 to 103%.

Gunnar et al. [161] presented a procedure for simultaneous semiquantitative/quantitative screening of 51 drugs of abuse or drugs potentially hazardous for traffic safety in serum, plasma or whole blood. Benzodiazepines (12), cannabinoids (3), opioids (8), cocaine, antidepressants (13), antipsychotics (5) and antiepileptics (2) as well as zolpidem, zaleplon, zopiclone, meprobamate, carisoprodol, tizanidine and orphenadrine and internal standard flurazepam, were isolated by high-yield liquid–liquid extraction. The dried extracts were derivatized by two-step silylation and analyzed by the combination of two different GC separations with both electron capture detection (ECD) and MS operating in a selected ion-monitoring (SIM) mode. Quantitative or semiquantitative results were obtained for each substance based on four-point calibration. Intra- and inter-day precisions were within 2.5–21.8 and 6.0–22.5%, and square of correlation coefficients of linearity ranged from 0.9896 to 0.9999. The LOQ varied from 2 to 2000 ng ml^{-1} due to a variety of the relevant concentrations of the analyzed substances in blood. The method is feasible for highly sensitive, reliable and possibly routinely performed clinical and forensic toxicological analyses. An analytical procedure was developed by the same authors for the simultaneous sensitive identification, screening and quantitation of 30 drugs of abuse using 250 μl of human oral fluid [162]. The method employs sequential mixed-mode SPE, optimized derivative formation and long-column fast GC/EI-MS. After sequential SPE elution, the most sensitive and stable derivatives were formed by taking careful account of the characteristics of the active functional groups and possible steric hindrances affecting derivatization chemistry. In addition, the following analytes were included: methadone, cocaine, alprazolam, midazolam, fentanyl and zolpidem. In GC separation, fast temperature ramping and high carrier gas flow-rate combined with long 30 m columns of i.d. 0.32 mm offered a reduction in analysis time and sharp peak shapes while still maintaining suffi-

cient resolution and high sample capacity. Validated parameters including selectivity, linearity, accuracy, intra- and inter-day precision, extraction efficiency and limit of quantitation were all within required limits. In contrast to previously published methods, this single procedure is suitable for the simultaneous toxicological determination of the most common illicit drugs and benzodiazepines, and also zolpidem, in a small amount of oral fluid.

A procedure based on GC–MS is described for determination of opiates (6-monoacetylmorphine, morphine and codeine) and cocaine and metabolites (cocaine, benzoylecgonine and cocaethylene) in human teeth [163]. After addition of nalorphine as internal standard, pulverized samples were incubated in HCl at 37 °C for 18 h. Then, after pH adjustment to 6, and the analytes were extracted with two volumes of 3 ml of chloroform/isopropanol (9:1). Chromatography was performed on a fused silica capillary column and analytes were determined in the selected-ion-monitoring (SIM) mode. The assay was validated in the range 7.5 (6.0 in case of codeine) to 500 ng g^{-1} with mean absolute recoveries ranged between 74.1 and 92.1% for the different analytes and precision and accuracy always better than 15%. The method was applied to the analysis of teeth from drug-addicts to assess past chronic consumption and verify self-reported declarations. Teeth can be a promising non-invasive biological matrix in biomedical analysis for both clinical and forensic purposes.

3.3. Capillary electrophoretic (CE)/electrokinetic methods

CE is probably the most rapidly growing analytical technology that has appeared in the last two decades. As it is well known, without changes in the instrumental hardware, 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).

Probably, the main application field of CE is, at present, the determination of drug substances, and, indeed, the penetration of this technique into the pharmaceutical industry, after a slow start, is now a reality. In the early 1990s CE, in the MECC mode, was introduced in the forensic field by the pioneering work of Weinberger and Lurie [164], who showed the potential of this technique for the separation of 18 illicit and/or controlled drugs.

Almost all the existing commercial CE hardware in its “standard configuration” features automated injection in both electrokinetic and hydrodynamic modes, thermostated separation under constant voltage or constant current conditions, and “in capillary” UV absorbance detection, by means of filter or monochromator single wavelength detectors or with more sophisticated fast scanning or diode array multiwavelength spectrophotometers.

This has oriented applications towards methods based on UV absorbance detection, which although characterized by a broad spectrum of applications, in CE show specific limitations in sensitivity and selectivity, due to the limited choice of wavelengths at which most of the compounds display sufficient molar absorptivity (i.e. around 200 nm) to allow sensitive detection. For this reason, most of the overall selectivity required by the method is based on that offered by the electrophoretic/electrokinetic separation.

Wernly and Thormann in 1991 [165], using MECC in a fully aqueous borate–phosphate buffer pH 9.1 containing 75 mM SDS and a 75 μm i.d., 90 cm long fused-silica capillary, first reported the qualitative analysis of many abused drugs and metabolites in urine, including benzoylecgonine, morphine, heroin, 6-monoacethylmorphine (MAM), methamphetamine, codeine, amphetamine, cocaine, methadone, methaqualone and benzodiazepines. Detection was “in capillary” by a fast scanning UV spectrophotometer. Thus peak identification was based not only on the migration times, but also on the on-line recorded UV spectra of the peaks. Urine purification and concentration was by “double mechanism” SPE, as discussed above, allowing a sensitivity of about 100 ng ml^{-1} in the biological matrix. The same authors [166] showed that also the major urine metabolite of heroin and morphine, morphine-3-glucuronide, can be determined in this biological fluid by either CZE (12 mM sodium tetraborate, 20 mM disodium hydrogenphosphate, pH 9.8) or MECC (75 mM SDS in phosphate–borate buffer, pH 9.2), with a detection limit of 1 μg ml^{-1} (spectral UV analysis), after SPE with C_8-silica cartridges.

A screening method for opiates (morphine, heroin, codeine), amphetamine and caffeine in urine and serum was reported by Hyotylainen et al. [167], by using MECC (quite surprisingly CZE did not provide an acceptable separation of the analytes) in an electrolyte system composed of 50 mM glycine and 50 mM SDS at pH 10.5. Short capillaries (50 μm i.d., 23 cm length) were used for fast screening (less than 2 min), and longer capillaries for quantification (50 μm i.d., 67 cm length). Detection was by UV absorption at 200 nm. The core of the paper, however, was the use of two carboxylic acids as markers of electrophoretic mobility, to determine “migration indices” of the analytes, which were used for the identification of the compounds, instead of the usual, but less precise, migration times. The marker technique, based on the use of two or more compounds of known electrophoretic mobility to calculate the effective field strength, the electroosmotic flow velocity and consequently the electrophoretic mobility of unknown compounds, allowed highly reliable identification in CZE [168]. This approach was then adapted to MECC, where the net mobility of an analyte is determined by its total mobility and the electrophoretic mobility while partitioned into the micelles.

CZE was reported to provide excellent quantitative determination of opiates, comprising pholcodine, 6-MAM, morphine, heroin, codeine and dihydrocodeine in urine, using a running buffer of 100 mM disodium hydrogenphosphate at pH 6 [169]. Electrokinetic injection with field-amplified sample stacking, after SPE of urine on “double mechanism” cartridges, allowed detection limits in the region of 4–9 ng ml^{-1}. Levallorphan was used as internal standard to limit imprecision inherent in the chosen injection method and the assay passed a careful validation procedure.

Hair analysis is gaining increasing popularity in forensic toxicology, as a tool for investigating past, chronic exposure to illicit

drugs and, in this field, CE could offer clear advantages over current chromatographic techniques, due to the minimal need of sample mass for analysis, which in the case of hair can be a crucial point.

CZE was adopted for morphine and cocaine determination in hair, using a basic background electrolyte consisting of 50 mM borate, pH 9.2 [170,171]. Hair samples (about 100 mg) were first incubated overnight in 0.25 M HCl at 45 °C, then the mixture was extracted by liquid–liquid extraction before injection. Detection was either at 200 nm for the simultaneous analysis of cocaine and morphine or at the absorbance maxima of each analyte (for cocaine: 238 nm; for morphine: 214 nm) for higher selectivity.

Petrovska et al. [172] used isotachophoresis, a special kind of microanalytical technique with capillary separation, to determine morphine in serum. However, an extraction was necessary to concentrate morphine and, furthermore, the minimum tested concentration was approximately 70 ng ml^{-1}.

Reddy et al. [173] used a CZE method for the qualitative and quantitative determination of morphine, codeine, thebaine, papaverine and narcotine in gum opium. A 50 μm × 70 cm capillary (55 cm effective length) was used with a running buffer consisting of 100 mM sodium acetate, pH 3.1, 70% (v/v) methanol. Separations were performed for 25 min, using an applied voltage of 15 kV at 25 °C. Quantification of the samples was carried out using the external standard method at 224 nm. The extraction method of the alkaloids was adopted from Ayyangar et al. [174]. Peak identification was done by sample spiking; in addition, spectral scanning was performed between 200 and 300 nm. Calibration curves were linear in the range of 2–20 μg ml^{-1}, with correlation coefficients ≥0.996 for all standard alkaloids. The limit of detection for each alkaloid was 850 ng ml^{-1} for morphine, 450 ng ml^{-1} for thebaine, 500 ng ml^{-1} for codeine and narcotine, and 550 ng ml^{-1} for papaverine. The peak area R.S.D. ($n = 5$) ranged between 1.03 and 3.56%, and the migration time R.S.D. ($n = 5$) ranged between 0.34 and 0.69%. Recoveries ranged from 98 to 102% for spiked samples.

Dynamic coating of a capillary consists of a two-step process, whereby, the capillary (after flushing with base) is first coated with a proprietary polycation (an initiator), then with a proprietary polyanion (an accelerator). Lurie et al. [175] used the above coating conditions for the separation of morphine, papaverine, codeine, noscapine and thebaine in opium alkaloids; however, this approach gave poor resolution. The addition of dual cyclodextrins (hydroxypropyl-β-cyclodextrin and dimethyl-β-cyclodextrin) to the run buffer, imparted excellent selectivity for the opium alkaloids. Excellent migration time and peak area (R.S.D. ≤0.12 and ≤1.2%, respectively) were obtained. Good agreement for the determination of opium alkaloids in opium gum and opium latex samples was obtained using CE and HPLC. CE afforded better resolution with significantly faster analysis time (12 min versus 29 min). The CE conditions reported were also applicable to the analysis of LSD exhibits. Excellent linearity and precision were obtained, with runs carried out within 8 min. Capillary performance was also examined with over 500 samples analyzed, using the same capillary. Migration times increased over time; however, resolution remained constant.

CE has been used to identify many drugs in a variety of biological samples. Blood and urine serve most frequently as sources of biological specimens for analysis, although analysis can be extended to other specimens, such as saliva, vitreous humor, hair, etc. The measurement of drugs in body fluids and tissues is necessary for the determination of specific drugs and/or metabolites and for the confirmation of illicit drugs for forensic interest. Capillary electrophoresis has been successfully applied to the determination of various analytes in biological samples, using UV and fluorescence spectroscopy methods of detection.

The capability of SDS to solubilise proteins signifies that plasma samples can be directly injected into an untreated fused-silica capillary containing a buffer with SDS, as demonstrated by Emara et al. [176]. MEKC was used for the determination of morphine in human plasma without the need of sample pre-treatment. Endogenous components present in plasma were also shown not to co-migrate with morphine. Methods for the separation and determination of a variety of drugs of abuse in biological fluids, using capillary electrophoresis with native fluorescence and LIF detection, were described by Alnajjar et al. [177]. Normorphine, morphine, 6-acetylmorphine and codeine were detected using fluorescence detection. Detection was performed at an excitation wavelength of 245 nm and a cut-off emission filter of 320 nm, with detection limits of approximately 200 ng ml^{-1}. LIF detection was used employing a two-step precolumn-derivatization procedure. Detection was performed with an excitation wavelength of 488 nm and an emission wavelength of 520 nm. Detection limits for the derivatized analytes were in the range of 50–100 pg ml^{-1}. Additionally, few endogenous compounds from the urine samples were extracted using the CE-LIF extraction procedure. An alternative approach from the same group used field amplified sample stacking [178]; for the detection of 6-acethyl morphine, morphine, codeine, and nor-morphine (heroin metabolites) in urine, a method was developed using a pH 6.0 running buffer containing 50 mM sodium phosphate and 15 mM b-CD and UV detection at 214 nm. The application of the mentioned sample stacking provided detection limits of about 40 ng ml^{-1} in the biological matrix with good reproducibility, precision, and accuracy.

CE with head-column field-amplified sample stacking was applied to the determination of opioids in urine by Wey and Thormann [179]. This method is based upon electro-injection of analytes from sample extracts of low conductivity, resulting in a sensitivity enhancement 1000-fold, using UV detection. Electro-injection, applied to CE-ion trap MS–MS and MS–MS–MS of two-fold diluted urines, urinary solid-phase and liquid–liquid extracts provided greater sensitivity compared to hydrodynamic injection of these samples. Unambiguous confirmation of free opioids and their glucuronic acid conjugates from solid-phase extractions with electroinjections was also observed.

Wey and Thormann [180] used CE-tandem MS and CE-triple MS with atmospheric pressure electrospray ionization for the analysis of morphine and related opioids in urine. Commencing with 2 ml urine and reconstitution in 0.2 ml sample

solvent, detection limits for free opioids of 100–200 ng ml^{-1} were obtained with hydrodynamic sample injection.

A novel multi-target antibody to morphine and derivatives was induced by designed morphine-3-site substituted and the polyclonal antibody was prepared with immunizing rabbits. A simple, specific and accurate method for the determination of morphine and related compounds, codeine, acetylcodeine, 6-monoacetylmorphine and morphine-3-glucuronide in urine of heroin abusers, has been developed using the multi-target immunoaffinity column (IAC) prior to capillary electrophoresis separation [181]. The analytes were extracted from the urine of drug addicts with the column, which was made by coupling CNBr-activated Sepharose 4B and multi-target polyclonal antibodies. The analytes of interest were extracted with a methanol/water mixture in one step. Baseline separation of these analogs was achieved by CE using β-cyclodextrin as additive and they were monitored at 214 nm. The assay presented very good reproducibility and precision with the recovery and detection limit between 91–105% and 10–20 ng ml^{-1} based on S/N = 2, respectively. The inter- and intra-day variation, capacity and elution conditions of the immunoaffinity column were also discussed. The metabolites in five heroin addicts' urine were measured by the present method. The experimental results indicated that the combination of IAC and CE was a useful technique for determination of heroin metabolites from urine samples.

4. Conclusions

Morphine and other opiate and cocaine abuse continues to represent significant health problems for much of modern society. As a result, analytical chemists and toxicologists are frequently called upon to analyse biological specimens and drug seizures for the presence of parent drug and breakdown products. The variety of metabolites originating from biotransformation and degradation processes makes this a formidable problem. The state of the art in the modern analytical instrument making allows reliable and highly sensitive systems for determinations based on individual analyte parameters to be designed. Among these characteristic parameters of the compound is its mass spectrum, which can be used for its identification. Combining various versions of chromatography with the mass-selective detection of the separated components is one of the promising trends in the analysis of composite mixtures of unknown composition.

The use of GC–MS for the identification and measurement of drugs of abuse is currently believed to be crucial to acceptance of evidence in legal proceedings because of its sensitivity and specificity.

Although micellar chromatography has been used for the determination of drugs in biological fluids since 1985, relatively few researchers have applied the technique to therapeutic monitoring.

Also, various versions of chromatographic analysis are among these techniques widely used in forensic expert examinations. Of these techniques, TLC, GC, and GC–MS are most popular among forensic expert examiners. Chromatographic analysis in combination with other techniques, such as optical spectroscopy (UV and IR), provides an opportunity to determine unambiguously the structure of a test substance, to identify analytes, to determine concentrations, and to reveal the identity or differences in the composition of trace components in test materials. TLC is one of the main techniques used for examining controlled compounds, such as narcotic and psychotropic agents. In the forensic examinations of these substances, TLC is used both in target-oriented (particular) examinations for the identification of a particular substance (to confirm or disprove the presence of this substance in the test material) and in the screening of materials of unknown nature. The methodological approaches to solving these problems are somewhat different from one another.

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