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Review

Tropane alkaloid analysis by chromatographic and electrophoretic techniques: An update☆

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ABSTRACT

Tropane alkaloids like atropine are antidotes applied against organophosphorus intoxications. Atropine is toxic itself and should be closely monitored during treatment. Hence, simple, fast, and sensitive determination methods for tropane alkaloids in serum are desirable. Mostly adopted methods of analysis are gas chromatography (GC); high performance liquid chromatography (HPLC), and capillary electrophoresis (CE). Various liquid and solid capillary fillings used in micellar electrokinetic chromatography, microemulsion electrokinetic chromatography, capillary electrochromatography, and enantioseparation provide high versatility to CE applications. In HPLC, specialised columns enhance separation efficacy. Ultraviolet light detection is common practise, but recently sensitivity and analyte identification were enhanced by coupling GC, HPLC, and CE to mass spectrometry. Apart from medical treatment, tropane alkaloids, cocaine in particular, are abused with various intentions. Forensic analysis of tropane alkaloids and their metabolites comprises the additional difficulty of unequivocal drug identification. Because of severe legal consequences, sophisticated analytical methods were developed and may provide additional techniques for therapeutic drug monitoring. Examples from forensic cocaine analysis and from doping analysis are included in this review.

Contents

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1. Introduction

1.1. Fields of tropane alkaloid analysis

For the treatment of intoxications with organophosphorus compounds, tropane alkaloids are important antidotes, given to victims of poisoning to counteract excessive acetylcholine effects. Dosage of therapeutic alkaloids in these cases is difficult, because the amount of organophosphorus toxicant that has been taken up and actually circulates in the body is usually unknown [1,2]. The tropane alkaloids are highly toxic themselves and should be dosed carefully; overdosing of the antidote may have a dramatic outcome. Also, individual patients appear to possess different sensitivity to tropane alkaloid treatment [3]. However, when no other means of monitoring are available, tropane alkaloid dosage is only adjusted following the visible intoxication symptoms. For practical atropine dosing schedules, a lack of evidence in the current recommendations leads to wide variations in clinical applications [4]. Therefore, simple, fast, and sensitive determination methods for alkaloids in serum are as highly desirable as for the organophosphorus toxicants.

Tropane alkaloids for therapeutic purpose are isolated from plants and applied as salts, e.g. atropine sulfate, or as semisynthetic derivatives such as homatropine bromide or *N*-butylscopolamine bromide (Fig. 1). The term tropane alkaloids derived from the 8-membered bicyclic ring system with a methylated bridge forming nitrogen that is called 8-methyl-8-azabicyclo[3.2.1]octane (IUPAC) or tropane (trivial name). Tropane alkaloids are obtained from Solanaceae, e.g., *Atropa belladonna*, *Hyoscyamus niger*, and *Datura stramonium*. The first pure compounds were atropine isolated from *Atropa belladonna* and hyoscyamine from *Hyoscyamus niger* [5]. Today, "atropine" is defined as the racemic mixture of (*S*)-hyoscyamine and (*R*)-hyoscyamine. (*S*)-hyoscyamine is genuine in plants and (*R*)-hyoscyamine forms under alkaline conditions. (*S*)-hyoscyamine possesses strong acetylcholine-inhibitory activity by blocking muscarine receptors, while the (*R*)-hyoscyamine is mostly inactive. Atropine, which is more often applied than (*S*)-hyoscyamine, exhibits approximately half of the pharmacological activity of (*S*)-hyoscyamine. Scopolamine, in contrast, is mostly applied as pure enantiomer, e.g. (*S*)-scopolamine bromide. Different tropane alkaloid derivatives and enantiomers also vary pharmacokinetically, so that actual blood levels will result from dosing and different turnover and excretion kinetics. High inter-individual variation in pharmacokinetic parameters was observed with tropane alkaloids after intravenous application [6]. When medication is to be monitored in short intervals, the major demands on the analytical methods are velocity and sensitivity. When drug metabolism in the body is to be investigated, degradation products with different physicochemical characteristics must be included into the analyses.

Tropane alkaloids, cocaine in particular (Fig. 2), apart from being applied for medicinal purposes are abused with various intentions. Forensic analysis of intoxication victims and of suspects comprises the additional difficulty of identification of the drugs and their metabolites, sometimes from a cocktail of various narcotics that were ingested [7]. In cases where a culprit is to be sentenced, analysis results must be quantitative and unequivocal in order to be litigable. Reliable drug identification and quantitation is also the task of doping analysis in sport contests [8]. Because of the high amount of money to be earned by drug dealers or by successful athletes, strategies to conceal drug trade and consumption are quite advanced. Because of the severe legal consequences and the high public concern, analytical methods to identify those drugs nevertheless are sophisticated and may provide techniques and strategies for therapeutic drug monitoring. Some examples from forensic analysis and from doping analysis are included in this review.

1.2. Intended coverage of the review

Irrespective of the purpose of the investigations, tropane alkaloids are predominantly analysed by gas chromatography (GC) and high performance liquid chromatography (HPLC). In addition, capillary electrophoresis (CE) is a versatile and fast developing analytical technique, and examples of CE separation of tropane alkaloids have been included in this review. Thin layer chromatography is of importance in fast analytical screening of complex samples, typically plant extracts, because they do not need intricate sample work up before separation. The technique is fast, robust, and costs for equipment and consumables are usually reasonable. Thin layer chromatography procedures for tropane alkaloids were included in a former review [9]. A recent monograph on thin layer chromatography in phytochemistry [10] and a review on advanced two-dimensional thin layer chromatography in the analysis of secondary plant metabolites include methods for tropane alkaloids [11]. In the present review, thin layer chromatography is not discussed in detail.

Methods for tropane alkaloid analysis in plant tissues and in human body fluids were recently summarised [12]. This review extensively describes procedures for tissue or fluid extraction and for sample preparation, which are of particular importance for all subsequent chromatographic measurements. Comprehensive comparisons of tissue work-up and sample preparation procedures for a large number of drugs and their metabolites allow generalisations that may be valuable for tropane alkaloid sample preparation in therapeutic drug monitoring by HPLC–MS [13] and in forensic urine samples by GC–MS [14]. Reduction of sample volume is considered as beneficial for both, faster analysis and the opportunity of direct coupling of the extraction device to the sample injector of the chromatography apparatus. Headspace air sampling for drugs like cocaine by direct introduction of polydimethyl siloxane fibres after solid phase microextraction of air was described [15] and belongs to the solvent-free techniques for extraction of analytes, which also have been summarised [16]. Microextraction by packed sorbent (MEPS) is the miniaturisation of conventional SPE packed bed devices from millilitre bed sizes to microlitre volumes. MEPS cartridges, like siloxane fibres, can be connected directly to a GC or a LC injector. The cartridges containing the solid packing material, after loading of the analytes and washing off contaminations, are placed as plugs between the glass syringe and the injection needle. The procedure can be fully automated [17]. A wide variety of sample preparation techniques before chromatographic analysis was comprehensively discussed in a recent survey [18]. As there are excellent summaries and surveys available in the recent literature, the present review does not focus on extraction and sample preparation.

Authors have tried to point out novel developments in tropane alkaloid chromatography and discussed velocity, specificity, and

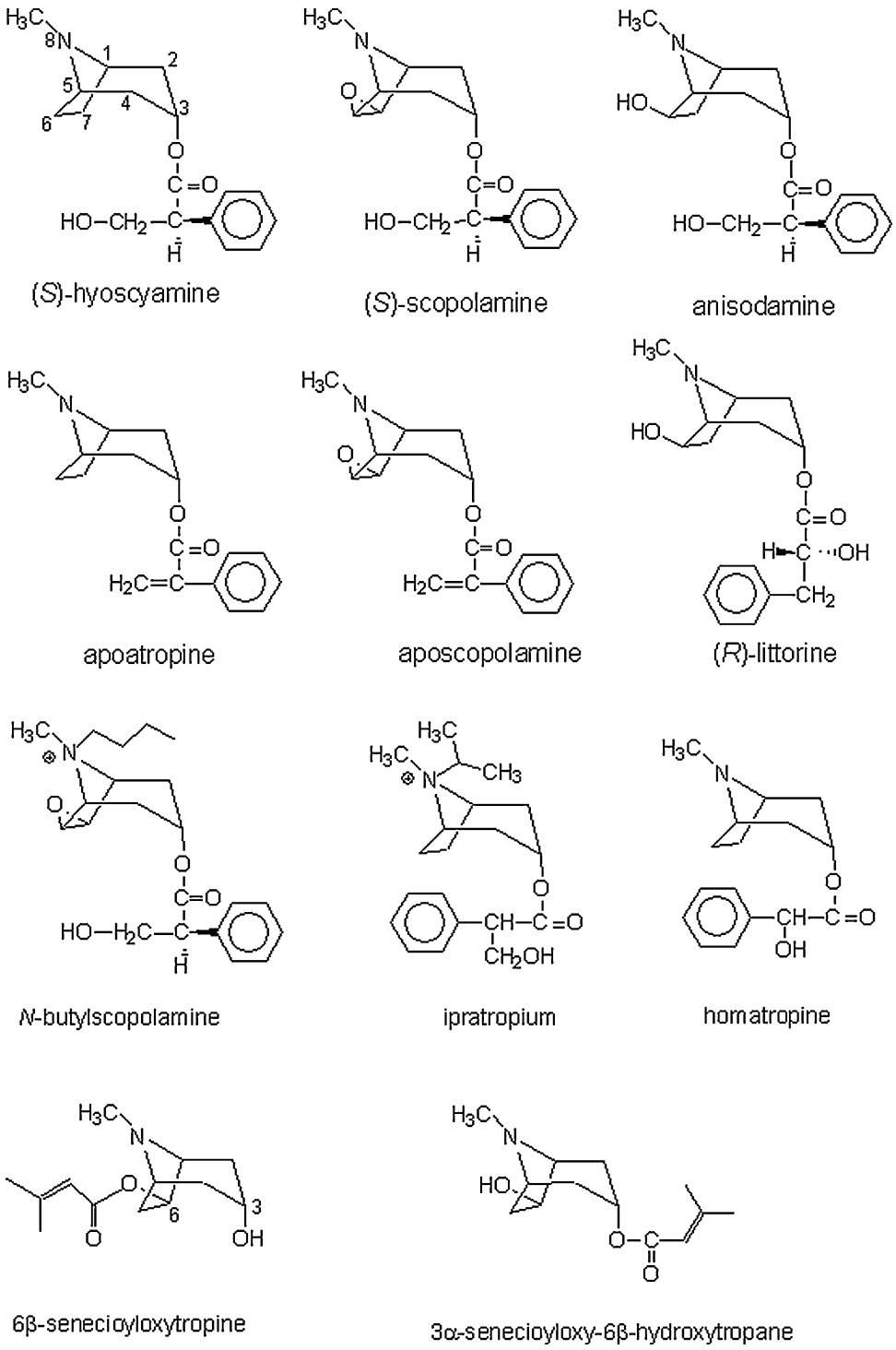

Fig. 1.

sensitivity of chromatographic and electrophoretic techniques. The literature was searched back to the year 2002. A complete survey of publications on atropine and scopolamine analysis was obtained, but in cases where similar methods were applied in several publications only examples were chosen and presented. Choice of literature examples was directed by a detailed description in chromatographic methods, by a report of limits of detection and quantitation, by originality of results, and the choice was limited to publications in English language. Reviews on chromatographic methods and materials were cited when they appeared recently (< 5 years) and their focus complemented the topics that are covered in this review. Literature on analysis of cocaine and its metabolites is broad and was not evaluated exhaustively, as cocaine is not in the focus of the present compilation. Due to the forensic interest in cocaine, novel and unique analytical approaches have been made. Some analytical approaches for cocaine and its metabolites may therefore serve as guidelines for optimisation of atropine and scopolamine measurements.

2. Gas chromatography

Analysis of tropane alkaloids was performed by gas chromatography long before high performance liquid chromatography (HPLC) methods were established [19]. GC with flame ionisation detec-

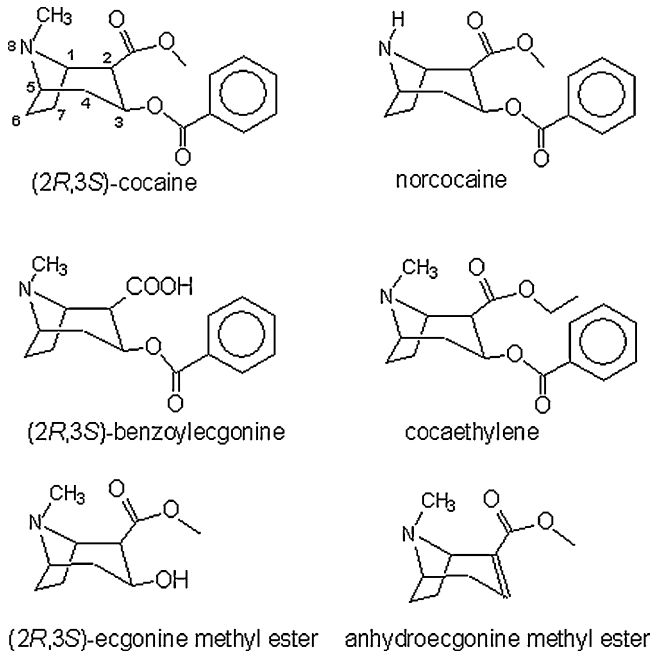

Fig. 2.

tion (FID) was applied to pharmaceutical preparations and tropane alkaloids in plant extracts. After HPLC became routine, tropane alkaloids were still analysed by GC due to their weak UV light absorption. Tropane alkaloid detection by GC and FID was more sensitive than by HPLC and UV detection. Also, many tropane alkaloids were sufficiently volatile for direct separation without derivatisation (summarised in ref. [9]). Nowadays GC is still applied as routine method for tropane alkaloid chromatography, in particular in combination with mass spectrometry (MS) detection. Major fields for GC–MS analysis are tropane alkaloids in plant extracts and in toxicological analysis. Also, when pharmacokinetic behaviour of tropane alkaloids is monitored, GC–MS together with HPLC (see below) are the methods of choice [20].

2.1. Alkaloids in plant extracts

Tropane alkaloids are more widespread in the plant kingdom than generally assumed. Only a few Solanaceae genera, *Atropa, Hyoscyamus, Duboisia,* and *Datura* (of which some species are also named *Brugmansia*) are used for extraction of hyoscyamine and scopolamine, but further Solanaceae genera such as *Schizanthus* species contain diverse mixtures of tropane esters. Also, angiosperm families distant from Solanaceae like Proteaceae, Euphorbiaceae, and Rhizophoraceae form various tropane derivatives [21,22]. Convolvulaceae are a sister family to Solanaceae, and many species within the Convolvulaceae were shown to contain tropane alkaloids. Tropane alkaloids from plants were mostly analysed by GC–MS in the past, and typical tropane fragmentation patterns were published for many major and minor compounds in the alkaloid mixtures. Typically, plant tissue is exhaustively extracted with methanol. The dried extract is dissolved in chloroform or dichloromethane and washed with alkalised water. After evaporation and solution in a GC-compatible solvent, e. g. methanol or hexane, this crude extract is subjected to GC–MS. For example, Jenett-Siems and colleagues [23] elucidated 74 tropane derivatives in an extended study on *Merremia* alkaloids and listed their fragmentation patterns in GC–MS. Ott and colleagues observed heterogeneous mixtures of compounds with typical tropane fragmentation pattern by GC–MS (quadrupole mass spectrometer) in crude plant extracts of African Convolvulaceae species [24]. They isolated tigloyl- und methylbutyryl esters of both α- and β-3-hydroxytropanes and confirmed the structures by ^{1}H-NMR and ^{13}C-NMR. Detailed tables of GC–MS fragment patterns in those and other publications enable identification of tropane alkaloids in further plant extracts by mass spectral data and Kovats retention indices [25–29]. Bieri and colleagues [30] applied fast gas chromatography for the separation of tropane alkaloids extracted from the stem-bark of *Schizanthus grahamii* (Solanaceae) on short narrow bore columns (3 m × 100 μm ID and 1.5 m × 50 μm ID) connected to a quadrupole mass spectrometer. The alkaloids to be separated were four isomeric esters, named 3α-senecioyloxy-7β-hydroxytropane, 3α-hydroxy-7β-angeloyloxytropane, 3α-hydroxy-7β-tigloyloxytropane and 3α-hydroxy-7β-senecioyloxytropane. According to their structural formula, all four compounds should be denoted as 3,6-dihydroxy tropane esters or 6-hydroxy tropine esters, e. g. 6β-senecioyloxytropine, CAS 877785-35-8 (Fig. 1) and 3α-senecioyloxy-6β-hydroxytropane (Fig. 1), CAS 77101-57-6. Using hydrogen as carrier gas, velocities of 150 m/s in the column were calculated after optimisation of stationary phase and internal column diameter. Baseline separation of the four alkaloids under these conditions was completed in 9 s. It is evident that this short analysis time is limited to isothermal analysis, as no reproducible temperature gradient is applicable.

2.2. Analysis of intoxications

GC–MS is often used when a fast and reliable identification of a wide possible range of compounds is necessary in intoxications. Plant poisonings may occur by unintended ingestions, intended ingestions, and poisoning due to abuse of plant material. Unintended ingestions are typical for children or in adults as a consequence of insufficient plant knowledge and adulterations of harmless herb preparations. Intended ingestions are common in homicides and suicides. Increasingly common is the abuse of plants for hallucinogenic effects. Atropine is the racemic mixture of (*R*)- and (*S*)-hyoscyamine, of which only (*S*)-hyoscyamine, the *laevo*-enantiomer of atropine, is formed in plants. Due to easy racemisation during extraction and processing, atropine is mostly applied in medicine. However, the endogenous plant compound (*S*)-hyoscyamine contained in plant samples is much more toxic than (*R*)-hyoscyamine. Therefore intoxications with plants samples can become severe with only small amounts of herbs, considering that 10 mg of atropine were reported to be lethal [31], and belladonna leaf contains ca. 0.5% (*S*)-hyoscyamine, i.e. 5 mg/g dried leaf.

Balikova [32] described a case where over 30 people were poisoned by a herbal tea mixture propagated as “support for lucid thinking” in a meditation session. The toxicological laboratory could rapidly identify atropine, scopolamine in addition to harmane alkaloids in samples of the herbal infusion and in the gastric lavage of many patients. Ether extracts of herbal tea and of gastric lavage were injected directly into a GC–MS (electron ionisation) equipped with a HP-5 fused silica MS column, dimensions 30 m × 0.25 mm internal diameter × 0.25 mm film thickness. The concentrations of hyoscyamine and scopolamine in the herbal infusion were 27 and 515 mg/l, resulting in a single dose of ca. 4 mg hyoscyamine and ca. 78 mg scopolamine per cup of tea. Serum samples from patients before GC–MS analysis were tenfold concentrated by evaporation after purification by solid phase extraction. In the serum samples atropine as detected in all cases, average 5 ng/ml. Scopolamine was detected in about half of serum samples, average 13 ng/ml. The example shows that a robust and versatile GC–MS allows fast identification of tropane alkaloids and enables physicians to specifically counteract the intoxication.

Direct injection of tropane alkaloids into GC is often performed, however, dehydration in the hot injection port may lead to apoatropine and aposcoploamine formation (Fig. 1). To improve stability and reliable quantitation, tropane alkaloids were analysed as trimethylsilyl derivatives [33]. Some silylation reagents, *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), trimethylchlorsilane (TMCS), *N*,*O*-bis(trimethylsilyl)acetamide (BSA), and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) were examined and derivatisation with a mixture of BSTFA and TMCS (99:1) achieved best results. The derivatives showed good chromatographic behaviour. Hyoscyamine (retention time 13.20 min) and scopolamine (retention time 13.70 min) were separated on HP-5 fused silica MS column, 30 m × 0.25 mm internal diameter (i.d.) × 0.25 μm film thickness. Deuterated atropine-d3 was used as internal standard. Calibration showed good linearity in the range of 10–5000 ng/ml, the limit of detection (LOD) was 5.0 ng alkaloid/ml for biological materials. In an intoxication case of an amateur herbalist, the methods turned out as fast and reliable. In 1996 already, Oertel et al. [34] determined scopolamine in serum by gas chromatography coupled to ion trap tandem mass spectrometry. The mass spectrometer was operated with positive ions in the selected reaction mode with chemical ionisation using methane. The limit of detection for scopolamine derivatised with MSTFA was 50 pg/ml in serum. GC–MS methods and other means for fast and reliable determination of toxic alkaloids from a variety of plants in human body samples have been reviewed [35].

Tropane alkaloids are among the most frequently abused hallucinogens, and they appear in clinical and forensic analysis together with cannabinoids, *N*,*N*-dimethyltryptamine derivatives, and synthetic or semisynthetic hallucinogens. Thin layer chromatography can detect higher concentrations of most hallucinogens, atropine and scopolamine included [36,37]. For specific and sensitive analysis of hallucinogens in biological material GC–MS with total ion monitoring (TIC mode) in *m*/*z* range of 40–450 was applied [37]. The column was Rtx5 MS (dimensions 15 m × 0.25 mm; film thickness 0.25 μm). Under optimised conditions, silylated atropine and scopolamine eluted after 11.8 and 12.5 min, respectively. LOD was at 0.2 ng/ml, and the limit of quantitation (LOQ) was at 1 ng/ml for both compounds. For trace analysis when searching for a specific compound, single ion monitoring (SIM mode) proved to be helpful. Ions typical for atropine and scopolamine after silylation appeared as signals at *m*/*z* 361 and *m*/*z* 375.

2.3. Cocaine in forensic analysis

Cocaine is a tropane alkaloid of specific interest in forensic cases. To furnish a proof of acute illicit cocaine intake is as important as the unequivocal analytical evidence of a history of cocaine consumption. In most laboratories GC–MS is the method of choice for cocaine analysis. Alternative methods of analysis comprise immunological tests marketed for drug screening in urine [38], in saliva [39] and in serum [40,41]. The advantages of immunoassays are rapid results and the opportunity for large-scale screening by automation. Various forms of immunoassay techniques are in use, among them fluorescence polarisation immunoassay and radioimmunoassay. In addition, immunoassays are used in many mobile screenings kits. The main disadvantages of immunoassays are false-positive results when a drug of the same structural class cross-reacts with the antibodies or false-negative results due to insufficient limits of detection. Cocaine screening immunoassays usually apply antibodies raised against benzoylecgonine, one of the two major cocaine metabolites in human blood (Fig. 2) [42]. They report cocaine only weakly, e.g. when 300 ng/ml benzoylecgonine are detected, cocaine would be visible in concentrations ranging from 10,000 to 80,000 ng/ml [43]. Thus, a very recent consumption of cocaine may escape immunological analysis, if too little time has elapsed for metabolism of the parent drug to benzoylecgonine, which then occurs in serum or urine. Other parts of the body can be used for drug analysis such as hair [44,45] or sweat [46] and offer less invasive ways of sampling than obtaining blood serum. They contain cocaine and methylecgonine, but only little benzoylecgonine. Possibly due to its zwitterionic structure it is hardly excreted by sweat or into hair follicles. Sweat was collected by cellulose patches that have to be worn by the persons observed for seven days [47]. GC–MS analysis of silylated patch extract allowed a LOD of 2.5 ng per patch for cocaine and its degradation products ecgonine methyl ester, benzoylecgonine, and anhydroecgonine methyl ester [47] and a LOQ of 5 ng of each compound per patch [48]. Saliva is increasingly used in drug testing as it is readily available and obtained by non-invasive, fast, and easy techniques of collection, including a lower possibility of sample adulteration compared with urine collection that required privacy [49]. Saliva can be analysed on the spot of apprehension of suspects by immunoassays. However, drugs that are basic in nature like cocaine accumulate to higher concentrations in oral fluid than in blood serum, whereas an acidic drug metabolite like benzoylecgonine, which is detected immunologically, has a much lower concentration [50,51]. Relationships between saliva and blood concentrations of cocaine and metabolites determined by GC–MS, however, were highly variable and do not allow calculation of the blood concentrations from saliva concentrations [52].

In consequence, results of immunoassays are always considered presumptive until confirmed by a chromatographic proof for the specific drug. Different structural properties of cocaine and its metabolite not only render immunological detection difficult For GC–MS, polar metabolites often need derivatisation for exact quantitation. Silylation by BSTFA-TMCS like for atropine and scopolamine was applied to cocaine and the metabolites benzoylecgonine and ecgonine methyl ester [53]. Several silylation steps [46] or derivatisation with several reagents according to the chemical nature of the metabolites [54] were successful for detection of methyl ecgonidine and thirteen further cocaine metabolites in human blood and urine, however, they demanded tedious sample preparation. A wide range of cocaine metabolites could be derivatised simultaneously by a mixture of pentafluoropropionic anhydride (PFPA) and 2,2,3,3,3-pentafluoro-1-propanol (PFPOH). PFPA reacts with hydroxyl groups (alcoholic and phenolic) and secondary amino groups to form pentafluoropropionyl ($-COC_2F_5$) esters and amides, respectively, while PFPOH reacts with carboxyl groups to produce pentafluoropropyl ($-C_3H_2F_5$) esters [55]. Derivatisation of benzoylecgonine by a mixture of PFPA and PFPOH (2:1) enabled a LOQ of 20 ng/ml [42]. A harmonisation of analytical procedures for cocaine and accompanying alkaloids by GC–MS in forensic laboratories between France and Switzerland was achieved. The comparability of results allows cross-border collaboration and data that are resilient in jurisdictional cases [56,57].

Consumption of cocaine worldwide is high, it is estimated consistently at 560–670 metric tons per year since 2000 [58]. In addition to the traditional application by sniffing, smoking of often impure cocaine base preparations (“crack”) has become widespread. This way of consumption causes pyrolysis products, e.g. anhydroecgonine methyl ester (Fig. 2) to appear in the human body as further metabolites [54,55]. Analytical procedures for the whole array of cocaine metabolites and further drugs in blood [59] and in saliva [60] were comprehensively summarised. The intensive cocaine consumption leaves traces in the environment. Waste water [61] and air particles in larger cities [62,63] contain quantifiable amounts of cocaine. Banknotes are contaminated with cocaine [64,65], and methods for determination of cocaine on banknotes from many countries by HPLC–MS, GC–MS, CE and other means were summarised [66]. Cocaine consumers cause problems to others when they drive cars or fly airplanes [40–42,42,55,67,68].

Table 1
Determination of tropane alkaloids (except cocaine) by GC and HPLC methods.

Method	Compound	LOQ/LOD	Reference
GC–MS	Atropine Scopolamine (silylated derivatives)	LOQ 1 ng/ml blood LOD 0.2 ng/ml blood	[37]
GC–MS	Hyoscyamine Scopolamine (silylated derivatives)	LOQ 10 ng/ml LOD 5 ng/ml in both serum and urine	[33]
HPLC-APCI-MS	Atropine Scopolamine	LOQ 5 ng/ml plasma	[98]
HPLC-ESI-tandem-MS	Atropine Scopolamine	LOQ 0.1 ng/ml plasma	[98]
HPLC-MS	(*R,S*)-hyoscyamine	LOQ 0.5 ng/ml plasma	[90]
HPLC-MS	Atropine Scopolamine	LOQ 100 ng/ml standard solution LOQ 1 ng/ml standard solution LOD 10 pg/ml standard solution LOD 100 pg/ml standard solution	[94]
HPLC-tandem-MS	Ipratropium *N*-Butylscopolamine Atropine Homatropine Littorine Scopolamine	LOQ 50–100 pg/ml plasma for all alkaloids, except *N*-butylscopolamine (800 pg/ml plasma) LOD 4–10 pg/ml plasma	[2]
HPLC-tandem-MS	Atropine Scopolamine	LOQ 5 ng/ml urine LOQ 10 ng/ml urine LOD 2 ng/ml urine LOD 10 ng/ml urine	[99]
HPLC-tandem-MS	Scopolamine	LOQ 20 pg/ml serum	[88]
HPLC-UV	Atropine Scopolamine	LOQ 5 ng/ml urine LOQ 8 ng/ml urine LOQ 9 ng/ml serum LOQ 10 ng/ml serum	[95] [95]
HPLC-UV	Atropine	LOQ 4 μg/ml parenteral preparation LOD 1.2 μg/ml parenteral preparation	[81]
HPLC-UV	Atropine, tropic acid, norhyoscyamine, 6-hydroxyhyoscyamine, 7-hydroxyhyoscyamine, hyoscine, littorine, apoatropine, atropic acid	LOD 20–30 ng/ml eye drop solution	[82]
HPLC-UV	Atropine Scopolamine	LOQ 10 μg/ml standard solution LOD 1 μg/ml standard solution	[94]
HPLC-UV	Atropine	LOQ 25 ng/ml plasma LOD 10 ng/ml plasma	[135]

Cocaine is consumed with various intentions, higher achievements in athletics being one [8]. Consumption with the aim of better performance in sport is not limited to human athletes but extended to racing horses, which react to cocaine by increased blood pressure and masking of fatigue [69]. Benzoylecgonine in horse urine was identified and determined in 21 racing horses with a LOQ of 5 ng/ml, arbitrarily taken from the lowest point on the standard curve. The range of the seven-point standard curve was 5–300 ng/ml, and the LOD was 1 ng/ml with a signal-to-noise ratio of 3:1.

2.4. Summary of GC methods

A survey of analysis of tropane alkaloids analysis by GC–MS and a comparison of sensitivity is given in Tables 1 and 2.

3. High performance liquid chromatography

Since the first separation of atropine, its dehydration product apoatropine, scopolamine, and semisynthetic homatropine (Fig. 1) in 1973 [70], analysis of tropane alkaloids by high performance liquid chromatography (HPLC) became steadily more common. Separations were usually performed on reversed phase (RP) columns, and separation methods were developed and applied for tropane alkaloids in pharmaceutical preparations, in plant material, and in clinical and forensic probes. Low UV light absorption of many tropane alkaloids and almost no UV light absorption of many of their degradation products was a draw-back for sensitive analysis in HPLC equipped with conventional fixed wavelength UV light or diode array detectors [71]. Also, tropane alkaloid metabolites of different chemical nature, e.g. hydrophilic or acidic, made separation and quantitative determination in one chromatographic run difficult. In recent years much progress has been made in the analysis of tropane alkaloid by HPLC, which is mainly related to three different areas:

- Analyte detection by mass spectrometry alternative to UV light overcomes sensitivity limitations.
- At the same time, HPLC coupled to MS, in particular tandem MS, offers enhanced identification reliance.
- Ultra performance liquid chromatography (UPLC) replacing common HPLC reduces analysis time maintaining satisfactory separation.

Table 2
Determination of cocaine and major metabolites.

Method	Compound	LOQ/LOD	Reference
GC-FID	Cocaine, BZE, EME (Silylated derivatives)	LOQ 400 ng/ml urine LOQ 100 ng/ml blood	[53]
GC–MS	Cocaine, BZE	LOQ 20 ng/ml blood	[42]
GC–MS	BZE	LOQ 5 ng/ml urine LOD 1 ng/ml urine	[69]
GC–MS	Cocaine (1), BZE (2), EME (3), norbenzoyl-ecgonine (4), norcocaine (5), ecgonine (6), m-Hydroxybenzoyl-ecgonine (7), anhydroecgonine methyl ester (8), cocaethylene (9), Norcocaethylene (10) Ecgonine ethyl ester (11)	LOD (1/2/3/5/9/10/11) 2 ng/ml LOD (4) 25 ng/ml blood LOD (6) 640 ng/ml blood LOD (7) 50 ng/ml blood LOD (8) 13 ng/ml blood	[55]
GC–MS	Cocaine (1), cocaethylene (2), norcocaine (3), norcocaethylene (4), benzoylecgonine(5), norbenzoylecgonine (6), m-hydroxy-benzoylecgonine (7), p-hydroxy-benzoylecgonine (8), methyl ecgonine (9), ethylecgonine (10), ecgonine (11), methyl ecgonidine (12), ethyl ecgonidine (13), ecgonidine (14), norecgonidine (15)	LOQ urine (1/2/7/8/9/12/15) 1 ng/ml (3/4/6/14) 2 ng/ml; (5) 4 ng/ml; (10) 0.5 ng/ml; (11) 16 ng/ml; LOQ blood (1/2/8/9/12) 1 ng/ml; (3/6/10/13/14/15) 2 ng/ml (4/5) 4 ng/ml; (11) 16 ng/ml	[54]
GC–MS	Cocaine, BZE, anhydroecgonine methyl ester, EME	LOQ 5 ng/sweat patch LOD 2.5 ng/sweat patch	[47]
HPLC-APCI- tandem-MS	Cocaine, EME, BZE, ecgonine, norcocaine, cocaethylene, ecgonine ethyl ester	LOQ 20–32 ng/ml blood	[110]
HPLC-tandem-MS	Cocaine	LOQ 50 pg/ml plasma LOD 4 pg/ml plasma	[2]
HPLC-tandem-MS	Cocaine, benzoylecgonine, cocaine *N*-oxide	LOQ 2.5 ng/ml plasma	[113]
HPLC-tandem-MS	Cocaine, benzoylecgonine	LOQ 14 pg/banknote LOD 4 pg/banknote	[131]
HPLC–UV	Cocaine, BZE, cocaethylene	LOQ 100 ng/ml plasma	[107]

BZE: benzoylecgonine, EME: ecgonine methyl ester.

- Diversification of chromatography material, such as hybrid column materials and restricted-access media columns, enables enhanced separation and saves sample preparation efforts.

3.1. Alkaloids in plant extracts and in pharmaceutical preparations

UV light and diode array detectors (DAD) are common laboratory equipment, and if the identity of the tropane alkaloids to be analysed is known and very low levels of analytes are no problem, HPLC-UV is widely practised. In plant tissue and cell culture analysis, hyoscyamine and scopolamine are measured by HPLC-DAD usually after separation on a RP18 column in an acetonitrile-buffer solvent system. Quantitation is mostly performed between 210 and 220 nm [72–80]. Control of pharmaceutical preparations containing atropine is possible by HPLC and DAD detection [81,82]. At 228 nm detection wave length, the LOQ for atropine was 4 μg/ml in parenteral injection devices [81]. Atropine and degradation products such as apoatropine and biosynthetic by-products such as anisodamine (=6-hydroxyhyoscyamine) and littorine were measured in eye drop solutions [82]. LOD for the contaminating alkaloids at 215 nm were determined to be 20–30 ng/ml. The differences in LOQ and LOD between the two studies, apart from instrumental peculiarities, may be caused by the differences in detection wavelengths. The UV spectra of atropine and other tropane alkaloids show a steep decrease in absorbance starting from 200 nm and going down to 235 nm [83].

3.2. Therapeutic drug monitoring and pharmacokinetic investigations

Therapeutic doses for atropine and scopolamine range from 0.1 to 1 mg for adults [31], therefore analyses in therapeutic drug monitoring and pharmacokinetic investigations must be able to determine low concentrations [84]. Also laboratory animals that serve for pharmacokinetic investigations are usually small rodents, e.g. rats or mice that provide only limited sample volumes of blood or urine. Therefore, analyses are mostly performed by mass spectrometry coupled to GC (see above) or to HPLC. The ionisation of analytes and the elimination of a surplus of solvent molecules in mass spectrometry coupled to HPLC (HPLC–MS) was the major obstacle during development of machines for routine analysis, and the problem was solved by various constructions. The most common ion sources at present are electrospray ionisation (ESI) in various modes (e.g. turbo ion spray, nano-electrospray, positive and negative ionisation) and atmospheric pressure chemical ionisation (APCI).

Atropine and eleven metabolites were determined in rat urine by liquid chromatography and tandem mass spectrometry (HPLC–tandem-MS) [85]. The metabolites were products of ester hydrolysis, *N*-demethylation, hydroxylation of the aromatic acid moiety, sulfation, and glucuronidation. Similarly, scopolamine metabolism was monitored in rat urine [86] and later in rat faeces, urine, and blood plasma samples [87]. Eight metabolites resulting from similar metabolism as observed for atropine (*N*-demethylated norscopolamine, dehydrated aposcopolamine, aponorscopolamine, hydroxyscopo-

lamine, hydroxyscopolamine *N*-oxide, norscopine, scopine, and tropic acid) were found together with the parent drug in the faeces or in plasma. Three additional metabolites (tetrahydroxyscopolamine, trihydroxy-methoxyscopolamine, and dihydroxydimethoxyscopolamine) were identified in rat urine. Numerous studies on the pharmacokinetic behaviour of scopolamine in the human body were summarised [20]. For example, for the detection of scopolamine from microdialysis, samples after solid phase extraction were injected into a HPLC–tandem-MS system [88]. The mass spectrometer was operated in the multi reaction monitoring (MRM) mode. Linear response was demonstrated from 20 pg/ml (LOQ) to 5 ng/ml scopolamine. The elimination half-life of scopolamine was compared between blood serum and subcutaneous adipose tissue of healthy volunteers [89]. Concentrations of scopolamine in adipose tissue resembled those in serum. (*R*,*S*)-Hyoscyamine was determined in human plasma after application of a total dose of 20 μg/kg (approximately 1.4 mg per patient) [90]. Two millilitre of human plasma were used per analysis and detected by quadrupole MS coupled behind an atmospheric pressure chemical ionisation interface. Single ion monitoring enabled a LOQ of 0.5 ng/ml. The problem of appropriate dosage of atropine after intoxication by organophosphates was addressed by Abbara et al. [91]. They used HPLC coupled with electrospray mass spectrometry for the simultaneous determination of three drugs often given simultaneously to victims of nerve gas intoxications. Diazepam liberated from the prodrug avizafone, pralidoxime, and atropine were measured in human plasma. Chromatographic separation on a C8 column (100 mm × 2.1 mm, 3.5 μm particle size) in formate buffer (2 mM, pH 3) and acetonitrile at a flow rate of 0.2 ml/min needed 9 min. The triple quadrupole mass spectrometer was operated in positive ion mode and multiple reaction monitoring (MRM) was used for drug quantification. The method was validated over the concentration range 0.25–50 ng/ml for atropine. John and colleagues extended the quantitation of tropane alkaloid levels in plasma by a similar HPLC–tandem-MS method to seven different compounds including the medicinally applied derivatives ipratropium and *N*-butylscopolamine. They achieved LOQs of 50–100 pg/ml for all tropane alkaloids included in the investigation [2]. The authors pointed out an important problem when dealing with serum or plasma samples: ester alkaloids will hydrolyse at different velocities and depending on the organism, in which they are monitored. Rabbit serum taken for example caused rapid degradation of atropine and scopolamine, but ipratropium and *N*-butylscopolamine remained intact over 160 min. Human serum will slowly hydrolyse cocaine. Stability investigations for drugs in biological samples were summarised [92].

3.3. Analysis of intoxications

While pharmacokinetic investigations mostly demand a sensitive and targeted analysis of known compounds, cases of intoxications additionally need confirmation or *de novo* identification of the toxic agents. Thus the requirements for the mass spectrometry results are more complex. Intoxications by tropane alkaloids are frequent, but reports on toxic doses of atropine and scopolamine for humans vary largely in the literature and are suspected to depend on individual parameters [93]. For both, atropine and scopolamine, 10–1000 mg were reported to be fatal. Many case reports of accidental poisoning by plants indicate that children are considerably more sensitive than adults. In a case of intoxication by *Datura* seeds, which had been identified by morphology, HPLC-DAD results were compared to HPLC-MS in a quadrupole instrument [94]. Columns were RP18, 150 mm × 2.1 mm, 5 μm particles, and elution was performed in acetonitrile mixed with a volatile buffer consisting of ammonium acetate pH 10.5. Detection by mass spectrometry for atropine and scopolamine was more than 1000-fold more sensitive; LODs for atropine and scopolamine by MS were 10 and 100 pg/ml, respectively, and 1 μg/ml for both compounds by DAD. But DAD detection may be more sensitive [95]. A screening method for the simultaneous determination of thirteen plant alkaloids (among them atropine and scopolamine) in serum and urine was developed applying solid-phase extraction and reversed-phase liquid chromatography. LOQ for atropine and scopolamine from serum and urine ranged from 5 to 10 ng/ml. Not only humans but also animals may become victims of tropane alkaloid intoxications [96]. One from two horses with colic died after unsuccessful treatment, while the other was less seriously affected, but exhibited mydriasis. Scopolamine was found in the urine of the horse for three days following the colic attack, and atropine was detected for two days by HPLC–tandem-MS. The hay fed to the animals had been contaminated by *Datura ferox*.

Plant-derived psychoactive compounds like atropine and scopolamine are often ingested intentionally; however, the dosing of frequently impure or adulterated or insufficiently known herbs is risky. Direct injection of urine from intoxicated patients diluted with three deuterated internal standards allowed separation of ten plant-derived psychoactive substances by reversed phase chromatography within 14 min [97]. Electrospray ionisation and positive selected reaction monitoring (SIM) by tandem-MS enabled identification and quantification down to LOD 2 ng/ml for atropine and 10 ng/ml for scopolamine. The detection and quantitation of toxic alkaloids in human blood plasma by HPLC-APCI-MS with HPLC-ESI-tandem-MS were compared [98]. Alkaloids were separated on a C8 column. Both MS methods were found to be selective for the tested compounds. The assays were linear for atropine and scopolamine from 5 to 100 ng/ml for LC-APCI-MS and 0.1 to 100 ng/ml for LC-ESI-MS/MS, respectively. LC-ESI-tandem-MS possessed greater sensitivity and higher identification power than LC-APCI-MS. The applicability of both assays was demonstrated by analysis of plasma samples from suspected poisoning cases. In an effort to evaluate screening possibilities in urine for psychoactive alkaloids Björnstad et al. [99] analysed urine samples from 103 suspected cases of intoxications. Samples were collected over four years from patients who either admitted or were suspected of ingestion of psychoactive plant materials. HPLC-tandem mass spectrometry was used for analysis of ten plant-derived compounds including atropine and scopolamine. When ingestion of any of the plant-derived substances covered in this study was admitted or suspected, 77% of the cases could be confirmed analytically. Psilocin, originating from hallucinogenic mushrooms, was the most frequent drug, and psychoactive plants and mushrooms were commonly obtained *via* internet acquisition.

Correct identification of unsuspected substances in addition to the analysis of particular compounds can be of the utmost importance in toxicology. Analytical procedures suitable for clinical and forensic toxicology need careful validation; appropriate recommendations for correct interpretation of toxicological findings were published recently [100]. Avoiding pitfalls includes, besides a lot of analytical experience, individual inspection and manual correction of results [101]. For the screening of atropine, an automatic optimisation procedure of selected ion monitoring (SIM) utilised two major fragments (*m*/*z* 124 and 93) of the pseudomolecular ion at *m*/*z* 290.1. Atropine-d3 was used as internal standard. Applying the method for a forensic urine sample, a peak with a retention time and two selected daughter ions very close to those of atropine-d3 was observed, but the ratio between the two SRM transitions was not acceptable when compared to the calibration compound [101]. A general screening procedure for unknown compounds was applied that operated the mass spectrometer in the information-dependent acquisition mode, switching between a survey scan acquired in the enhanced mass spectrometer mode with dynamic subtraction of background noise and a dependent

scan obtained in the enhanced product ion scan mode [102]. This approach did not find any atropine in the sample but identified the interfering substance as benzoylecgonine resulting from cocaine intake.

A history of atropine and scopolamine consumption can be proved by hair analysis. Kintz and colleagues reported a case of a patient with hallucinations, mydriasis, tachycardia, and a systolic blood pressure to 180 who had ingested six flowers of *Datura innoxia* in a hot water infusion [103]. Three weeks after recovery, 200 mg hair was collected from the person and tested for atropine and scopolamine. Sample preparation comprised superficial decontamination with dichloromethane, segmenting into parts, cutting each segment into pieces smaller than 1 mm, incubation overnight in 1 ml phosphate buffer pH 8.4 containing atropine-d3 as internal standard, and extraction with 5 ml dichloromethane/isopropanol/*n*-heptane (50:17:33). The residue was reconstituted in 100 μl of methanol, from which 10 μl were separated on a C18 column (100 mm × 2.1 mm, 3.5 μm particles). The detector was a triple-quadrupole mass spectrometer connected to electrospray in the positive ionisation mode. For each compound, detection was related to two daughter ions (atropine: *m*/*z* 290.2–124.0 and 92.9; atropine-d3: *m*/*z* 293.1–127.0 and 92.9; scopolamine: *m*/*z* 304.1–138.0 and 156.0). Scopolamine, 14–48 pg/mg, was identified in the hair segments. Absence of atropine (LOD for atropine 2 pg/mg) in hair samples was consistent with its low concentration in *Datura innoxia* flowers. The hair segmentation (human hair grows ca. 1 cm per month) indicated that the patient had previously taken this or other tropane alkaloid containing plant preparations. Scopolamine was also abused for keeping children calm and compliant [104]. Even when parents denied to have administered the drug to their children, hair analysis by UPLC–tandem-MS revealed a continuous exposure to scopolamine for several months. Analytical procedures and precautions for the detection of chemicals in hair in cases of drug-related and drug-facilitated crimes were comprehensively reviewed [44,105,106].

3.4. Cocaine in forensic analysis

Cocaine as a drug of abuse is usually consumed in higher amounts than atropine. An adulteration of street-traded cocaine by atropine can therefore have a fatal outcome [93]. When a high number of suspected drug-using patients were presented to an emergency department, urine samples were positive for benzoylecgonine resulting from cocaine metabolism, but some clinical symptoms of the patients did not match to acute effects caused by cocaine. Symptoms such as tachycardia, dilated pupils, hot dry red skin, dry mouth, severe thirst, rather pointed to anticholinergic drugs. One patient's cocaine powder sample could be retrieved for analysis and contained 25% atropine sulfate monohydrate, approximately 60% cocaine hydrochloride, and the rest of the sample was dextrose.

The usually higher single doses of cocaine (ca. 50 mg up to 500 mg) and the established and court-approved GC–MS based proof for cocaine appear to have delayed the introduction of HPLC–MS for analysis of cocaine and its metabolites in drug samples and in human body fluids. Also, HPLC–MS instrumentation is more costly in both, purchase and maintenance, than GC–MS. HPLC with UV detection worked satisfactory for the simultaneous determination of cocaine, benzoylecgonine, cocaethylene, and further illicit drugs in plasma [107]. Chromatography after solid phase extraction was performed on a RP8 column (250 mm × 4.6 mm, 5 μm particle size) using gradient elution with acetonitrile-phosphate buffer pH 6.53. The detector response was linear at concentrations over the range 0.1–10 μg/ml in plasma. The average extraction recovery from plasma was 60% for benzoylecgonine and 89% and 94% for cocaethylene and cocaine. In a review of 2003 [108], the introduction of HPLC–MS for drug screening in forensic toxicology was already highly recommended. In spite of the high purchase costs for HPLC–MS machines, many analytical procedures for cocaine and similar drugs of abuse were published during the last years and seem to confirm this view. Cocaine and metabolites were analysed in hair by HPLC–APCI-tandem-MS [109]. Cocaine and its degradation products were found in post-mortem body fluids by HPLC–tandem-MS [110]. Analytical procedures for post-mortem analysis of drugs were reviewed [111,112]. HPLC–MS was found particularly useful, when cocaine metabolites were searched that cannot be separated by GC–MS. Cocaine-N-oxide will decompose in the hot GC-injector [67,113]. The compound was directly measured in rat plasma and in human plasma by HPLC with electrospray ionisation and mass spectrometry. Similarly, anhydroecgonine methyl ester *N*-oxide that is formed during crack smoking, was analysed in whole blood, serum, and urine samples from crack users [114]. In the recent years, a large number of further analytical approaches for cocaine and metabolites by HPLC-MS were published. Various biological materials were investigated, for example whole blood and blood serum [115,116], oral fluid [117], meconium (first stool from a newborn child, typically black coloured, contains compounds taken up during the intrauterine phase from the amniotic fluid) [118], and human fetal post-mortem brain [119]. Sensitivity and specificity of HPLC–MS analysis was proven by the measurements of cocaine metabolites from human excretions in surface water and waste water [120–122]. All methods employed tandem–MS for detection and measurement after HPLC or UPLC separations. Preliminary screening of cocaine and other drugs is often performed by immunoassays. Subsequent chromatographic analyses require additional time and effort, therefore combing screening and confirmation in a single analysis appears attractive. HPLC-tandem–MS and an automated MS fragment library search were proposed recently to fulfil these goals [123]. The advantages of HPLC-MS in drug analysis for forensic toxicology have been reviewed [68,124,125].

3.5. Ultra performance liquid chromatography

Narrow-bore (ca. 2 mm internal diameter) and micro-bore (1 mm internal diameter) columns packed with particles of 2 μm diameter or smaller are characteristic for ultra performance liquid chromatography (UPLC), which are operated with ultra-high pressure up to 1000 bar for separation. UPLC allows better resolution, largely due to the small particle size and, with flow rates that approach common HPLC (0.1–1 ml/min), UPLC offers fast separations. These advantages are important for sensitivity improvement and sample throughput. In particular, when columns were heated up to 90 °C during separation, decreased viscosity allowed very fast separations without loss of efficiency. A change in solvent selectivity due to temperature increase must be considered during method development [126]. Also, with the wish to save time and solvents by the application of UPLC columns, the purchase of new pumps if not a complete new chromatography system may turn out as necessary when conventional HPLC was applied before. Some of the more recently built machines, however, will operate both, UPLC and common size HPLC columns.

For tropane alkaloids from plant extracts, Russo and colleagues [127] compared the separation and detection of scopolamine, 6β-OH-hyoscyamine, hyoscyamine, and littorine by UPLC-UV and UPLC-ELSD (evaporative light scattering detector) on RP-C18 columns of various dimensions (2.1 mm × 30 mm, 2.1 mm × 50 mm, 2.1 mm × 100 mm, 1 mm × 50 mm) filled with 1.7 μm particles. The solvent gradient rose from 10 to 15% acetonitrile in aqueous solution (trifluoroacetic acid 0.1% v/v) within 4 min for the 2.1 mm × 50 mm column and within 8 min for the

2.1 mm × 100 mm column. Flow rates were 500 μl/min, injection volume was 2 μl. Compared to UPLC-UV at 210 nm, the sensitivity for tropane alkaloids in the optimised separation was improved by a factor of 2–3 with the ELSD. Separation on the shorter column was possible in less than 4 min, while sufficient resolution was maintained. UPLC was also applied for doping drug analysis[128]. Due to the sensitivity of the method, urine samples could be diluted 2-fold prior to injection. Compounds from various classes prohibited in sports such as stimulants, diuretics, narcotics, and anti-estrogens were analysed on a C18 reversed-phase column in two gradients of 9 min with positive and negative electrospray ionisation and compounds were detected in the MS full scan mode. The automatic identification of analytes was based on retention time and mass accuracy, with an automated tool for peak picking. For reliable quantitation, matrix effects on MS response were determined for all investigated analytes in spiked urine samples. LOD ranged from 1 to 500 ng/ml, allowing the identification of all selected 103 compounds in urine. When a sample was reported positive during the screening, confirmatory analyses [129] by ultra-high-performance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer (UHPLC-QTOF-MS) was used for individual doping agents. Basic, neutral, and acidic compounds were extracted by solid-phase extractions and detected by MS in the tandem mode to obtain precursor and characteristic product ions. The mass accuracy and the elemental composition of precursor and product ions were used for unequivocal compound identification.

3.6. HPLC columns

Innovative column materials also enabled improvements in tropane alkaloid analysis by HPLC. A hydrophilic embedded RP-C18 column was used to separate atropine, its hydrophilic degradation products and additional by-products occurring in plants, namely, noratropine, tropic acid, 6-hydroxyhyoscyamine, hyoscine, and littorine [82]. The employed C18 column (125 mm × 4 mm, 5 μm particle size, Aquasil, Thermo Hypersil) is characterised by a hydrophilic endcapping. Gradient elution with 25% acetonitrile to 45% acetonitrile in 20 mM phosphate buffer, pH 2.5, at a flow rate of 1.0 ml/min separated the alkaloids, which were detected by UV light at 215 nm. Under those conditions, all compounds were eluted within 12 min.

Porous graphite carbon as column material offers good chromatographic selectivity and was applied to separate four isomeric 3,6-dihydroxy tropane esters from the stem-bark of *Schizanthus grahamii* (Solanaceae) [130]. Elution conditions were optimised for a capillary column with porous graphite (15 cm × 300 μm, 7 μm particle size, 250 Å pore size). The acidic aqueous mobile phase contained 0.05–0.4% (v/v) formic, acetic, or trifluoroacetic acid. For basic eluent solutions, 50 mM ammonium formate aqueous solution was adjusted to the required pH (i.e. 8.0 and 9.5). The organic mobile phase consisted of pure methanol. Elution was isocratic; flow rate was 4 μl/min for both columns. Optimal conditions were obtained at 60° C with 0.1% (v/v) formic acid in 30% methanol.

For the analysis of cocaine and benzoylecgonine on banknotes, monolithic and particle packed (sub-2 μm particles) silica columns were evaluated with tandem MS detection and quantitation [131]. Monolithic reversed-phase columns of 100 mm × 3 mm and 200 mm × 3 mm size were compared with a 50 mm × 2.1 mm column filled with 1.8 μm reversed-phase particles. Column backpressure versus plate number showed an advantage of using the monolithic phases. The 200 mm monolithic column exhibiting a maximum 15,000 plates at a column backpressure of ca. 70 bar, compared to ca. 7000 plates at 150 bar for the 1.8 μm particle packed column. The monolithic column was subsequently applied to the determination of illicit drug contaminations on banknotes demonstrating a LOD of 4 pg per banknote and a LOQ of 14 pg per banknote for both, cocaine and benzoylecgonine, when 20 μl sample were injected.

Zerzanova et al. [132] compared the separation of cocaine and other model drugs on various HPLC columns. Monolithic columns included bonded C18 or C8 chains. Particle-packed columns included alternative reversed phase chemical surfaces, i.e. pentafluorophenylpropyl (HS F5) and palmitamidopropyl (RP-Amide) chains. UV light detection was at 230 nm. RP-Amide was chosen as the best column for analyses of cocaine and benzoylecgonine, the compounds were separated with satisfactory resolution in an optimised mobile phase consisting of 50 mM phosphate buffer, pH 3, and acetonitrile 82:18 (v/v) as isocratic elution mixture. Under those conditions, the amide column appeared optimal, but the C8 column gave satisfactory results as well.

In general, retention and peak shape, and overall chromatographic efficiency of basic compounds on RP columns is better in high-pH mobile phases. However, most silica materials do not withstand elevated pH of solvents for longer times. A large number of various basic drugs covering a wide range of hydrophobic (log *P*: 0.09–7.6) and basic (pKa 6.8–10) characteristics were separated on a RP18 silica column with a silica-organic layer grafted onto the 5 μm silica particles (Gemini, Phenomenex). Compounds were separated in aqueous 0.1% formic acid and in 10 mM ammonium hydrogencarbonate buffers of different pH (7.8–11) and acetonitrile as organic phase [133]. The column was used with both, low- and high-pH mobile phases over a period of more than a year without any noticeable change in performance. Another interesting observation was that the usually expected decrease in sensitivity with electrospray mass spectrometric detection in positive ion mode (ESI+-MS) did not occur. The high-pH mobile phases did not compromise the responses of basic compounds in ESI+ mode. Analyte responses and limits of detection were comparable or most often better in high pH compared to acidic mobile phases. The MS instrument (API 3000 triple quadrupole MS, interfaced via a turboIon spray source with the HPLC system) tuning was performed with the autotune function by infusing groups of basic compounds with a syringe pump.

A novel approach to HPLC column design and chemistry was the introduction of restricted-access media (RAM) supports and direct analysis of biological fluid samples without prior purification. Frequently, the clean-up or extraction of the compounds of interest from the matrix is tedious and time consuming. Coupled-column separation using restricted-access media as the first dimension in order to exclude macromolecules and retain small molecules has been successfully used for a number of biological fluids and reviewed [134]. A restricted access sorbent application for the determination of atropine in human plasma was established as a fully automated procedure [135]. Sample clean-up was performed in a cation exchange RAM pre-column coupled to HPLC by means of a column switching system. After direct injection of a 200 μl plasma sample, the biological matrix was washed out for 10 min. Subsequently, atropine was eluted after rotation of the switching valve for 2 min and transferred to the analytical column packed with RP8 silica using the LC mobile phase acetonitrile and potassium phosphate buffer (pH 3.0; 50 mM) containing 2 mM sodium heptanesulfonate (16:84; v/v). The UV detection was performed at 220 nm. The method was validated over a concentration range from 25 ng/ml (taken as LOQ) to 1000 ng/ml. The method was then applied for the determination of atropine in plasma after intravenous administration to hospitalised patients. Similarly, a column-switching method for the determination of cocaine and benzoylecgonine in human blood plasma samples was developed [136]. A 50-μl plasma sample was introduced to an alkyl-diol-silica ADS-C18 extraction precolumn. The fraction containing cocaine and benzoylecgonine was back-flushed and transferred to a mixed-mode C18 cation-exchange analytical column (Alltech) for final

separation. UV light detection at 235 nm enabled a LOD (signal to noise ratio >3) of 0.03 μg/ml for both compounds with an injection volume of 50 μl. LOD decreased further when larger plasma samples up to 200 μl were applied. Constructing a work overlap of sample preparation, analysis, and reconditioning of the extraction column enabled a throughput of 5 samples per hour. The method was successfully applied to human blood plasma samples from subjects suspected of cocaine abuse.

Novel column materials also improved enantiomer separation of tropane alkaloids. Separation of (*R,S*)-hyoscyamine was achieved on a Chirobiotic V chiral stationary phase, which uses the antibiotic vancomycin as chiral selector [137]. Analytes were quantified by a quadrupole mass spectrometer in the positive ion mode behind the APCI interface [90]. Single ion monitoring enabled a LOQ of 0.5 ng/ml for both, (*R*)- and (*S*)-hyoscyamine. The pharmacologically active enantiomer (*S*)-hyoscyamine was eliminated faster than (*R)*-hyoscyamine. Alternatively, enantiomer separation of atropine could be achieved by a Chiral AGP- column (150 mm × 2 mm) packed with α1-acid glycoprotein-coated silica (5 μm particles) [138]. Anisodamine, the 6β-hydroxyl derivative of (*S*)-hyoscyamine (Fig. 1) was separated from its synthetic enantiomer and diastereomers by a Chiralpak AD-H column as chiral stationary phase, which uses an amylose derivative as chiral selector [139]. Acetonitrile, 2-propanol, and diethylamine 97:3:0.1 (v/v/v) were used as mobile phase. A novel chemistry on chiral phases for enantiomer separation was chlorine-containing substituents on polysaccharide basis; the separation was performed with polar organic solvent liquid chromatography (POSC). In the polar mode, only mixtures of polar organic solvents such as acetonitrile, methanol, and higher alcohols (ethanol, propanol, butanol) are used as mobile phase in combination with a *quasi*-reversed-phase stationary phase. Various chiral drugs used as test compounds demonstrated the effectiveness of the POSC strategy [140]. Optimal separation was achieved by a screening using solvent mixture strategies adapted from normal phase liquid chromatography and supercritical fluid chromatography. POSC was an attractive separation mode because of the many fast (>10 min) baseline separations obtained. The separation of atropine and cocaine was tested on Sepapak-2 and Sepapak-3 columns (Sepaserve, Germany) containing cellulose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(5-chloro-2-methylphenyl-carbamate), respectively [141]. Atropine enantiomers were separated by the Sepapak-2 phase using acetonitrile, diethylamine, and trifluoroacetic acid (100/0.1/0.1) (v/v/v) as mobile phase. Cocaine, however, was not separated by the new phases under the examined conditions.

3.7. Summary of HPLC methods

A survey of analysis of tropane alkaloids analysis by HPLC and a comparison of sensitivity is given in Tables 1 and 2.

4. Capillary electrophoresis

Capillary electrophoresis (CE) is performed using several techniques such as capillary zone electrophoresis (CZE), non-aqueous capillary electrophoresis (NACE), micellar electrokinetic chromatography (MEKC), micro-emulsion electrokinetic chromatography (MEEKC), and capillary electrochromatography (CEC). The common element is an apparatus that applies a voltage between both ends of a silica glass capillary and causes a current through the ions in the buffer, with which the capillary is filled. The capillary dimensions typically are 250–900 mm × 50 μm with an internal volume of ca. 40–150 μl. Therefore the minute sample amounts (a few nanolitres) that can be applied limit the analyte loaded onto a CE capillary and consequently decrease the sensitivity of CE expressed in concentration in the sample. Typically, detection limits of CE-UV methods hardly reach below the micromolar range, which is about 100 times higher than what can be achieved with HPLC–UV or HPLC–MS. Yet, CE compared with HPLC has a number of advantages, high resolution of analytes, fast analysis, comparatively cheap and easy instrumentation and consumables, high versatility and, by coupling to mass spectrometry, also good analyte identification power. It is therefore not surprising that some authors regret the still reluctant application of CE techniques in pharmaceutical drug analysis [142] and in drug analysis for forensic purposes [143]. Applications for capillary electrophoresis of tropane alkaloids occurring in Solanaceae plants have been summarised [144].

4.1. Tropane alkaloid separation by capillary zone electrophoresis

Capillary zone electrophoresis (CZE) is the most basic form of CE [145]. Analytes are dissolved in a buffer, thus the techniques is also termed “free-solution CE”. A constant field strength is applied throughout the length of the capillary. Separation relies on the pH-controlled dissociation of the analyte molecules, thus on their charge and on their size including the hydration shell. Non-dissociated, neutral molecules migrate by the electro-osmotic flow. The buffer may contain organic solvents modifying the analyte migration or the liquid phase in the capillary may consist of organic solvents only (non-aqueous capillary electrophoresis, NACE). Non-aqueous media enable the use of high ionic-strength electrolytes, which results in increased resolution and in a reduction of capillary wall interactions. NACE as an alternative to aqueous CE is also applied, when analytes are hardly soluble in aqueous buffer. NACE applications focussing on pharmaceuticals and phytochemicals were expertly reviewed [146,147]. An example for plant tissue analysis by CZE is *Flos Daturae*, a herb preparation of Chinese medicine. CE was used for the separation and determination of tropane alkaloids contained in the herbs [148]. Scopolamine, atropine, and anisodamine were separated in a fused silica capillary (421 mm × 50 μm) in a buffer of 50 mM phosphate (pH 5.0) containing 20% (v/v) tetrahydrofuran with a voltage of 20 kV. A linear response was obtained for 2.4–21.8 μg/ml scopolamine, 4.0–36.0 μg/ml atropine, and 2.6–23.7 μg/ml anisodamine, respectively. Atropine and scopolamine were also determined in seeds of *Datura metel* (Solanaceae) by CZE [149]. The pH of the 50 mM phosphate buffer containing 10% (v/v) tetrahydrofuran was adjusted to pH 8 for better peak shape. LOD were 3.2 μg/ml for atropine and 3.5 μg/ml for scopolamine with UV light detection at 210 nm. The seeds of *Datura metel* under investigation had been carried aboard a retrievable satellite and exposed to extraterrestrial space, but effects of the space environment (weightlessness and ionising radiation) on the contents of atropine and scopolamine were non-significant. Optimised procedures for CZE for analysis of phytochemical bioactive compounds were summarised [150]. Recently, CE was used to identify mixtures of toxic alkaloids, among them atropine, scopolamine, and anisodamine, in human blood and urine [151]. The separation employed a fused-silica capillary of 600 mm × 75 μm (effective length: 502 mm), a buffer containing 100 mM phosphate pH 4.0 and 5% acetonitrile, and a voltage of 16 kV. The alkaloids were detected by UV absorbance at wavelengths of 195 and 235 nm. After a concentration step by solid phase extraction, the LOQ for the tropane alkaloids were 40–150 ng/ml blood and 90 ng/ml urine.

In order to improve the sensitivity of detection for tropane alkaloids, whose UV light absorbance is weak, electrochemiluminescence was applied. The determination of the two major active ingredients, atropine and scopolamine, in *Flos Daturae* was based on electrochemiluminescence detection with tris(2,2′-bipyridyl)

ruthenium(II) chloride hexahydrate after CE separation [152]. A three-electrode configuration was used in the detection system consisting of a 500 μm platinum disc as a working electrode, silver/silver chloride a reference electrode and platinum wire as a counter electrode. The electrochemical detection cell contained the end of separation capillary that was inserted into a stainless steel tube serving as the ground electrode of electrophoresis. The working electrode for detection was adjusted in a fixed distance to the capillary. The lower layer of the detection cell made of UV white optic glass through which the photons were captured by the photomultiplier tube. The cell contained about 300 μL 50 mM phosphate buffer with 5 mM tris(2,2′-bipyridyl) ruthenium(II). Reference electrode and counter electrode were inserted into the solution above the capillary and the working electrode to avoid blocking the flow of photons into the photomultiplier. The total complex set-up is illustrated in a former publication [153]. The solution in detection cell had to be replaced every 2 h. Separations were performed in fused-silica capillaries (500 mm × 25 μm) at a voltage of at 15 kV. When the crude extracts in 80% aqueous ethanol were directly injected into the capillary, the large amount of ethanol reduced the electroosmotic flow, resulting in long migration time for atropine and scopolamine. A five-time dilution of the herbal extract with water was satisfactory. The alkaloid separation efficiency was strongly influenced by the buffer pH value, and buffers from pH 4.06 to 6.08 resulted in a complete overlap of the atropine and scopolamine peaks. An increased pH of 7.54 separated the peaks partially, and the maximum separation was achieved at pH 8.48. Detection limits of 5×10^{-8} mol/l for atropine and 1×10^{-6} mol/l for scopolamine were obtained, i.e. ca. 14 ng/ml atropine and ca. 300 ng/ml scopolamine. Separation, however, was still non-complete, and further alkaloids were suspected to be present in the herb extract. Atropine, scopolamine, and in addition anisodamine from *Flos Daturae* were completely separated by introduction of 4 mM β-cyclodextrin into the running buffer [154]. Quantitation ranged from 0.2 to 100 μM for anisodamine and atropine (corresponding to a LOQ of ca. 60 ng/ml), and from 20 to 200 μM scopolamine (corresponding to a LOQ of ca. 600 ng/ml). The determination of tropane alkaloids in *Przewalskia tangutica* (Solanaceae) by capillary electrophoresis with electrochemiluminescence detection was performed with a platinum electrode, which was modified by a film made from europium (Eu^{3+}) salt and potassium hexacyanoferrate [155]. The average amounts of anisodamine and scopolamine in the herbal rootstalk sample were 27.8 and 4.43 g/kg, respectively. The procedure for separation of tropane alkaloids was further modified by non-aqueous capillary electrophoresis (NACE) coupled with electrochemiluminescence and electrochemical dual detection [156]. The non-aqueous electrophoretic buffer consisted of acetonitrile and 2-propanol containing 1 M acetic acid, 20 mM sodium acetate, and 2.5 mM tetrabutylammonium perchlorate. Because of the organic buffer, the working platinum electrode (Pt) did not need frequent reactivation. The linear ranges for quantitation of atropine, anisodamine, and scopolamine were 0.5–50 μM, 5–2000 μM, and 50–2000 μM, respectively (corresponding to 0.145–14.5 mg/l, 1.5–610 mg/l, ands 15.2–606 mg/l) The LOQ are assumed as the lowest concentrations of the respective calibration range. Electrochemical detection was successful for improving LOD for atropine and scopolamine as model compounds [157]. A comparison of detection techniques after separation in NACE demonstrated improved LOD in narrow capillaries for both, electrochemical detection and UV light detection. For electrochemical detection a 25 mm platinum microdisk electrode served as working electrode. Absolute LOD for scopolamine ranged from 2.4 fmol to 79 attomol (2.4×10^{-15} to 7.9×10^{-18} mol) in capillaries from 50 to 2 μm diameter with electrochemical detection. With UV light detection, capillaries from 75 to 5 μm diameter provided slightly improved absolute LOD for scopolamine of 213–120 nmol. Thus, CE with electrochemical detection appears promising for samples of very small volume.

4.2. Micellar electrokinetic chromatography and microemulsion electrokinetic chromatography

The strength of CE is not in sensitive detection and quantitation, but in the versatility provided by largely different chromatographic media that can be filled into the separation capillary. Micellar electrokinetic chromatography (MEKC) and microemulsion electrokinetic chromatography (MEEKC) are electroseparation techniques that use aqueous buffers containing surface-active compounds (surfactants). In MEKC, the concentration of the surfactants is above the critical micellar constant (CRC), and micelles of, e.g. sodium dodecylsulfate (SDS) are formed in the buffer solution, which partially include the analytes. The rate of inclusion of the analytes into the micelles depends on the analyte structures, providing the basis of the separation and the selectivity. In MEEKC, organic solvents immiscible with water, typically hydrocarbons like heptane and octane and also butanol, are added to the liquid phase, and the surfactant ensures the formation of an emulsion. Small oil droplets with a few nanometres in diameter, i.e. microemulsions are formed by sonication. CE with microemulsions was first reported by Watarai in 1991 [158]. The separation mechanism of MEEKC is similar to MEKC. MEEKC separations rely on the hydrophobicity and electrophoretic mobility of the analytes in the given buffer. Differential migration times of the neutral compounds are primarily governed by the electroosmotic flow (EOF) and the distribution of the uncharged analytes between the aqueous buffer phase and the hydrophobic phase constituted of the oil droplets. Ionic analytes display their own electrophoretic mobility. The overall separation results from both, partitioning between oil droplets and aqueous phase, and different electrophoretic mobility. Additionally, electrostatic interactions between the analytes and microemulsion droplets depend on the pH, i.e. on the relative charges of analytes and the surfactant.

A microemulsion electrokinetic chromatography (MEEKC) method for simultaneous analysis of atropine, its major degradation products, and related substances from plant materials used an organic-in-water (O/W) microemulsion consisting of 0.8% (w/w) octane, 6.62% (w/w) 1-butanol, 2.0% (w/w) 2-propanol, 4.44% (w/w) SDS and 86.14% (w/w) 10 mM sodium tetraborate pH 9.2 [159]. Fused-silica capillaries (400 mm length to detector × 50 μm) and a voltage of +15 kV were employed. In order to speed up the apoatropine migration a voltage gradient was applied characterised by an increase of the applied voltage for 0.5 min from +15 to +25 kV. The MEEKC method was specific, robust, and provided baseline separation allowing the detection and quantification of all impurities at concentrations of 0.02% relative to atropine sulfate at a concentration of the test solution of 1.0 mg/ml. A cyclodextrin-modified microemulsion buffer allowed the enantioseparation of atropine, scopolamine, ipratropium, and homatropine [160]. The O/W microemulsion background electrolyte solution remained similar, 0.8% w/w octane, 6.6% w/w 1-butanol, 2.0% w/w SDS and 90.6% w/w 10 mM sodium tetraborate buffer (pH 9.2). Enantioseparation with high resolution and short migration times of all tropane alkaloids were achieved using heptakis(2,3-di-O-methyl-6-O-sulfo)-β-cyclodextrin and sulfated β-cyclodextrins in the microemulsion background electrolyte and appeared superior to corresponding cyclodextrin-modified CE methods.

The detection sensitivity for cocaine, benzoylecgonine, norcocaine, and cocaethylene was enhanced by a preconcentration step called sweeping micellar electrokinetic chromatography (MEKC) and combined with a cation-selective exhaustive injection (CSEI)-technique [161]. When only the sweeping-MEKC mode was

Table 3
Analysis of tropane alkaloids by capillary electrophoresis.

Method	Compound	CE capillary	Migration time [min]	Linear calibration range [μg/ml]	LOD [μg/ml]	Reference
CE-ECL	Anisodamine Atropine Scopolamine	50 cm total length 25 μm i.d.	ca. 8 ca. 10 ca. 11	0.061-30 0.058-29 0.61-61	0.0031 0.0046 0.06	[154]
CE-ECL	Scopolamine Atropine	50 cm total length 25 μm i.d.	5.07 5.16	3-303 3-289	0.30 0.014	[152]
CE-ESI-TOF-MS	Tropine Belladonnine Norhyoscyamine Apoatropine Hyoscyamine 6β-OHS Scopolamine	85 cm length 50 μm i.d.	10.1 11.1 11.4 11.7 12.1 12.5 15.2	Qualitative analysis only		[176]
CE-ESI-TOF-MS	Cocaine Benzoylecgonine	100 cm length 75 μm i.d.	ca. 18 ca. 21	0.010–2	0.002 0.005	[181]
CE-UV	Scopolamine Atropine Anisodamine	42.1 cm total length 37.3 cm effective length 50 μm i.d.	ca. 11.2 ca. 11.5 Ca. 11.9	2–22 4–36 3–24	N.A.	[148]
O/W-MEEKC-UV	7-OH-hyos Tropic acid Scopolamine 6-OH-hyos Atropic acid Noratropine Littorine Apoatropine	48.5 cm total length 24.0 cm length to detector 50 μm i.d.	0.94 1.01 1.08 1.11 1.31 1.79 1.92 2.14	0.20–25 0.15–20 0.30–35 0.20–25 0.15–20 0.35–40 0.30–35 0.35–40	0.10 0.08 0.15 0.10 0.08 0.20 0.15 0.20	[159]
O/W-MEEKC-UV (*chiral separation*)	Homatropine Scopolamine Ipratropium Atropine	32.5 cm total length 24.0 cm length to detector 50 μm i.d.	2.41/3.34 2.81/3.37 5.32/5.72 6.05/7.24	N.A. The racemic alkaloids were analysed at 0.2 μg/ml		[160]

ECL, electrochemiluminescence. i.d., internal diameter. N.A. no data available. OH-hyos, hydroxyhyoscyamine. O/W-MEEKC, oil-in-water microemulsion electrokinetic chromatography.

applied, the capillary was filled with a micellar background electrolyte, 75 mM SDS in 100 mM phosphoric acid pH 1.8 containing 10% 2-propanol and 10% tetrahydrofuran, and a negative voltage (20 kV) was applied. The samples obtained from solid phase extraction cartridges were diluted in non-micellar background electrolyte to provide the same conductivity as the background solution in the capillary and were pressure-injected. When the anionic micelles entered the sample zone, sweeping and separation were achieved through MEKC. In the CSEI-sweeping-MEKC procedure, the capillary was conditioned with non-micellar background electrolyte. High conductivity solution, 150 mM phosphoric acid pH 1.8, was injected hydrodynamically, followed by the sample of low conductivity (diluted with pure water) injected electrokinetically. Finally, the inlet and outlet of the capillary were placed in micellar background electrolyte and a negative voltage (20 kV) was applied to effect separation. The detection wavelength was always set at 230 nm. In CSEI-sweeping-MEKC the ranges of linearity in quantitation of the alkaloids were 0.2–10 ng/ml for cocaethylene, 0.2–10 ng/ml for norcocaine, 0.2–10 ng/ml for cocaine, and 1–50 ng/ml for benzoylecgonine. Many more novel MEKC procedures including on-line sample concentration such as stacking and sweeping, in-capillary derivatisation and the coupling of flow-injection systems with MEKC for numerous kinds of analytes were expertly summarised [162]. Applications were listed including natural products such as saponins from *Panax ginseng* and aristolochic acids, pesticides in fruits and vegetables, paraben preservatives in cosmetic products, and non-steroid anti-inflammatory drugs in mineral water.

4.3. Capillary electrochromatography

Capillary electrochromatography (CEC) can be considered as a hybrid between CE and HPLC. A fused silica capillary, typical dimensions 500 mm × 75 μm–100 μm, is filled with a solid separation phase, which may consist either of small particles or a resin that is instilled as liquid into the capillary and set to polymerise inside. CEC is performed on CE instruments and uses an electrically driven flow to transport the analytes through the capillary. Avoiding the use of pressure results in advantages for CEC over conventional HPLC. The pressure-driven flow rate through a packed column depends on the particle size and on column length. In contrast, the electrically driven solvent transport is achieved by a high endoosmotic flow. The flow rate is largely independent from particle diameter and column length so that small particles and long columns can be used, which offer high chromatographic selectivity. However, when RP silica particles that are widely applied in HPLC are used as stationary phase in capillary electrochromatography (CEC), unacceptable peak tailing may occur due to electrostatic absorption to acidic groups on the silica particles. Attempts to use high pH solvents for the separation of alkaline analytes may degrade the chromatographic material. Therefore alkali-resistant polymers appear attractive and are often applied in CEC, because pressure stability that is required in HPLC is less important. Polymer-filled CEC columns were obtained by *in situ* polymerisation of acrylamide, methylene bisacrylamide used as crosslinker, and negatively charged monomers of 2-acrylamido-2-methyl-1-propanesulfonic acid that provide negatively charged

interaction sites [163]. The resulting monolithic strong cation exchange column was employed in CEC separation of basic drugs such as atropine and of further alkaloids extracted from natural products. Separation of ten basic compounds of interest in forensic science, among them cocaine, was achieved on a stationary phase with cyano group-derivatised silica particles [164]. Chromatographic retention, selectivity, and efficiency were optimal with aqueous sodium phosphate buffer pH 2.5/acetonitrile (80/20, v/v) as the mobile phase and 10 kV as voltage applied. Under these conditions all the ten analytes were baseline resolved within 20 min. LOD for cocaine was 10 ng/ml and LOQ was 20 ng/ml.

4.4. Enantioseparation

The easy applicability of additives to the separation buffers in CE enables soluble chiral selector compounds such as cyclodextrins to be used for enantioseparation. Cyclodextrins are cyclic oligosaccharides consisting of typically six to eight D-(+)-glucopyranose units joined by α-1,4 linkages. Cyclodextrins with six, seven, or eight glucose units are referred to as α-, β-, and γ-cyclodextrins, respectively. The three-dimensional shape of cyclodextrins is a ring with an open cavity, whose size depends of the number of the ring units (ca. 5.7–9.5 Å diameter and ca. 8–9 Å height). The outside of the rings is hydrophilic, providing solubility of cyclodextrins in water. The cavity inside is less hydrophilic than the surrounding water and may bind organic molecules which otherwise are scarcely soluble in water. Cyclodextrins are derivatised yielding compounds with altered charge and polarity. Current derivatives include methylated-, ethylated-, hydroxypropylated-, and acetylated cyclodextrins; and anionic cyclodextrins with e.g. methylamino-sulfobutylether-, carboxymethyl ether-, sulfate-, and phosphate groups, which are frequently used for enantiomer separation. Chiral selectivity in analysis results from various degrees of inclusion of the analytes into the cyclodextrin cavity and from differential hydrogen bonds of the polar functionalities of the cyclodextrins. Chiral separations by CE applying cyclodextrins and other chiral selectors were reviewed [165]. The wide variety of cyclodextrin derivatives for enantioseparations has attired particular interest and was reviewed comprehensively [166,167]. Various cyclodextrins were initially evaluated for the enantiomer separation of tropane alkaloids in 1999 [168]. The racemic mixtures of atropine, homatropine, and ipratropium were resolved with short migration times in a basic medium at low concentrations of sulfated cyclodextrins. Scopolamine and butylscopolamine, however, could not be separated with any of the various cyclodextrin derivatives applied. (*S*)-Hyoscyamine exerts about double of the acetylcholine-inhibitory effect of racemic atropine, therefore enantiomeric purity for (*S*)-hyoscyamine and the complete racemisation in atropine preparations are important issues in pharmaceutical control. Baseline separation of atropine in sulfated β-cyclodextrin enabled the quantitation of 0.5% (*R*)-hyoscyamine (45 μg/ml) in (*S*)-hyoscyamine [169]. The same sulfated β-cyclodextrin proved useful for separation of cocaine enantiomers and diastereomers, i.e. (−)-cocaine (=(2*R*,3*S*)-cocaine, the active enantiomer formed in coca plants), (+)-cocaine, (−)-pseudococaine, and (+)-pseudococaine [170]. Zandkarimi and colleagues [171] explored highly sulfated cyclodextrins for CE enantioseparation of different drugs including atropine. The general conditions for resolution of all drugs were 25 mM sodium phosphate buffer pH 2.5 containing 1.25% (w/v) of highly sulfated cyclodextrins with an applied voltage of +15 kV. Chiral resolution of atropine was only achieved by sulfated β-cyclodextrin, sulfated α-cyclodextrins and γ-cyclodextrins did not separate atropine enantiomers. The application of charged derivatives of cyclodextrins in capillary electrophoresis for chiral analysis has recently been reviewed [172].

4.5. Capillary electrophoresis-mass spectrometry

Most commercially available CE instruments employ UV light absorbance detectors because of their simplicity and versatility. An overview of relevant detection limits for tropane alkaloids is given in Table 3. The coupling of capillary electrophoresis and mass spectrometry (MS) promises to combine efficiency and speed of CE separation with information on the mass and the identity of the analytes provided by MS. For CE–MS procedures only volatile buffers can be used, typically aqueous or aqueous-organic solutions containing, e.g., acetic acid, formic acid, and ammonium hydroxide at low concentrations. The use of non-volatile components like cyclodextrins, inorganic salts, or surfactants are precluded since they inhibit ESI efficacy, increase the noise, and reduce the sensitivity of the system. Sample pretreatment procedures were reviewed that were adopted to these requirements [173]. Non-aqueous capillary electrophoresis (NACE) appears to be ideally suited for online coupling with mass spectrometry due to high volatility and surface tension of many organic solvents. Humam and colleagues [174] combined NACE with both, UV and MS detection to separate four isomeric tropane alkaloids from *Schizanthus grahamii*, namely 3α-senecioyloxy-6β-hydroxytropane, 3α-hydroxy-6β-senecioyloxytropane, 3α-hydroxy-6β-angeloyloxytropane and 3α-hydroxy-6β-tigloyloxytropane. An electrolyte consisting of 1 M trifluoroacetic acid and 25 mM ammonium trifluoroacetate in methanol:ethanol (40:60, v:v) provided separation of the four positional isomers in a fused-silica capillary (64.5 cm total length, 56 cm effective length, 50 μm internal diameter). The volatile background electrolyte could be used for peak detection via UV and MS detection. The UV detector was used for alkaloid quantification, and MS confirmed the identity of the isomers. CE-ESI–MS measurements were carried out in the positive ionisation mode and were performed with a single quadrupole using a CE–MS adapter kit from Agilent. The sheath liquid was composed of water:isopropanol (50:50, v:v) and 0.1% formic acid. A comprehensive review summarises applications of NACE–MS for the analysis of drugs, stereoisomers, peptides, alkaloids, and polymers [175].

Arraez-Roman and colleagues [176] developed a CE-electrospray interface to connect a time-of-flight (TOF) mass spectrometer (ESI-TOF-MS). The sheath liquid was optimised. At a flow rate of 6 ml/min isopropanol/water 50:50 (v/v) provided highest TOF-MS signals, while 0.5% (v/v) formic acid enabled better sensitivity. Tropane alkaloids from pharmaceutical preparations of *Atropa belladonna* leaf extracts were separated in an alkaline buffer of 60 mM ammonium acetate, pH 8.5 containing 5% isopropanol, and several alkaloids such as tropine, belladonnine, norhyoscyamine, apoatropine, hyoscyamine, 6β-hydroxyhyoscyamine, and scopolamine could be simultaneously identified.

The specific and sensitive CE–MS coupling was applied to the analysis of cocaine in hair samples. A CZE–MS method for quantitative determination of several drugs of abuse and their metabolites, among them cocaine and benzoylecgonine required only 100 mg hair [177]. CZE separations were carried out in a fused silica capillary (1000 mm × 75 μm) in a buffer composed of 25 mM ammonium formate, pH 9.5 at a voltage of 15 kV. ESI-ion trap MS detection was performed in the ESI positive ionisation mode and the ion trap MS operated in a selected ion monitoring mode (SIM) focussing on positive molecular ions for each drug and metabolite. Because of its zwitterionic structure, benzoylecgonine was less amenable to sensitivity enhancement by field amplified sample stacking and could not be detected below 0.1 ng/mg hair. Cocaine was measured with an LOD of 15 pg/mg hair and an LOQ of 50 pg/mg hair. A similar CE-ESI-ion trap MS set-up was used for measurement of cocaine and five major metabolites in human urine [178]. LODs were 100 ng/ml urine for cocaine and cocaethylene and 250 ng/ml

for benzoylecgonine, anhydroecgonine, anhydroecgonine methyl ester, and ecgonine methyl ester. LOQs were 250 ng/ml urine for cocaine and cocaethylene and 500 ng/ml for all other compounds.

Coupling of CE with a time-of-flight (TOF) mass spectrometer provided the exact mass and isotopic pattern of the analytes and avoided the need for reproducible mass fragment formation, which is a major difficulty in ion trap mass detectors [179]. Again, hair analysis was performed for cocaine and benzoylecgonine. The LOD for cocaine was 20 pg/mg hair, and LOQ was 67 pg/mg hair. Zwitterionic benzoylecgonine migrated in a broad zone and was detected with a LOD of 0.1 ng/mg hair; LOQ was 0.33 ng/mg hair. Capillary zone electrophoresis (CZE) coupled to time-of-flight mass spectrometry was applied to the analysis of cocaine together with further illicit drugs in blood [180]. Compounds were separated by CZE in a fused-silica capillary (100 cm × 75 μm) using a 25 mM ammonium formate buffer pH 9.5 and applying 15 kV. Under optimised conditions, linearity was assessed in the range 10–2000 ng/ml, and LODs were in the range of 2–10 ng/ml and LOQs of 10–30 ng/ml. Basic concepts, instrumental details, and applications of CE coupled to time-of-flight (TOF) mass spectrometer were recently reviewed [181].

A compound data base containing more than 50,000 major drugs and their phase I and phase II metabolites was employed to screen for unknown drugs using mass spectrometry coupled to CE or RP-HPLC in a 'metabolomic' approach [182]. Urine, blood, and hair samples collected from real cases were submitted to a screening procedure using CE-ESI-MS-TOF (positive-ion mode). Detected peaks with their exactly determined molecular mass were searched against the database, where a list of candidates was retrieved. Starting from the mass of unknown, mass shifts corresponding to pre-defined biotransformations (e.g. demethylation, glucuronidation, etc.) were calculated and corresponding mass chromatograms were extracted from the total ion current (TIC) to search for metabolite peaks. The presence of metabolites in the TIC was matched with functional groups data for exclusion of candidates with structures not compatible with observed biotransformations. The procedure was tested on 108 pharmaco-toxicologically relevant compounds and their phase I metabolites were detected in real positive samples. The mean list length of candidates retrieved from the database was 7.01 ± 4.77 before the application of the 'metabolomic' approach, and after the application it was reduced to 4.08 ± 3.11. High resolution mass spectrometry allows a much broader screening than other screening approaches, e.g. library search on mass spectra databases. The impressive progress during 22 years of CE–MS coupling was reviewed [183]. Possible future directions for CE–MS are seen in routine and easy-to-use applications in drug analysis enabling simultaneous identification and quantitation of drugs and their metabolites in small volumes that require only simple sample preparation procedures [9].

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