— Review —

Analysis of Drugs of Abuse in Biological Specimens

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(Received September 1, 2011)

Recent reports of the analysis of drugs of abuse in biological specimens are reviewed herein. Different perspectives from reviews so far published and the need for and the background of drug analysis in biological specimens, and difficulty of drug testing in biological specimens are introduced. Comprehensive biological specimens for the analysis of drugs of abuse in forensic science, including oral fluid (saliva), hair, umbilical cord, placenta, meconium, cadaver tissue (brain, adipose), sweat, breath, and nail clippings, in addition to the commonly used blood and urine specimens in clinical chemistry, are described along with their outlines, advantages/disadvantages, and actual examples. Today, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the method of choice for the analysis of drugs of abuse. A simultaneous screening method for multiple types of drugs has also become popular recently. However, because qualitative determination remains important in forensic science, gas chromatography-mass spectrometry (GC-MS) is still in use even if it requires complicated specimen preparation and derivatization procedures. This is because GC-MS is reliable and has been employed ever since for the appraisal of trials.

Key words —— drug of abuse, forensic science, biological specimen, liquid chromatography-tandem mass spectrometry, gas chromatography-mass spectrometry

INTRODUCTION

Drugs are classified into legal drugs and illegal (or illicit) drugs, and the abuse of drugs has become one of the most serious social problems in the world. In Japan, 32 psychotropic substances were listed as designated substances by the Pharmaceutical Affairs Law in April 2007 to prevent abuse of those substances. Subsequently, other psychoactive compounds were added and as of July 2009, 40 substances are listed as designated substances. However, an annual survey has indicated that new designer drugs still find their way into illegal drug markets.[1)]

The Japanese government is looking into specifying the part of a chemical structure that produces a narcotic effect and regulating drugs having chemical structures similar to those of abused drugs, as narcotics. However, the problem of designer drugs cannot be solved to this day. Clearly, there is an urgent need to develop analytical methods that would identify such drugs in order to prevent the increasing incidence of drug abuse. Major representative abused drugs are designated as narcotics or stimulants and the possession of such drugs is regulated.

Numerous publications have described the analysis of amphetamine-like drugs and demonstrated the significance of improving the methods of analysis of such drugs in biological specimens. In the last decade, liquid chromatography coupled to mass spectrometry (LC-MS) has seen rapid development for forensic and clinical applications.[2–4)] LC-MS offers high sensitivity and specificity and reduces the specimen preparation time compared with gas chromatography-mass spectrometry (GC-MS) because relatively non-volatile compounds can be analyzed and no derivatization is necessary.

Many reviews have focused on the drugs of abuse themselves,[5–8)] specific biological specimens, such as hair,[9, 10)] the methodology,[11, 12)] or the analytical instruments.[2–4)]

In this review, on the basis of reports published in the last three years (2008–2010) describing the

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analyses of drugs of abuse in biological specimens, different perspectives, such as the need for and the background of drug analysis in biological specimens, and difficulty of drug testing in biological specimens, are introduced together with some actual examples. Furthermore, comprehensive biological specimens for the analysis of drugs of abuse in forensic science, including oral fluid (saliva), hair, umbilical cord, placenta, meconium, cadaver tissue (brain, adipose), sweat, breath, and nail clippings, in addition to the commonly used blood and urine specimens in clinical chemistry, are described along with their outlines. At that time, amphetamines, cocaine, opiates, and cannabis were selected as the target drugs because they are the most frequently used illicit drugs.

NEED FOR AND BACKGROUND OF DRUG ANALYSIS IN BIOLOGICAL SPECIMENS

Drug analysis in biological specimens, such as urine, hair, blood, sweat, or oral fluid (saliva), which is used to determine the presence or absence of specified parent drugs or their metabolites, is necessary in forensic science. The background of drug analysis in biological specimens is shown below.

Testing for Drugs of Abuse in Saliva of Drivers

In forensic science, the study of the detection of drugs of abuse extends to different fields. In the interest of road safety, driving under the influence of drugs has social and legal repercussions and is a cause of grave concern. Drugged drivers pose a serious threat to other drivers as well as to themselves.[13] To obtain information about drug abuse in drivers, a comprehensive screening test for such drugs is necessary, even under normal situations where the drivers are not involved in any accidents.[14]

In Sweden, a person's driving license is suspended after being convicted for petty drug offense or driving under the influence of drugs.[15] To regain the license, the person has to prove that he or she has been drug-free during a specified observation period. Reliable oral fluid screening devices for on-site screening of drugged drivers are both useful and convenient for maintaining road safety.[13]

Fernández *et al.*[14] screened 632 saliva specimens collected from drivers in Catalonia, Spain for drugs of abuse through an immunoassay test (Cozart® kit; Cozart Bioscience Ltd., Abingdon, UK) and the drugs detected by the screening test were cocaine, cannabis (Δ^9-tetrahydrocannabinol), opiates (6-monoacetylmorphine), amphetamine, and metamphetamine. Specimens that were tested positive were subjected to GC-MS for confirmation. As a result, more than 80% of the specimens were positive for a combination of two drugs. The most common combination was cocaine and cannabis.

Workplace Drug Testing

Workplace drug testing is a well-established application of forensic toxicology and its aim is to reduce workplace accidents caused by employees who are working under the influence of drugs or alcohol.[16] Several drugs of abuse may be involved, including alcohol, amphetamines, cannabis, cocaine, opiates, and prescription drugs, such as benzodiazepines. The use of alternative biological specimens, such as hair, oral fluid or sweat, in workplace drug testing presents several advantages over urinalysis, mainly because specimen collection can be performed easily without infringing on the workers' privacy. However, the drugs are usually present in these alternative specimens at low concentrations and the amount of specimen available for analysis is small. In this regard, the use of highly sensitive techniques is necessary.

Drug Abuse among College Students

Drug abuse among college students is characterized by low academic performance and long-term negative consequences.[17] Screening for students who are at a high risk of drug abuse is of primary importance to ensure early identification and appropriate care.

The study of Garcia-Jimenez *et al.*[17] aimed to determine current or past use of drugs of abuse through a questionnaire distributed to students at the Universidad Autónoma del Estado de Morelos. The actual test results of that study showed that 16% of the specimens were positive for Δ^9-tetrahydrocannabinolic acid-A (Δ^9-THCA-A). The results were not different from those of the National Questionnaire on Addiction. On the basis of that finding, they established a program for detecting drug abuse in students. This type of study is important in order to implement programs to decrease the incidence of drug abuse in college students.

Drug-induced Seizures

Approximately 6% of new-onset seizures are

said to be drug-related. However, there is currently no reliable way to determine whether a seizure is indeed drug-induced or not. Drees *et al.*[18] investigated whether the results obtained by liquid chromatography-tandem mass spectrometry (LC-MS/MS) from a seizure panel could affect patient care as LC-MS/MS is a powerful tool that allows the simultaneous detection of numerous analytes of diverse chemical nature in patient specimens. On analysis of 157 serum or plasma specimens from patients who underwent seizures, 17 were found to be positive for a drug of abuse on the seizure panel. The team of experts determined that the test results probably or definitely would have affected treatment in seven of those cases. Therefore, a test that detects the presence of drugs implicated in drug-induced seizures can help physicians determine if an unexplained seizure is drug-related, thus leading to potentially better direct patient care.

Body Packing

The illicit transport of cocaine and heroin, either swallowed or inserted into the rectum and/or vagina of individuals, which is defined as "body packing," is becoming an increasingly common occurrence. The assessment of smuggling by body packers through urinalysis has been little reported and on-site rapid screening methods are essentially lacking. Marchei *et al.*[19] screened for cocaine and heroin metabolites in the urine specimens of suspected body packers with an on-site immunochromatographic test and confirmed the obtained results by GC-MS and X-ray examination. From the obtained results, the on-site detection of cocaine and heroin metabolites in the urine specimens of suspected body packers appears to be a reliable screening test to disclose internally concealed drugs and justify subsequent radiological investigations.

DIFFICULTY OF DRUG TESTING IN BIOLOGICAL SPECIMENS

When a drug is taken, the drug itself and/or its metabolites are excreted in the urine. Urinary concentration corresponds to the non-metabolized drug and its metabolites at the time of specimen collection. Meanwhile, hair is known to reflect the accumulation of drugs. Thus, in the case of an arrest for drug abuse, "use up to the last minute" can be determined by analyzing urine specimens and chronic use over the past few months can be determined by analyzing hair specimens.

In Japan, news of drug abuse among celebrities has appeared. Some have tried to conceal past use of drugs by cutting their hair. The difficulty of drug testing in biological specimens arises from not only the technical problem that the residual level is extremely low, but also the problem of multiple drug use and thus, the test results may not match the self-report made by such abusers of the drugs they used.

Discrepancies between simple test methods, such as biological assays, and the self-report of illicit drug use could undermine epidemiological research findings. Ledgerwood *et al.*[20] examined the extent of agreement between self-reported illicit drug use and the results of hair analysis in a community sample of middle-aged men and tried to identify factors that may be used to predict the discrepancies between self-report and hair analysis. Hair specimens were analyzed for marijuana, cocaine, opiates, phencyclidine, and methamphetamine using radioimmunoassay (RIA) and GC-MS techniques. As a result, self-report and the results of hair analysis generally showed good, but not excellent, agreement.

BIOLOGICAL SPECIMENS COMMONLY USED IN FORENSIC SCIENCE

Blood and urine are the typical specimens for analysis in clinical chemistry. However, in addition to these, a variety of biological specimens are analyzed in forensic science. In this review, as the major specimens used in the analysis of drugs of abuse for the period of 2008–2010, blood, urine, oral fluid (saliva), hair, umbilical cord, placenta, meconium, tissue (adipose and brain), sweat, nail clippings, and breath will be taken up.

Urine

There are a great many studies that use urine as the biological specimen. As drugs for analysis, amphetamine-type stimulants[21–26] and cocaine and its metabolites[27, 28] have been used. Amphetamine-type stimulants, such as amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA), are known as central nervous system stimulants and their abuse has recently increased throughout the world.

It is presumed that the increase in the number of drug analysis examples is correlated to the increase in the number of criminal cases related to drug abuse. Notable progress in the overall analysis of drugs of abuse in biological specimens would be the fruit of the development of numerous LC-MS (/MS) methods. The successful interface of LC-MS has brought new light to bioanalytical and forensic sciences as it allows the detection of drugs and metabolites at concentrations that are difficult to analyze with commonly used GC-MS based techniques.[16)]

LC-MS/MS has become very popular and today, it is the leading tool for the simultaneous multicomponent analysis of drugs of abuse. However, in the analysis of amphetamine-type stimulants in urine specimens in particular, a method that uses GC-MS and requires either acetylation or pentafluorobenzyl (PFB) derivatization is also very much in use. This is because the method that uses GC-MS is reliable and has been employed ever since in forensic science.

On the other hand, in screening tests for the target drug and its metabolites, the LC-MS (/MS) method is advantageous. More drug metabolites exist in urine than in any other biological specimens. However, in the case of drug abusers who use multiple similar drugs, it is difficult to determine the types of drugs used.

For example, as it is difficult to physically distinguish amphetamine-type stimulants, analysts may not be aware of the drug taken by drug abusers. Therefore, a rapid and sensitive method that uses LC-MS/MS was developed for the identification and classification of amphetamine-type stimulants used by drug abusers.[24)] This method detects specific metabolites [such as *p*-hydroxy amphetamine, *p*-hydroxy methamphetamine, *p*-hydroxy *N*,*N*-dimethylamphetamine (DMA), and DMA *N*-oxide] of amphetamine, methamphetamine, and DMA as biomarkers in drug abusers' urine specimens. The determination of *p*-hydroxy amphetamine, *p*-hydroxy methamphetamine, amphetamine, methamphetamine, DMA, and DMA *N*-oxide was accurate and reproducible, with the limit of quantitation being 5 ng/ml in urine. When applied to urine specimens of suspected amphetamine-type stimulant abusers, the drugs used by methamphetamine and DMA abusers were precisely identified.

The utility of LC-MS/MS in cocaine urinalysis is also remarkable. Cocaine is a potent central nervous system stimulant that is metabolized to benzoylecgonine and further to minor metabolites, such as *m*-hydroxybenzoylecgonine. Cocaine is also metabolized to norcocaine. Cocaethylene is formed when cocaine and ethanol are taken simultaneously. Anhydroecgonine methyl ester is a unique marker in cocaine smokers, and anhydroecgonine ethyl ester is found in cocaine smokers who also use ethanol.

Numerous methods are available for the identification of cocaine or its metabolites in urine.[27–29)] Langman *et al.*[28)] developed an LC-MS/MS method for the detection and quantitation of cocaine, benzoylecgonine, norcocaine, cocaethylene, *m*-hydroxybenzoylecgonine, anhydroecgonine methyl ester, and anhydroecgonine ethyl ester in urine. Urine specimens (200 individuals) were cleaned by solid-phase extraction (SPE) and then subjected to LC-MS/MS measurement and confirmation by GC-MS. The results of GC-MS and LC-MS/MS measurements of cocaine and benzoylecgonine showed good correlation.

Shakleya *et al.*[30)] reported that opiates, cocaine, and their metabolites were quantified by LC-MS in 284 urine specimens collected thrice weekly, to monitor possible drug relapse in 15 drug-dependent pregnant women. As a result, 165 urine specimens (58%) from all the 15 subjects were positive for one or more cocaine analytes. Ecgonine methyl ester and/or benzoylecgonine were the major cocaine biomarkers in 142. Anhydroecgonine methyl ester, a biomarker in cocaine smokers, was positive in six and cocaethylene and/or ecgonine ethyl ester, biomarkers of cocaine and ethanol co-ingestion, were found in 25.

A simple deproteinization step involves mixing each specimen with an acetonitrile-internal standard mixture.[29)] The method has excellent precision across a wide linear range (*i.e.*, 25–2000 ng/ml) for each analyte. Clearly, the LC-MS/MS method for urinalysis is a significant improvement over conventional GC-MS methods. Another point of interest is that different patterns of cocaine biomarkers were observed after LC-MS as compared to GC-MS analysis.

As other examples of urinalysis, the 2-(2,5-dimethoxy-4-(methylthio)phenyl)ethanamine (2C-T) series,[31)] the 2-(2,5-dimethoxy-4-methylsulfanylphenyl)-1-methylethylamine (ALEPH) series,[32)] and cannabinoid[33)] analysis have been reported. The 2C-T series and the ALEPH series are psychedelic phenethylamines of the 2C family. 2C is a general name for the family of psychedelic phenethylamines containing methoxy groups on

positions 2 and 5 of the benzene ring. 2C-T-4 is the 2-carbon homologue of ALEPH-4 and its full chemical name is 2-4-(isopropylthio)-2,5-dimethoxyphenylethanamine. The structural and pharmacodynamic properties of 2C-T-4 are similar to those of 2C-T-7 and 2C-T-9.

An analytical procedure for the simultaneous determination of four thiophenethylamine designer drugs (2C-T series) in human urine has been reported. Quantitative analysis was performed by capillary electrophoresis with mass spectrometry (CE-MS),[31] using 2,5-dimethoxy-4-methylthiophenethylamine-D_4 as the internal standard. In order to minimize interference by matrix components and preconcentrate the target analytes, SPE was introduced as a cleanup step. The method was validated according to international guidelines and demonstrated to be specific, sensitive, and reliable for the analysis of these derivatives in urine specimens.

Hidvegi *et al.*[33] tried to identify cannabigerol (CBG), which, in its acid form, is one of the main intermediates in the biosynthesis of cannabinoids in hemp, in urine specimens of confirmed cannabis users. It was shown that enzymatic hydrolysis is necessary for the formation of free neutral cannabinoids from conjugates. After extraction, derivatization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and/or bis-trimethylsilyl derivatization, GC-MS was performed for the identification of CBG and its metabolites in the urine specimens. It was shown that CBG enters the body through cannabis smoking and is excreted in urine in the conjugated form, similar to other neutral cannabinoids. The presence of CBG metabolites, such as its glucuronated form, was examined by analyzing the chromatograms of hydrolyzed and trimethylsilylated extracts.

Oral Fluid (Saliva)

Some problems arise when urine specimens are collected at several occasions. For instance, the risk of manipulation and the risk of false-negative urine specimens are high. In addition, many suspected drug users find it difficult or embarrassing to urinate while being observed.[15]

The collection of oral fluid for drug analysis is easy and non-invasive. Oral fluid has demonstrated its usefulness for the analysis of drugs of abuse. Laboratory tests of oral fluid for drugs of abuse have continued to expand in the workplace as well as in legal and medical settings.[34] One of the purposes is to evaluate physical alterations in individuals suspected to be under the influence of drugs.

For instance, oral fluid is gaining prominence as an alternative matrix for monitoring drugs of abuse in the workplace, investigating criminal cases, and screening drivers who may be driving under the influence of drugs. There is a great need for a reliable and rapid on-site oral fluid test that can be used by police authorities to detect drivers suspected of being under the influence of drugs or alcohol. The European Driving under the Influence of Drugs, Alcohol and Medicines (DRUID) project called for the analysis of oral fluid specimens collected randomly and anonymously at the roadside from drivers in Denmark from 2008 through 2009.[35]

On the other hand, significant development has occurred in oral fluid drug analysis in the last few years. New pharmacokinetic studies have been conducted, optimal cutoffs have been proposed, and new studies have examined the correlation between oral fluid drug concentration and impairment.[36]

A recent study, *e.g.*, the detection of THC-COOH in oral fluid, can contribute to resolving the issue of false-positive results caused by passive exposure to marijuana.[36]

Analytical methods for measuring multiple licit and illicit drugs and metabolites in oral fluid require high sensitivity, specificity, and accuracy. As the volume available for analysis is limited, a comprehensive method is needed for the simultaneous measurement of multiple analytes in a single aliquot.

For instance, Fritch *et al.*[37] reported the validation of a semi-automated method for the simultaneous extraction, identification, and quantitation of 21 analytes in a single oral fluid aliquot. The target compounds were amphetamine, methamphetamine, MDA, MDMA, 3,4-methylenedioxyethylamphetamine (MDEA), pseudoephedrine, cocaine, benzoylecgonine, codeine, norcodeine, 6-acetylcodeine, morphine, 6-acetylmorphine, hydrocodone, norhydrocodone, dihydrocodeine, hydromorphone, oxycodone, noroxycodone, oxymorphone, and phencyclidine. Oral fluid specimens were collected with the Intercept® device (OraSure Technologies, Inc., Bethlehem, PA, USA) and subjected to SPE. Thirty-nine patients were tested positive for various combinations of licit and illicit drugs and their metabolites.

There are many reports of the use of LC-MS/MS for oral fluid specimens.[38–40] A screening system to analyze multiple drugs simultaneously has be-

come the mainstream. In this regard, matrix effects should be taken into consideration. Although ion suppression and enhancement were observed for cocaine, opiates, and their metabolites, the performance of the LC-MS/MS method was not influenced by matrix effects when deuterated internal standards were used.[40)]

In the case of urinalysis, in addition to LC-MS/MS, recently, rapid test kits, such as immunoassay, have been widely used.[14, 41)] The target drugs of the test kits are marijuana, methamphetamine, cocaine, opiates, and so on. However, most of the target drugs require confirmatory tests by LC-MS/MS and/or GC-MS, in case the immunoassay gives positive results.

So far, urine and saliva are the major specimens for the analysis of drugs of abuse. However, sometimes, the use of both seems to be best. For example, tests for sports doping fall under this category.

Stimulants are banned by the World Anti-Doping Agency (WADA) if used "in competition." As the analysis of stimulants is presently carried out on urine specimens only, for better interpretation of analytical data, it may be useful to discriminate between early intake of the stimulant and administration specifically aimed at improving sport performance.

Strano-Rossi *et al.*[42)] investigated differences in the excretion/disappearance of drugs between urine and oral fluid, a specimen that can reflect plasma concentration. As a result, both the absolute concentrations and their variations as a function of time were markedly different between urine and oral fluid; drugs were eliminated much more slowly from urine than from oral fluid.

In all those cases, the concentration of a drug metabolite in urine was very low and the parent compound was not detected at all. It was suggested that it is indeed impossible to discriminate between recent administration of small doses and past administration of larger doses, if one relies on only urine data. In addition, the results also suggested that the analysis of oral fluid could be used to successfully complement data obtained from urinalysis.

Hair

Urine or saliva sampling is noninvasive and has been widely used because of ease of collection. However, the main disadvantage of urinalysis is the short window of detection. On the other hand, hair is becoming as important as urine and saliva as the specimen used in drug analysis.[9, 10)]

Hair has become an important matrix for drug analysis, complementing blood and urine specimens. The prolonged detection window makes hair analysis suitable for the detection of exposure to illegal drugs for a period of up to 12 months.[43)] Hair sampling, which features easy sampling and minimal risk of manipulation, may therefore be a welcome alternative. The prolonged detection window may also provide more reliable information to the physician.[44)]

In order to evaluate the usefulness of hair specimens and investigate practical and interpretive problems or advantages with the use of hair specimens, Kronstrand *et al.*[15)] conducted hair analysis for drugs prior to re-issuing drivers' licenses as a Swedish pilot study. Ninety-nine hair specimens and 198 urine specimens were collected from 84 individuals during the 12-month study period. The hair specimens were screened for 20 drugs with LC-MS/MS and confirmation of positive results was accomplished with GC-MS or LC-MS/MS. As a result, hair analysis identified illegal drugs in seven different occasions, whereas urinalysis failed to identify any illegal drugs in all occasions. This indicates that the physician must have an understanding and knowledge of the limitations of the screening methods used.

An LC-MS/MS method was developed for the determination of ketamine (and its metabolite norketamine) and some amphetamines (amphetamine, methamphetamine, MDA, and MDMA). Hair specimens were washed and pulverized and then extracted with aqueous formic acid solution. Deuterated analogs of the analytes were used as internal standards for quantification. The analysis by LC-MS/MS was simpler and faster than that by conventional GC-MS methods that usually require more laborious extraction procedures and, in most cases, an additional derivatization process.[45)]

As hair cocaine concentration is a reliable marker of exposure to the drug, an original liquid chromatographic method has been developed for the determination of cocaine in human hair specimens by HPLC with fluorescence detection.[46)] The method was validated and successfully applied to hair specimens collected from cocaine users. Specimen pre-treatment was carried out by incubative extraction with 0.1 M HCl, followed by SPE with C2 cartridges. For detection, native cocaine fluorescence was monitored at 315 nm with excitation at 230 nm.

Miller *et al.* used phosphate buffer (pH 5.0) as the optimum hair incubation medium because of the high stability of cocaine and 6-monoacetylmorphine in this buffer. Then, two different SPE methods [Bond Elut Certify® (Agilent Technologies, Inc., Santa Clara, CA, USA) and Clean Screen® (United Chemical Technologies, Bristol, PA, USA)] were employed for specimen cleanup before LC-MS/MS analysis. However, the matrix effect, demonstrating ion suppression, was observed in most analytes.[47)]

As regards the solution for extraction and incubation of hair specimens, other than phosphate buffer, 25 mM formic acid: acetonitrile mixture (5 : 95),[43)] 0.1 M HCl,[48)] and methanol[49)] have been used. Considering the influence of matrix effects, deuterated internal standards were usually added during the specimen extraction procedure.

Cocaine and its metabolites are included in the proposed United States federal regulations for hair analysis. The suggested cut-off concentration for the metabolites is 50 pg/mg, which is difficult to achieve routinely with electron impact GC-MS.[50)] This may be due to the inability to derivatize cocaethylene to improve its response and the disturbance in the detection of co-eluted norcocaine and cocaethylene, or potentially similar ions as the derivatives of norcocaine and benzoylecgonine.

Barroso *et al.*[51)] developed and validated an analytical method for the simultaneous determination of cocaine and its main metabolite, benzoylecgonine, in human hair specimens by GC-MS. After extraction of the hair matrix with a mixture of methanol/HCl (2 : 1) at 65°C for 3 hr, followed by specimen cleanup by mixed-mode SPE, the extract was analyzed by GC-MS after derivatization with MSTFA with 5% chlorotrimethylsilane. The developed method may be useful for the analysis of cocaine and benzoylecgonine in hair specimens in forensic toxicology laboratories, taking into account the speed and sensitivity achieved with a single quadrupole MS that is available in most laboratories.

Unlike cocaine, the analysis of amphetamines in hair specimens by GC-MS has been widely practiced. As examples of specimens for analysis using GC-MS, amphetamine-type stimulants[52–54)] and marijuana[55,56)] are mentioned. The procedure involves liquid-liquid extraction of hydrolyzed hair specimens spiked with deuterated internal standards, followed by derivatization with *N*-methyl-bis(trifluoroacetamide) (MBTFA),[52)] heptafluorobutyric anhydride (HFBA),[54)] or perfluorooctanoyl chloride.[53)]

As regards cannabinoid analysis by GC-MS, a similar alkaline hydrolysis of hair specimens was employed, and for derivatization of the analytes, MSTFA was used for silylation.[55)]

Hair analysis in forensic and clinical toxicology has focused on drugs of abuse and comprehensive, drug-class-independent screening methods based on MS have not been applied to date.

Pelander *et al.*[44)] investigated a qualitative basic drug screening method for hair specimens that uses liquid chromatography coupled to time-of-flight mass spectrometry (LC/TOF-MS), which has been developed and evaluated for forensic toxicological urinalysis. The method included alkaline hydrolysis and purification by mixed-mode SPE prior to LC/TOF-MS analysis. Identification was based on accurate mass, isotopic pattern fit, and retention time, if available. The drug classes identified included antidepressants, antipsychotics, antiepileptics, amphetamines, opioids, beta-blockers, a benzodiazepine, a hypnotic, a local anesthetic, an antiemetic, and an antipyretic analgesic.

Analysis of 32 hair specimens from deceased drug addicts revealed the intake of 35 different drugs, and the findings were in good agreement with the findings in blood and urine specimens by other methods. Moreover, information about previous drug use, which is not evident in the analysis of other matrices, was obtained in the majority of the cases. However, one apparent false-positive result was identified.

Two points should be noted when conducting hair analysis. First, discriminative analysis to differentiate the incorporation of abused drugs into hair from external contamination of the hair surface is necessary. Hoelzle *et al.*[57)] investigated the utility of discriminative analysis in differentiating between incorporation of cocaine and its congeners into hair and external contamination, when dealing with compounds that are administered by sniffing or inhalation (*e.g.*, cocaine). The statistical discriminant analysis made a prediction for each cocaine-positive hair specimen as regards the likelihood of its belonging to the group of cocaine users or being contaminated.

As the second point, in hair analysis, particularly cocaine analysis, there is a possibility of contamination during sample preparation and hair analysis. In other words, "hair porosity" should be considered in evaluating external contamination. Hill *et al.*[58)] applied a method for determining hair porosity to hair specimens, and the porosity charac-

teristics of hair are discussed in relation to experimental and actual contamination of hair.

Umbilical Cord, Placenta, and Meconium

As regards biological specimens specific to pregnant women, the umbilical cord, placenta, and meconium will be discussed as specimens for the analysis of drugs of abuse. The exposure of pregnant women to recreational drugs, that is, in utero drug exposure, may adversely impact fetal and neonatal development. In this regard, detecting drug exposure during pregnancy is the most effective way to improve pre- and post-natal care in the mother and the newborn as it permits implementation of adequate treatment and follow-up.[59)]

Umbilical Cord: The umbilical cord is considered to be a waste product after childbirth. Nevertheless, the umbilical cord also is suitable for anonymous epidemiological studies. Montgomery *et al.*[60)] suggested that the umbilical cord could be an alternative to meconium for detecting fetal drug exposure after demonstrating that comparable results were obtained in those two matrices.

De Castro *et al.*[59)] developed and validated an LC-MS method for the simultaneous quantification of methadone, cocaine, opiates, and metabolites in umbilical cord tissue. In their method, homogenization of the specimen in acidic conditions and subsequent extraction with mixed-mode cation-exchange cartridges allowed for adequate sensitivity and selectivity for quantification of the analytes of interest.

The quantification of methadone and its metabolite 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine in umbilical cord specimens from women receiving known doses of methadone permits determination of potential correlations between drug concentration in the umbilical cord and the administered dose, and whether umbilical cord drug concentration can predict maternal and neonatal outcomes.

In general, umbilical cord tissue is available in abundance and thus specimen acquisition is not an issue. Therefore, the simultaneous determination of other common drugs of abuse will help us understand the usefulness of umbilical cord analysis for the identification of in utero drug exposure.

Jones *et al.*[61)] reported the use of LC-MS/MS to confirm amphetamines in umbilical cord specimens presumed to be positive for amphetamine and methamphetamine. The study using matched pairs of meconium and umbilical cord demonstrated a high degree of agreement.

Placenta: The placenta may serve as an alternative specimen to urine for testing drugs of abuse during the first trimester of gestation. The advantage of placenta tissue, obtained as discarded material in the event of pregnancy termination, over such conventional matrices as urine and blood is that the collection is almost noninvasive and relatively easy to perform.

Joya *et al.*[62)] reported the development and validation of a method for the quantification of drugs of abuse, such as amphetamine, methamphetamine, MDMA, methadone, cocaine, benzoylecgonine, cocaethylene, morphine, 11-nor-9-carboxy-delta-9-tetrahydrocannabinol, nicotine, and cotinine, in human placenta tissue, using GC-MS. Placenta specimens were stored at $-80^{\circ}C$ until analysis and the analytes were stable even after three freeze-thaw cycles (stored at $-20^{\circ}C$). The analytical method was used to evaluate the effect of the consumption of drugs of abuse in women who voluntarily terminated their pregnancy at the 12th week of gestation. The use of placenta as a non-conventional matrix showed that it was able to provide information about early in utero exposure to the drugs of abuse.

In another study, cocaine and its metabolites, and opiates in placenta have also been analyzed by LC-MS/MS. A method for the simultaneous quantification of methadone, cocaine, opiates, and metabolites in human placenta by LC-MS was developed by De Castro *et al.*[63)] Specimens were homogenized and subjected to SPE in the conventional manner. The applicability of the method was demonstrated in the analysis of five placenta specimens from opioid-dependent women receiving methadone pharmacotherapy, with methadone doses ranging from 65 to 95 mg on the day of delivery. The detection of drugs of abuse in the placenta may also improve knowledge of the usefulness of this matrix for detecting in utero drug exposure and studying the disposition of drugs in the maternal-fetal dyad.

Meconium: The meconium offers several advantages for detecting prenatal drug exposure and has become the specimen of choice for the past two decades. Accumulating substances from the 12th gestational week until birth, the meconium acts as a reservoir for exogenous compounds, such as drugs and metabolites. Drugs are incorporated into the meconium via fetal biliary excretion or through ingestion of drug-contaminated amniotic fluid. Meconium analysis is thought to enable the

detection of maternal drug use during the second and third trimesters of pregnancy, whereas neonatal hair and urine analyses indicate more recent exposure. Meconium is usually passed in the first 1 to 3 days of life but may be delayed in premature infants. Its complex composition of mucopolysaccharides, water, bile salts, bile acids, epithelial cells, and other lipids makes drug isolation difficult.[64]

Reviews by Gray *et al.*[64] summarize current literature on meconium analysis. A brief overview is provided as follows: illicit drugs, therapeutic drugs, ethanol metabolites, pesticides, and other xenobiotics have been detected in meconium using conventional toxicological techniques, including immunoassay, and confirmed by GC-MS or LC-MS.

As regards analytical methodology, because of the complexity of the meconium matrix, extraction and specimen preparation prior to instrumental analysis may prove difficult and time-consuming. Gunn *et al.*[65] described a method for the rapid extraction and quantification of amphetamine and methamphetamine from meconium, which uses SPE (ISOLUTE® HM-N supported liquid extraction columns containing a modified form of diatomaceous earth; Biotage Inc., Charlottesville, VA, USA) and GC-MS.

Lopez *et al.*[66] reported the case of a newborn with symptoms of hyperexcitability. After it was confirmed that the mother had consumed drugs during pregnancy using an enzyme multiplied immunoassay technique, the newborn's urine and meconium specimens were analyzed by GC-MS. A GC-MS method for the simultaneous analysis of cocaine, benzoylecgonine, codeine, morphine, and 6-acetylmorphine in meconium is described. Confirmation of urine and meconium results by GC-MS showed consumption of cocaine and codeine during pregnancy and the gradual decline of the levels of those drugs in the newborn, totally disappearing by the third day of life.

Gunn *et al.*[67] described the simultaneous determination and quantification of cocaine and its major metabolite, benzoylecgonine, in meconium using ultra performance liquid chromatography (UPLC)-MS/MS. The extraction of cocaine and benzoylecgonine from the homogenized meconium matrix was achieved with a preliminary protein precipitation or protein "crash" procedure employing cold acetonitrile, followed by mixed-mode SPE. Following elution from the SPE cartridge, the eluents were concentrated and injected onto the UPLC-MS/MS for analysis.

Tissue (Brain, Adipose)

Brain Tissue: Postmortem drug screening in cases of presumed overdose as cause of death is routinely carried out in toxicology laboratories. The most common specimen used for the analysis is blood due to its temporal relation with the effect of a drug on the individual and his/her status at the time of death. Other specimens, such as vitreous humor, saliva, and urine, are also analyzed to complement blood analysis or in cases where blood extraction is difficult for certain reasons.

There are solid data demonstrating that drugs and their metabolites distribute mainly in tissues rich in lipids, including brain and adipose. Brain tissue provides excellent information relevant to the status of the individual and the possible physical alterations and conduct at the time of death.[68] The analysis of brain tissue specimens may provide such valuable information as the type of drug administered when a postmortem investigation is carried out to determine the potential cause and manner of death.[69]

In the analysis of brain tissue specimens, SPE is carried out, followed by the semi-quantitative determination of cocaine levels by GC-MS/MS using deuterated cocaine as the internal standard.[68]

Shakleya *et al.*[69] developed an LC-MS/MS method for isolating drugs of abuse, such as cocaine, benzoylecgonine, cotinine, ecgonine methyl ester, 6-acetylmorphine, trans-3-hydroxycotinine, codeine, and nicotine, in brain tissue specimens.

On the other hand, Reich *et al.*[70] pointed out the difficulty of detecting drugs in brain tissue, which typically requires extensive specimen preparation followed by drug extraction before the extracts are finally analyzed by LC-MS. Therefore, in order to eliminate any complications introduced by specimen pretreatment, a method for directly analyzing drugs in intact tissue was developed.

Matrix-assisted laser desorption/ionization tandem mass spectrometry [MALDI-MS(n)] demonstrated the ability to quantify cocaine present in postmortem brain tissue of a chronic human cocaine user.[70]

Adipose Tissue: Adipose tissue can be used to detect absorbed chemicals in living organisms, provided that the chemicals are highly lipophilic and bind to it.[71] In mammals, an increased storage of chemicals in adipose tissue indicates a longer exposure of the tissue to the chemicals even after the cessation of external exposure; that is, chemicals that are adsorbed by the body tend to persist in adipose

tissue on the basis of their lipophilicity. The extent of chemical accumulation in adipose tissue is correlated with the adipose tissue/blood partition coefficient. For instance, THC is highly soluble in lipids and rapidly distributed in tissues, including adipose tissue.

Adipose tissue is also an optimal specimen in cases with extended postmortem time lag and whenever body fluids cannot be obtained. Similar to hair specimens, adipose tissue provides long-term information about an individual's drug use, which is especially useful when the history of drug abuse is not easily obtained. Drugs identified in postmortem adipose tissue are accumulated because of antemortem deposition and not any postmortem changes.[71] In this connection, adipose tissue may be an optimal matrix in forensic toxicology.

Unfortunately, adipose tissue is a complex biological matrix that necessitates several preanalytical preparation steps to separate drugs and metabolites from the lipophilic matrix. Adipose tissue is not considered to be an optimal specimen in analytical toxicology, primarily because of the difficulties in specimen handling and processing for cleanup. It is for these reasons that few case reports on drug detection in postmortem adipose tissue have been published.

Colucci *et al.*[71] developed a GC-MS method for the determination of cocaine, its metabolites, methadone, and morphine in postmortem adipose tissue. The method involves the aqueous acid extraction of analytes, the alkalinization of the extract, SPE with chloroform, and derivatization with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide before GC-MS analysis. Deuterated compounds were used as internal standards for the determination and quantification of analytes.

Sweat, Breath, and Nail Clippings

Next, relatively minor specimens for the analysis of drugs of abuse are introduced.

Sweat: Sweat has attracted attention in recent years as an alternative matrix in forensic toxicology as it can be sampled easily and non-invasively. Sweat can be collected with patches over a long period, thereby permitting drug monitoring over a longer period than urine.

Concheiro *et al.*[72] developed an LC-MS/MS method for the quantification of buprenorphine, norbuprenorphine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, cocaine, benzoylecgonine, ecgonine methyl ester, morphine, codeine, 6-acetylmorphine, heroin, 6-acetylcodeine, cotinine, and trans-3′-hydroxycotinine in sweat specimens. Buprenorphine and methadone are medications used in the clinical setting to help opiate-addicted individuals withdraw from and stop using drugs. Sweat patches were mixed with acetate buffer at pH 4.5 and the supernatant was subjected to SPE (Strata-XC-cartridges, Phenomenex, Inc., Torrance, CA, USA). The method was applied to sweat patches collected weekly from an opioid-dependent buprenorphine-maintained pregnant woman. As a result, buprenorphine, cocaine, opiates, and methadone were detected from the sweat specimens.

Breath: Berchtold *et al.*[73] tried to detect such narcotics as morphine, fentanyl, norfentanyl, naloxone, cocaine, hydroxybutyrolactone, and nicotine from breath. They challenged the diagnosis by online breath analysis using MS, because of the low concentrations of the pertinent compounds in breath.

Nail Clippings: Nail clippings were used as analytical specimens for the detection and quantitation of illicit drugs.

Kim *et al.*[74] developed a GC-MS method for the simultaneous qualification and quantification of amphetamine, methamphetamine, MDA, MDMA, ketamine, and norketamine in nail clippings. Similar to hair analysis, methods for the surface decontamination and extraction of the specimens are critical for the analysis. Water and methanol washes, followed by alkaline hydrolysis and liquid-liquid extraction with ethyl acetate, were selected for use in the study. The extracts were evaporated to dryness, derivatized with HFBA at 60°C for 30 min, and analyzed by GC-MS. The results indicated that nail clippings are potentially useful specimens for the detection of past illicit drug use.

Although it is not a recent report, Lemos *et al.*[75] demonstrated the analysis of nail clippings for drugs of abuse, *i.e.*, they determined cannabis in the nail clipping specimens by RIA and GC-MS.

Dried Blood Spot

For the last section of biological specimens, we would like to discuss "blood." Blood is the most important and versatile specimen for drug analysis not only in clinical chemistry, but also in forensic science. In fact, in cases of the analysis of drugs of abuse, blood specimens have often been employed in detecting cocaine,[76–78] amphetamines,[79] and opiates.[80]

Table 1. Overview List of Analytical Methods for Abused Drugs in Various Biological Specimens

Specimens	Analytes	Pre-treatment (extraction, cleanup, and/or derivatization)	Analysis Instruments	References
Urine	amphetamines, 2Cs	SPE	LC-MS	21
	amphetamines (TMA series)	SPE	LC-MS	22
	amphetamines, ephedrines	SPE	GC-MS	23
	amphetamines	filtration	LC-MS/MS	24
	amphetamines	LLE	GC-MS	25
	amphetamines	SPE	GC-MS	26
	cocaines	SPE	LC-MS/MS	27
	cocaines	SPE	LC-MS/MS	28
	cocaines	deproteinaization	LC-MS/MS	29
	cocaines, opiates	no-cleanup (direct injection)	LC-MS	30
	2C-T series	SPE	CE-MS	31
	ALEPH series	SPE	CE-DAD	32
	cannabinoids	enzymatic hydrolysis, silylation	GC-MS	33
Oral fluuid (saliva)	amphetamines, cocaines, opiates, cannabinoids, benzodiazepines	SPE	LC-MS/MS	35
	amphetamines, opiates	SPE	LC-MS/MS	37
	amphetamines, cocaines, opiates	LLE, SPE	LC-MS/MS	38
	amphetamines, cocaines, opiates, benzodiazepines	dilution	LC-MS/MS	39
	cocaines, opiates	deproteinaization	LC-MS/MS	40
	amphetamines, cocaines, opiates, cannabinoids	no-cleanup	immunoassay, GC-MS	14
	amphetamines, cocaines, opiates	no-cleanup	immunoassay, LC-MS/MS	34
	cannabinoids	no-cleanup	immunoassay, GC-MS	41
Hair	amphetamines, cocaines, opiates, benzodiazepines, nicotines	acid extraction	LC-MS/MS	43
	amphetamines, opiates, benzodiazepines, others	alkaline hydrolysis, SPE	LC-TOFMS	44
	amphetamines, opiates	acid extraction	LC-MS/MS	45
	cocaine	SPE	LC-FL	46
	amphetamines, cocaines, opiates, benzodiazepines	SPE	LC-MS/MS	47
	cocaines	SPE	LC-MS/MS	48
	cocaines, opiates	methanol extraction	LC-MS/MS	49
	cocaines	SPE, sylilaton	GC-MS	51
	amphetamines	alkaline hydrolysis, LLE, acylation	GC-MS	52
	amphetamines	alkaline hydrolysis, LLE, acylation	GC-MS	53
	amphetamines, opiates	LLE, acylation	GC-MS	54
	amphetamines, cocaines, opiates, cannabinoids	SPME, sylilation	GC-MS	55
	cannabinoids	SPME	GC-MS/MS	56
	cocaines	enzymatic digestion, SPE	LC-MS/MS	58
Unbilical cord	amphetamines	SPE	LC-MS/MS	61
	opiates, cocaines	SPE	LC-MS	59
	amphetamines, cocaines, opiates, cannabinoids	no-cleanup	immunoassay	60
Placenta	amphetamines, cocaines, opiates, cannabinoids	SPE, sylilaton	GC-MS	62
	cocaines, opiates	SPE	LC-MS	63
Meconium	amphetamines, cocaines, opiates	SPE	LC-MS/MS	64
	amphetamines	SPE	GC-MS	65
	cocaines	SPE	LC-MS/MS	67
	cocaines, opiates	no-cleanup	immunoassay, GC-MS	66
Tissue (Brain)	cocaines	SPE	GC-MS	68
	cocaines, opiates, nicotines	SPE	LC-MS/MS	69
	cocaines	no-cleanup	MALDI-MS(n)	70
Tissue (Adipose)	cocaines, opiates	SPE, sylilaton	GC-MS	71
Sweat	cocaines, opiates, nicotines	SPE	LC-MS/MS	72
Breath	cocaines, opiates, nicotines	no-cleanup	MS	73
Nail clippings	amphetamines, opiates	alkaline hydrolysis, LLE, acylation	GC-MS	74
	cannabinoids	alkaline hydrolysis, LLE	RIA, GC-MS	75
Blood	cocaines	SPE	LC-MS/MS	78
	amphetamines	deproteinaization	LC-MS/MS	79
	amphetamines, opiates	SPE	LC-UV, LC-FL	80
Dried blood spot	cocaines	SPE	LC-MS/MS	76
	cocaines	LLE	LC-FL	77

amphetamines: including amphetamine derivatives and metabolites, cocaines: including cacaine derivatives and metabolites, opiates: including natural and synthetic alkaloids, and their metabolites, cannabinoids: including $>^9$-THC and synthetic cannabinoids, 2Cs: a family of psychedelic phenethylamines, 2C-T series: thiophenethylamine desinger drugs, ALEPH series: thioamphetamine desinger drugs, TMA series: trimethoxy amphetamines, ephedrines: including related homologues, benzodiazepines: including related homologues, nicotines: includeing metabolites, LLE: liquid-liquid extraction, SPME: solid-phase microextraction, CE-DAD: capillary electrophoresis with diode array detector, LC-FL: liquid chromatography with fluorescence detector.

However, blood *per se* is not interesting as a biological specimen. "Impregnated filter paper blood" or the so-called "dried blood spots (DBS)," which increase the shelf life of a drug in blood collected in the field and facilitate transport and storage, are presented here as examples of the analysis.

Alfazil *et al.*[76] investigated the stability of benzodiazepines and cocaine in DBS. Previous studies have shown that drug concentrations in blood can change during storage especially at room temperature. However, even labile drugs, such as cocaine, may be stable in DBS. A new method has been developed for the analysis of hydrolytically labile drugs in DBS in order to assess their degradation during a storage period of one month. As the test drugs, flunitrazepam, temazepam, oxazepam, lorazepam, nitrazepam, diazepam, and cocaine were selected. A piece of filter paper was spotted with 100 μl of blood containing the drugs at the concentration of 1000 ng/ml and left overnight to dry at room temperature. The drugs were then extracted by suspending the filter paper in phosphate buffer. The extract was subsequently cleaned up by SPE and analyzed by LC-MS/MS. In their experiment, DBS were stored in duplicate at room temperature, 4°C, and −20°C for up to one month. The degradation of the drugs in DBS at all storage conditions was minimal compared to the degradation in the corresponding liquid blood specimens stored under similar conditions and more than 80% of each analyte could be recovered from the specimens.

Mercolini *et al.*[77] also employed DBS for the analysis of cocaine and its two metabolites by liquid chromatography with fluorescence detection. A solvent extraction technique was implemented for fast and feasible specimen pre-treatment. The method was successfully applied to DBS collected from some cocaine users, both with and without concomitant ethanol intake. The results were in good agreement with those obtained from plasma specimens that were subjected to an original SPE procedure on C8 cartridges. The method was suitable for monitoring cocaine/ethanol use by means of DBS or plasma testing.

CONCLUSION

Various biological specimens have been used in the analysis of drugs of abuse in the field of forensic science, as described in this review. Table 1 shows the overview list of analytical methods for abused drugs in various biological specimens mentioned in this review. Although the analytical techniques used in forensic science are very similar to those used in clinical chemistry as well as pharmacokinetics and metabolic monitoring, there are marked differences in the purpose, specimen, and time required for analysis. In forensic science, because the analytical results greatly affect the outcome of criminal investigations and court decisions, qualitative ability is thought to be more important than quantitative ability. Of course, if cutoff values are set for drugs of abuse, high reliability of the quantitatively determined values is required.

Further advances in analytical instrumentation have paved the way for the development of sophisticated trace analysis methods that enable possible use of a specimen containing trace amounts of a drug and small amounts of specimen, such as hair and nail clippings. In the analysis of drugs of abuse, the proliferation of designer drugs has become a serious problem and there is an ongoing cat-and-mouse game between the police authorities and drug abusers. By further advancing analytical methods for drugs of abuse in the future, it is hoped that drug abuse would be completely eradicated.

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