RECOMMENDED METHODS FOR THE DETECTION AND ASSAY OF **HEROIN, CANNABINOIDS, COCAINE, AMPHETAMINE, METHAMPHETAMINE AND RING-SUBSTITUTED AMPHETAMINE DERIVATIVES** IN BIOLOGICAL SPECIMENS

MANUAL FOR USE BY NATIONAL LABORATORIES

UNITED NATIONS

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INTERNATIONAL DRUG CONTROL PROGRAMME

Recommended Methods for the Detection and Assay of

Heroin, Cannabinoids, Cocaine, Amphetamine, Methamphetamine and Ring-Substituted Amphetamine Derivatives

in Biological Specimens

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Introduction

A. Background

Over the last decade there has been an enormous increase not only in the production and supply of illicit drugs, reflected by the huge and mounting quantities of drugs seized by national and international authorities, but also in the rate of drug abuse, i.e. the illicit demand for drugs. Drugs seized are not only traditional drugs already under national and international control, but also include unexpected new illicit drugs or combinations of drugs prepared by chemists working in clandestine laboratories. At the same time there are reports of expanding misuse/abuse of drugs used for medical purposes, such as barbiturates and benzodiazepines.

What used to be traditionally a problem of developed countries, is no longer confined to these countries. Drug abuse is now a global problem affecting developed and developing countries alike, and today no nation is free from this threat.

The extent and diversity of abuse have put increasing demands on nations to intensify regulatory efforts, in some cases with the introduction of stringent legislation which may have serious consequences on the individual charged with drug offences. Ultimately, the final outcome of such legislative procedures rests upon the results of laboratory tests. This has placed greater pressures on national laboratories which are now required not only to identify seized materials but also to detect drug abuse. In addition, while in the past the laboratory was often only required to perform qualitative analyses, it is now required to produce reliable qualitative and quantitative results.

In the field of drug abuse, laboratories have now to be able to deal with more substances and to use methods of detection and analysis which are faster and yet, at the same time, are more accurate and specific. The analysis of biological specimens like urine and blood presents additional challenges because of the need to separate target substances from interferences in blood and urine which are complex biological matrices.

In addition, the international nature of the drug abuse problem requires speedy exchange of analytical data between laboratories as well as between the laboratories and law enforcement agencies on national and international levels. Development of internationally acceptable methods of detection and assay would contribute greatly towards the achievement of these objectives.

While working on recommended methods for testing seized cannabis and amphetamine/methamphemtamine an Expert Group Meeting in Kuala Lumpur in 1986 recognized that an issue of increasing importance is the development of methods for the analysis of abused drugs and their metabolites in body fluids. It was recommended that the United Nations should explore the most appropriate means of addressing this problem [1].

This proposal was endorsed by the Commission on Narcotic Drugs at its thirty-second session in February 1987, which encouraged the United Nations Laboratory to extend its assistance to Member States by establishing and providing guidelines on methods of analysis of controlled drugs in body fluids.

In response to that suggestion, the Division of Narcotic Drugs convened an expert group meeting in 1987 to establish guidelines for national testing programmes and laboratories performing assays for drugs of abuse in body fluids. The group, in assessing the world's needs in this specific area, noted that the list of drugs to be tested for should at least include opiates (heroin, morphine, codeine), cannabinoids, cocaine, methadone, methaqualone, amphetamine, methamphetamine and phencyclidine, and recommended the "publication of follow-up working manuals on the subject to serve as guidelines for laboratory and programme development" and "the setting up of an expert review group that would periodically review methodology and drug-testing procedures" [2].

The Commission on Narcotic Drugs, at its tenth special session endorsed the recommendations of the Group and placed particular emphasis on "the development of recommended laboratory testing methods and international standard criteria for national body fluid testing programmes, including proficiency testing and method/ procedure validation" [3].

The International Conference on Drug Abuse and Illicit Trafficking in June 1987 had similarly suggested that "The Division of Narcotic Drugs, in collaboration with the World Health Organization (WHO) and the International Labour Organisation (ILO), should promote and harmonize national efforts by developing internationally acceptable guidelines, criteria and methodologies for national testing programmes". The Conference also proposed "that a central source of reference standards of major drug metabolites should be established to serve national laboratories" [4].

In response to the Commission's request and at the invitation of the Government of Singapore, the Division of Narcotic Drugs convened an Expert Group Meeting on the Detection and Assay of Controlled Drugs in Body Fluids and Recommended Methods for the Detection and Assay of Heroin/ Morphine and Cannabinoids in Biological Specimens. A subsequent meeting was held in Madrid on Methods for the Detection and Assay of Cocaine, Amphetamine, Methamphetamine, and Ring-Substituted Amphetamine Derivatives in Biological Specimens.

B. Purpose of the Manual

This revised manual combines the United Nations manuals on *Recommended Methods for the Detection and Assay of Heroin and Cannabinoids* [5] and on *Recommended Methods for the Detection and Assay of Cocaine, Amphetamine, Methamphetamine and Ring-Substituted Amphetamine Derivatives in Biological Specimens* [6]. Special emphasis has been laid on properly conducted and supervised sample collection, transport and storage, and strict maintenance of the chain-of-custody process. In performing assays on biological specimens, it is important that guidelines for the submission of samples are strictly adhered to. This is necessary because the results may have serious legal implications on the individual.

The present manual, prepared by the Laboratory Operations, Technical Services Branch of the United Nations International Drug Control Programme (UNDCP), reflects the conclusions of the expert groups and has been designed to provide practical guidance to national authorities and analysts by describing recommended methods for use in forensic and toxicological laboratories for the detection and assay of heroin/morphine, cannabinoids, cocaine, amphetamine, methamphetamine and ring-substituted amphetamine derivatives in biological specimens.

In selecting the methods, the experts were aware that many laboratories in existence today utilize methods which meet or may even exceed legislative requirements. However, it was noted that there was great diversity with regard to the structure of

national programmes, laboratory equipment and methodologies employed in the detection of drug abuse. In general, this manual is an attempt to help promote and harmonize national efforts by providing internationally acceptable guidelines and a selection of methods that may be used in the laboratories. More importantly, it is meant to provide assistance to laboratories which may not have access to sophisticated equipment and methods. Each method has been used for a number of years in reputable laboratories and can be expected to provide reliable results. In the process of identifying these methods, the expert groups were of course aware that there are many other useful and acceptable methods.

C. Use of the Manual

Methods recommended in the manual are chosen on the basis of proven reliability, an important requisite if the results are to be used for legal or punitive objectives. The final choice of methodology and approach to analysis remains in the hands of the analyst working in his own country. This may necessarily depend on the availability of instrumentation, reference materials and trained personnel. However, it is recommended that for the purpose of establishing illicit consumption of drugs, two methods be used: an initial screening method (usually an immunoassay technique) followed by a confirmatory method using different chemical or physical principles (usually a chromatographic technique). Where only thin-layer chromatography (TLC) is available, it is suggested that a second thin-layer chromatography procedure utilizing a different solvent system be performed as well.

It is emphasized that whatever the methods selected, attention must be paid to proper equipment maintenance and environmental control, particularly for the transport and storage of specimens and unstable reagents, and that reliance is placed only on adequately trained and skilled personnel. Attention is also drawn to the importance of the availability of textbooks on drugs of abuse and analytical techniques. Furthermore, the analyst is expected to keep abreast of developments in the field of toxicological analyses by following current literature on the subject. Useful adjuncts to this manual would be the United Nations manuals on Recommended Methods for Testing Heroin [7], Opium/Crude Morphine [8], Cannabis [9], Cocaine [10], Amphetamine and Methamphetamine [11] and Illicit Ring-Substituted Amphetamine Derivatives [12]. Although these manuals were originally intended for the analysis of seized materials, they contain information which is also relevant to the analysis of biological specimens.

The United Nations International Drug Control Programme would welcome observations on the contents and usefulness of the present manual. Comments may be addressed to:

United Nations International Drug Control Programme
Technical Services Branch
Laboratory Operations
United Nations Office at Vienna
Vienna International Centre
P.O. Box 500
A-1400 Vienna
Austria.

I. General aspects of assay of controlled drugs in biological specimens

A. Purpose and strategy

There are generally two purposes for analysis of biological fluids/specimens:

For forensic purposes, for example the analysis of biological samples for the presence of controlled drugs. A positive analytical result for a sample taken in this context would usually result in criminal proceedings and a punitive outcome for the defendant whose sample was analysed.

For diagnostic, treatment and rehabilitative purposes, for example the analysis of samples from a clinical context to assess the cause of an intoxication or to determine if the sample donor has abstained from drug use within the previous few days. A positive analytical result in this context would not necessarily involve subsequent legal proceedings but might serve as a reliable indicator on which to base future medical treatment of the sample donor.

Because punitive action may be the consequence of positive analytical results, procedures and methods used must follow strict standards based on the principles of forensic toxicology. The generally recommended strategy is that an initial screening test should be performed to establish potential positive samples and this must be followed by a confirmatory test on such presumptive positive samples.

For the initial screening of specimens, laboratories should consider using immunoassay techniques such as radio immunoassay (RIA), enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA) and latex agglutination inhibition (LAI). This would provide a rapid means of eliminating negative specimens. A positive result with immunoassay should then be followed by confirmation analysis using a method based on a different chemical or physical principle like thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) or gas chromatography (GC).

B. General guidelines for the submission of specimens for drug detection

The purpose of these guidelines is to describe procedures that will fulfil the necessary criteria in order to guarantee optimum validity of results. As recommended by the Brussels Expert Group, urine is the sample of choice. Apart from being readily accessible by non-invasive procedures, practically all drug metabolites are excreted in urine and the metabolites can be detected for a longer period than in blood. The use of blood and other biological materials like hair, saliva and sweat for the present purposes, i.e. establishing the illicit consumption of drugs, has not yet been generally accepted.

In order to maintain the validity of analytical results in the forensic context, particular care should be given to the **supervision** of sample collection,

transportation and storage. Supervision must be done by trained personnel having a clear understanding of the legal implications of the procedure and this ought to be carried out by direct visual observation. Proper surveillance must be maintained at all times but every effort must be made to maintain the privacy and dignity of the individual. The supervisor should also ensure that no attempt is made to add contaminating or reactive substances to the urine. When it is necessary to transport samples to an analytical laboratory, security and a clearly established chain of custody must be maintained.

These guidelines are applicable to situations where the collection of urine samples is conducted at sites which are located away from the analytical laboratories. This situation may not apply to all countries or different geographical locations within the same country. The guidelines should therefore be adapted or modified to suit the local situation, for example, in the storage of samples; if freezer facilities are not available, the analyst should incorporate stability checks in his quality control programme.

C. Sample collection: detailed procedure

1. At the collection site

- The **collection site** is responsible for the collection, labelling, packaging and transporting of samples, ensuring that the collection and storage procedures have the proper documentation and necessary security methods.
- The collection site must provide all staff with sufficient training to understand the collection process and the significance of the laboratory results.
- The collection of specimens must be supervised and witnessed by trained authorized personnel.
- Suitable toilet facilities for the purpose of urine collection must be available before collection of urine is considered.
- The collection room must be surveyed for any substance which could be used to invalidate the sample and should be set up without soap dispensers or cleaning agents.

Possible ways to invalidate urine samples

Introducing various chemical substances to the sample. Table salt, detergents or some commonly used household items such as hypochlorite (bleach) can destroy the drugs or affect the assay to generate false negative results.

In certain circumstances, adding illicit substances to the urine to produce positive results.

Placing a pinhole in the bottom of the container which results in leaks.

Using a fluid filled bulb placed under the arm, with a tube leading to the genital area. The bulb can be squeezed to release water or other substances which could dilute or contaminate the urine.

Obtaining urine from friends not using drugs.

Scooping water from the WC into the collection container to dilute the urine.

- The urine samples should be collected in duplicate in two 50 ml bottles. Each bottle should be filled at least 2/3 full. Plastic containers and rubber stoppers should be avoided whenever possible as non-polar drugs and their metabolites such as the cannabinoids are very prone to absorb to some plastic and most rubber surfaces. If, for practical reasons, disposable plastic containers are used, individual laboratories should conduct tests to ensure that the plastic containers do not alter the composition or concentration of the drug(s) or metabolite(s) in the urine.
- Immediately after collection, the temperature (32°-38° C within 4 min) and the pH of the fresh sample should be measured and recorded. If adulteration is suspected the laboratory should be notified. In such cases a careful visual check (colour, precipitation, foam etc.) and the monitoring of creatinine (180 ± 80 mg/100 ml: "normal"; 10-30 mg/100 ml: "probably diluted"; < 10 mg/100 ml: "diluted") and specific gravity (1.007-1.035: "normal") is recommended [13, 14].
- The bottles should be securely stoppered, sealed and labelled. Steps should be taken to ensure that the integrity of the sample is maintained, for example, by use of a security seal consisting of sealing wax imprinted with a departmental seal or some other measure to indicate that tampering with the sample has occurred. It is important that the donor witness the sealing of the bottles and sign or initial the seal or label.
- Specimen labels should be affixed to the urine containers and not to the lids. This will prevent accidental or intentional switching of specimens and/or identifying labels.

The label should contain at least the following information:

Name: _____

I.D number: _____

Date and time of collection: _____

Place of collection: _____

Name of person supervising the collection: _____

Drug(s) to be tested for: _____

Sample number: _____

- Personal details of each specimen donor are filled in a request form for analysis. This form will accompany the specimen to the laboratory.
- The specimen donor should not be allowed to have any involvement in the post-collection handling of the sample-labelling, packing and transportation to the laboratory.
- Strict security should also be observed in the storage and dispensing of empty cups, request forms, labels and packing materials.

2. *Transport and storage*

- After the request form has been completed, the sample and request form are given to the dispatch person for transmission to the laboratory. Samples should be protected from direct light and heat during transportation and storage and should therefore be kept cold during transport, preferably in an insulated box containing ice or some other cooled packing.

  It is important that samples are kept cool and in the dark for the entire period between collection and analysis.

- The designated dispatch person is responsible for transporting the samples to the laboratory and maintaining appropriate chain-of-custody records for ensuring that the samples are not tampered with during transit.

3. *At the laboratory*

- At the laboratory an authorized person should receive and carefully check the samples and documents. One bottle of each urine sample should be used for analysis and the other stored frozen for further analysis if necessary.
- After ensuring that the samples and request forms are in order, a written acknowledgement of receipt should be made, signed and given to the dispatch person.
- The laboratory should maintain well-documented records and strict security to ensure integrity of samples and confidentiality of the results.
- If the analysis is delayed beyond one or two days, urine specimens should be stored frozen, if possible in a locked refrigerator. When frozen, specimens will generally be stable for several months.

4. *Request form for drug analysis*

- A drug analysis request form which accompanies the urine samples will allow the laboratory to check the individual urine samples against the form to confirm the identity of the donor and that all specimens collected have actually reached the laboratory.
- The form should contain, at least, identification data of the donor, of the person supervising the collection and of the dispatch person, sample number, date and time of collection, and temperature and pH of the sample at the time of collection.
- Additional information which may be included on the form is a specification of the drugs for which the sample is to be screened and a note of any suspicions concerning the validity of the sample.
- After completion the form should be signed by an authorized person and stamped with an official seal.

D. Confidentiality of results

It is important to maintain complete security and confidentiality at all times.

- Any information related to the donor and to the results of the analyses must be kept locked and secure.
- Reports should be accessible only to authorized persons.

E. Safety of laboratory personnel

The handling of biological materials exposes personnel to infection hazards from, amongst others, hepatitis and AIDS. All personnel should therefore take the necessary precautions and adhere to safety procedures such as wearing gloves and other protective clothing.

F. Summary of security precautions

- In addition to the samples, strict security should also be observed in the storage and dispensing of empty cups, request forms, labels and packing materials.
- The specimen donor should not be allowed to have any involvement in the post-collection handling of the sample labelling, packing and transportation to the laboratory.
- It is important that the donor witness the sealing of the bottle and sign or initial the seal or label.
- Accurate and complete records of all individuals involved in the urine collection, storage and transport should be maintained.
- Specimen labels should be affixed to the urine container and not to the lid. This will prevent accidental or intentional switching of specimens and/or identifying labels.
- Information on sample donors and results of analyses should be kept strictly confidential and be accessible only to authorized persons.

G. Methodology

As recommended earlier, both screening and confirmatory tests are required.

A screening test should be able to identify potential positives with a high degree of reliability and should be sensitive, rapid and inexpensive. These criteria are generally met by immunoassays. However, the antibodies used in immunoassays have relatively low specificities and may result in cross-reactivity. All positive results obtained by an immunoassay screen should be confirmed by a second assay based on different chemical principles. Thin layer chromatography (TLC) may also be used as a screening test.

Confirmatory tests should be at least as sensitive as, but more specific than, screening tests. They generally involve chromatographic techniques and may include TLC, gas-liquid chromatography (GC), high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC/MS).

1. Immunoassay methods

Immunoassays are the methods of choice when large numbers of samples must be assayed within a limited time. Several immunoassay kits are available commercially for the screening of drugs of abuse. The most commonly used methods are

radio immunoassay (RIA), enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA) and latex agglutination inhibition (LAI). RIA, EIA, FPIA, and LAI (instrumental, online version) require instrumentation that is relatively expensive. Any of the above assays may be used by laboratories which have access to them.

The choice of technique would depend in most instances on the workload (number of samples/day) handled by the laboratory. The EIA and RIA techniques for instance are available in either the single- or multi-test versions. For laboratories with small numbers of samples, the single-test versions, or LAI (non-instrumental, onsite version) may be utilized but are expensive when considered in terms of the cost of analysis per sample. For large workloads, the multi-test enzyme immunoassays or FPIA are more appropriate.

Adequate consideration must be given to equipment maintenance, environmental control (temperature stability), and supply and (cold) storage of relatively unstable reagents to minimize inaccuracies in results. False results may also be the consequence of specimen adulteration, for example by addition of pH-modifying (vinegar, ascorbic acid, lemon juice, lime solvent etc.), oxidizing (sodium hypochlorite), surface-active (detergent, soap etc.) and enzyme-desactivating (glutaraldehyde) agents, medicaments (such as tetrahydrozoline containing eye or nose drops), sweeteners (saccharin) and sodium chloride. Most popular manipulations are endogenic (excessive drinking, use of diuretics) and exogenic dilution (addition of water) as well as exchange or substitution of urine specimen.

Training and experience requirements may be less for some immunoassay techniques, which facilitates laboratory staffing, but supervising analysts with extensive experience of the techniques should be present.

Some of the characteristics of the main immunoassays are summarized in table I.1.

Table I.1 General characteristics of the main immunoassays

Feature	*RIA*	*EIA*	*FPIA*	*LAI*
Requirement for special instrumentation	Yes	Yes	Yes	No[c]/Yes[d]
Stability of reagents	3-4 weeks	Months	Months	>1 year[c,d]
Costs of reagents	+	+++[a]/++[b]	++(+)	+++[c,d]
Possibility of automation	Yes	Yes	Yes	No[c]/Yes[d]
Tests per technician per 8 h shift	200-400	100-400[b]	250-300	200-350[c]/>500[d]

[a]Single test (st).

[b]Drug abuse urine assay (dau), multi-test.

[c]Non-instrumental onsite test.

[d]Instrumental online test.

2. *Thin-layer chromatography*

Thin-layer chromatographic methods are inexpensive in terms of capital equipment and other initial set-up costs. They are labour-intensive, generally less sensitive than other techniques and require considerable experience for accurate application due to the subjective nature of their interpretation. They are recommended as a confirmation assay for immunoassay screening results and as the primary test where labour expenses are of less importance than capital outlays, but where adequately trained staff are available.

In situations where resources limit the laboratory to TLC methodology alone, the assay result should not be used as the sole proof of drug presence or use when the consequences impact severely on the individual. In the absence of more sophisticated equipment, an acceptable solution can be a confirmation using at least one alternative TLC solvent system and/or detection reagent.

3. Gas chromatography and high performance liquid chromatography

Gas chromatography and high performance liquid chromatography offer high sensitivity and specificity for confirmation of presumptive positive results in screening assays. The equipment is however, relatively expensive in comparison to TLC or immunoassay and training and experience for these highly technical systems are critical.

4. Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry is the most sensitive and specific method available for confirmation of drug presence in a biological specimen. It requires the greatest outlay in capital, training and maintenance costs. It is the method least likely to be challenged in court and should be considered as a necessary and important asset in national programmes where the control laboratory will be final source of confirmation for questioned assays.

5. Sample preparation

In general, very little sample preparation is required for initial immunoassay tests. It is unnecessary to hydrolyse the urine samples because immunoassays measure both the free and conjugated forms of the drug and/or metabolites. It may be necessary to adjust the pH or centrifuge the urine to remove turbidity. For optimum results, the manufacturer's instructions should be followed.

For chromatographic procedures, good sample preparation is extremely important. This is necessary because urine is a complex matrix containing a mixture of large amounts of numerous organic and inorganic compounds in which the specific target analytes are found in minute amounts. Sample preparation usually involves hydrolysis of urine and the extraction and purification of the analytes. The procedure should be efficient, since a good recovery is necessary to extract the small amounts present, and selective, to ensure that interfering substances in the urine are removed.

Sample preparation for GC and GC/MS often involves the preparation of chemical derivatives of the target analytes. Although this additional step may require additional time and expense because of the reagents used, nevertheless derivatisation is frequently recommended for the following reasons:

It provides greater sensitivity.

The derivatized compounds are thermally more stable than the underivatized forms.

Chromatographic properties are improved i.e. peak shape, retention times and separations.

The mass spectra contain ions which are more suitable for GC/MS in the selected ion monitoring mode (SIM) than those of the underivatized forms.

6. *Quantitative analysis*

For the purpose of establishing the illicit use of drugs, it is not absolutely necessary to use quantitative analytical methods. However, there are many advantages in measuring the quantities of drugs and their metabolites identified in the screening method(s), particularly with respect to problems of interpretation.

Chromatographic methods generally give reliable quantification of analytes. TLC methods may be used as a quantitative procedure but would require a plate scanner or densitometer and may not be reliable or cost effective. Also, immunoassay methods generally do not give reliable quantification in this context because of the inherent possibilities of unidentified cross-reacting substances being present in the sample.

Quantitative analysis by GC, HPLC or GC/MS requires an internal standard to be added to the sample prior to extraction. An internal standard also permits the measurement of relative retention times. Internal standards should resemble the target analytes such that they can be extracted, derivatized and analysed under the same conditions as the target analytes, but be readily distinguished from them during the chromatographic procedure. Care must be taken, however, to avoid using substances which might occur in urine such as other drugs or endogenous materials.

For quantitative analysis performed by GC/MS a deuterium-labelled analogue of the analyte is usually the best choice for internal standard. However, deuterium-labelled analogues are expensive and may not be readily available. Other analogues of the target compound are also generally satisfactory internal standards.

If the decision is taken to establish quantitative methods, an immediate consequence is the need to validate the method with respect to accuracy and precision, as discussed below. The coefficient of variation of a chromatographic method should certainly be less than 10% and preferably less than 5%.

The concentration of an analyte can be calculated using the general formula:

$$\text{Concentration of analyte } X = \left[\frac{A_X/A_{IS} \text{ in sample chromatogramme}}{A_{RS}/A_{IS} \text{ in standard chromatogramme}} \right] \bullet C_{RS}$$

Where:

A_X = Peak area for analyte X obtained from the sample chromatogramme

A_{IS} in sample chromatogramme = Peak area of internal standard obtained from the sample chromatogramme

A_{RS} = Peak area for reference standard obtained from the standard chromatogramme

A_{IS} in standard chromatogramme = Peak area of internal standard obtained from the standard chromatogramme

C_{RS} = Concentration of analyte X in the reference standard solution

H. Quality assurance

Properly trained and skilled personnel are basic requisites for reliability of results. Adherence to good laboratory procedures and practices (GLP), standard operation procedures (SOP) and regular retraining of staff will help maintain quality and reliability of the laboratory.

1. Internal quality control

A good and well-documented quality assurance programme must be an integral part of the drug laboratory set-up and it should at least incorporate some means of assessing the accuracy and precision of all analyses done. The precision of methods should be assessed either by multiple analyses of individual samples and/or the inclusion of a sufficient number of quality control specimens (with different concentrations of the drug or metabolite in the relevant body fluid). This will enable the analyst to conduct statistical evaluations of precision within batches and over a period of time.

2. External quality assessment

Where possible, the laboratory should participate in an external proficiency programme. Ideally, such a programme should be conducted by an independent external agency such as the United Nations and laboratories in Member States should be invited to participate. In the absence of such a programme, laboratories within a country can adopt the following strategy:

Inter-laboratory proficiency programme: this is done by laboratories submitting samples to each other for analysis and checking on each other's performance.

The main central laboratory should be designated as the reference laboratory. This centre should send samples containing different concentrations of the analyte(s) to all laboratories for analysis. Results of analyses would then be evaluated by the reference laboratory.

I. Interpretation of results

The qualitative or quantitative analysis of a biological sample will provide evidence that a subject has or has not used a controlled drug. The presence of metabolites can show that a drug has been absorbed into the body.

A positive result on initial screening means that a drug or metabolite is present in the urine at a level above or equal to the cut-off level. Elimination from the body and drug concentrations in urine depend on such factors as the route of administration, frequency and duration of use, function of organs, rate of drug metabolism, subject's physical condition, age, sex, food, collection time, endogenous dilution etc. It is important to note, however, that the concentration of drug in urine can in no way be related to the level of mental impairment.

II. Recommended methods for the detection and assay of heroin (morphine) in biological specimens

A. Common types of illicit opium, morphine and heroin products [7, 8]

1. Opium

Opium is a natural product, obtained by incision of the unripe poppy capsules. The milky latex which oozes from the incisions is scraped by hand and air dried to produce the opium gum. Raw opium is a complex mixture containing sugars, proteins, lipids, other gummy substances and water such that the active alkaloid fraction makes up only 10-20% of the total weight. About 40 alkaloids have been reported. Four or five of these can be considered as the main constituents, falling into two general categories, the phenanthrene alkaloids, represented by morphine, codeine and thebaine, and the isoquinoline alkaloids represented by papaverine and narcotine (noscapine).

The relative amounts of the different alkaloids can vary greatly depending on such factors as the climate, the altitude, the fertility of the soil, the amount of available moisture, the age of the plant, the time of lancing and the variety of *Papaver somniferum.*

Morphine is the principal alkaloid of opium, ranging in concentration from 4-21%, the usual range being 8-14%. Licitly produced raw opium, known as Indian Opium, contains not less than 9.5% morphine, calculated as anhydrous morphine.

Narcotine is the second most abundant alkaloid, usually present in 2-8%. It is non-narcotic and sometimes is present in crude morphine as an impurity.

Codeine is found in raw opium in concentrations ranging from 0.7-3%. Its presence, as an impurity in crude morphine used for the preparation of heroin, results in the formation of acetylcodeine.

Thebaine is a minor alkaloid in *Papaver somniferum,* present at the 0.2-1% level.

Papaverine is usually present at the 0.5-1.3% level.

Another characteristic substance for opium is meconic acid, present in amounts up to 15%. Depending upon the extraction technique, this influences the purity of the crude morphine isolated from opium.

(a) Raw opium

When fresh, raw opium is a sticky tar-like dark brown substance which can be moulded into any shape or form depending upon the method of packaging and country of origin. As it ages it loses its consistency, becoming brittle and hard. It

has a characteristic licorice-like odour which intensifies when the product is dissolved in water. It is a non-homogeneous substance containing poppy capsule fragments and sometimes is adulterated with banana pulp or rosin. It is usually wrapped in vegetable leaves in the first instance followed by plastic wrapping tied with string.

(b) Prepared opium

Prepared opium, also known as "chandu" in South East Asia, is a product obtained by the application of various methods to raw opium including water extraction, filtration and evaporation of the water. This treatment is carried out in order to obtain a product suitable for smoking.

(c) Opium dross

The product that remains in the pipe after opium has been smoked is called opium dross. Because of incomplete combustion and volatilization the product still has some characteristics of opium including the presence of morphine in considerable amount. Mixtures of opium dross with raw and prepared opium have been reported in South East Asia.

(d) Medicinal opium

Medicinal opium, also called powdered opium, is opium that has been dried at a moderate temperature, reduced to a fine or moderately fine powder and whose morphine content is adjusted to the parmacopoeial requirement of 9.5-10.5% by the addition of powdered lactose, cocoa husk or rice starch. It is usually a light brown powder, consisting of yellowish-brown or reddish-brown particles and has the characteristic odour of opium.

From a chemical point of view, the differences between all of the opium products mentioned above are relatively small.

2. Crude morphine

The crude morphine obtained on the illicit market, can be of very high or very low quality, depending upon the purification procedures used, the indended purpose of the material and the habits, knowledge and professional skill of the illicit chemist.

3. Heroin

It must be stressed that no two heroin samples have exactly similar physical appearances. Produced from a highly variable natural product, by a batch process capable of wide variation, and subsequently subjected to adulteration and transformation for trafficking purposes, it is not surprising that heroin occurs in such a multitude of forms. Those listed here are just a selection, albeit the most common. Because material submitted for forensic examination bears no physical relationship to any type described here, that does not mean, of course, that it is not heroin or a heroin containing product.

(a) Two types of South West Asian heroin

Type 1: Very variable in colour and consistency and has been encountered in virtually every shade from beige to dark brown. It is virtually always a powder, often fine, but occasionally small aggregates are present in the powder. These are soft and yield to slight pressure. This category constitutes by far the major type from this region. The physical variation is paralleled by a wide range of chemical composition but samples seized more recently indicate that a more consistent product is being made. Typically it is a fine light-brown powder with a characteristic opium-derived odour, the heroin purity is typically 60%, and all alkaloids and derivatives are present as the base. Typical contents of the other alkaloids and derivates are:

Acetylcodeine	5%
O^6-monoacetylmorphine	3%
Narcotine	10%
Papaverine	4%

Type 2: A white, off-white or creamy coloured fine dry powder, has less odour than type 1, the purity is in the range 80-90%, and the heroin is present as the hydrochloride salt. Some samples of this type are indistinguishable from "pharmaceutical grade" heroin. Typical contents of the other alkaloids and derivatives are:

Acetylcodeine	3%
O^6-monoacetylmorphine	2%
Narcotine	NOT DETECTED
Papaverine	NOT DETECTED

(b) Two types of Middle East heroin

Type 1: Beige coloured or very light brown coloured fine powders; rarely are aggregates present. Samples containing more than 70% heroin are rare and the average purity is about 50%. The alkaloids and derivatives are present as hydrochloride salts. The contents of the other alkaloids and derivatives are:

Acetylcodeine	3%
O^6-monoacetylmorphine	2%
Narcotine	NOT DETECTED
Papaverine	NOT DETECTED

This type very frequently contains an adulterant, often a pharmaceutical such as procaine.

Type 2: White or off-white coloured fine powders. Some samples contain 70-80% heroin, while other types would appear to be a diluted form of the high purity product, containing an equivalent amount of caffeine, such that the typical heroin level is reduced to 30-40%. The alkaloids and deratives are present as hydrochloride salts. These diluted forms contain only trace amounts of acetylcodeine, O^6-monacetylmorphine, papaverine and narcotine, but the highly pure forms typically contain:

Acetylcodeine	2-3%
O^6-monoacetylmorphine	2%

(c) Two types of South East Asian heroin

Smoking heroin "Chinese No. 3"

A hard granular material. The granules are usually 1-5 mm in diameter, and unlike the aggregates in South West Asian heroin they are hard and unyielding to pressure. Only a small amount of powder will be present in the material. The most frequent colour of the material is grey, although dirty brown frequently occurs and there is a special variation when the granules are red or pink coloured—"Penang Pink". A typical assay will be:

Grey or dirty brown material—heroin 20%, caffeine 40%, trace amounts of the other alkaloids in freshly made material, although in this type of heroin as much as 5% O^6-monoacetylmorphine may be quickly formed by hydrolysis. The alkaloids may be present as hydrochloride salts or bases; some samples would appear to consist of both salt form and base form, that is to say that hydrochloric acid has not been added in a stoichiometric amount.

Red or pink material—assay similar to grey or dirty brown material but barbitone replaces caffeine.

Injecting heroin "Chinese No. 4"

A fine white powder with little odour and no aggregates. Virtually the entire material will consist of heroin. Narcotine and papaverine are not detectable; O^6-monoacetylmorphine usually below 3%. The acetylcodeine content is usually appreciably higher than in the equivalent high purity product from South West Asia. All alkaloids are present as hydrochloride salts.

For all the types of heroin of whatever origin, it must be noted that O^6-monoacetylmorphine levels occasionally are higher than those quoted. Poorly manufactured heroin samples frequently hydrolyse with heroin converting to O^6-monoacetylmorphine. Non stoichiometric addition of (usually excessive) hydrochloric acid is the most frequent cause of such hydrolysis.

It is rare for the hydrolysis to result in high morphine contents—at least in illicit heroin in solid form. High morphine contents in recently seized material are most likely an indication of poor manufacturing procedure.

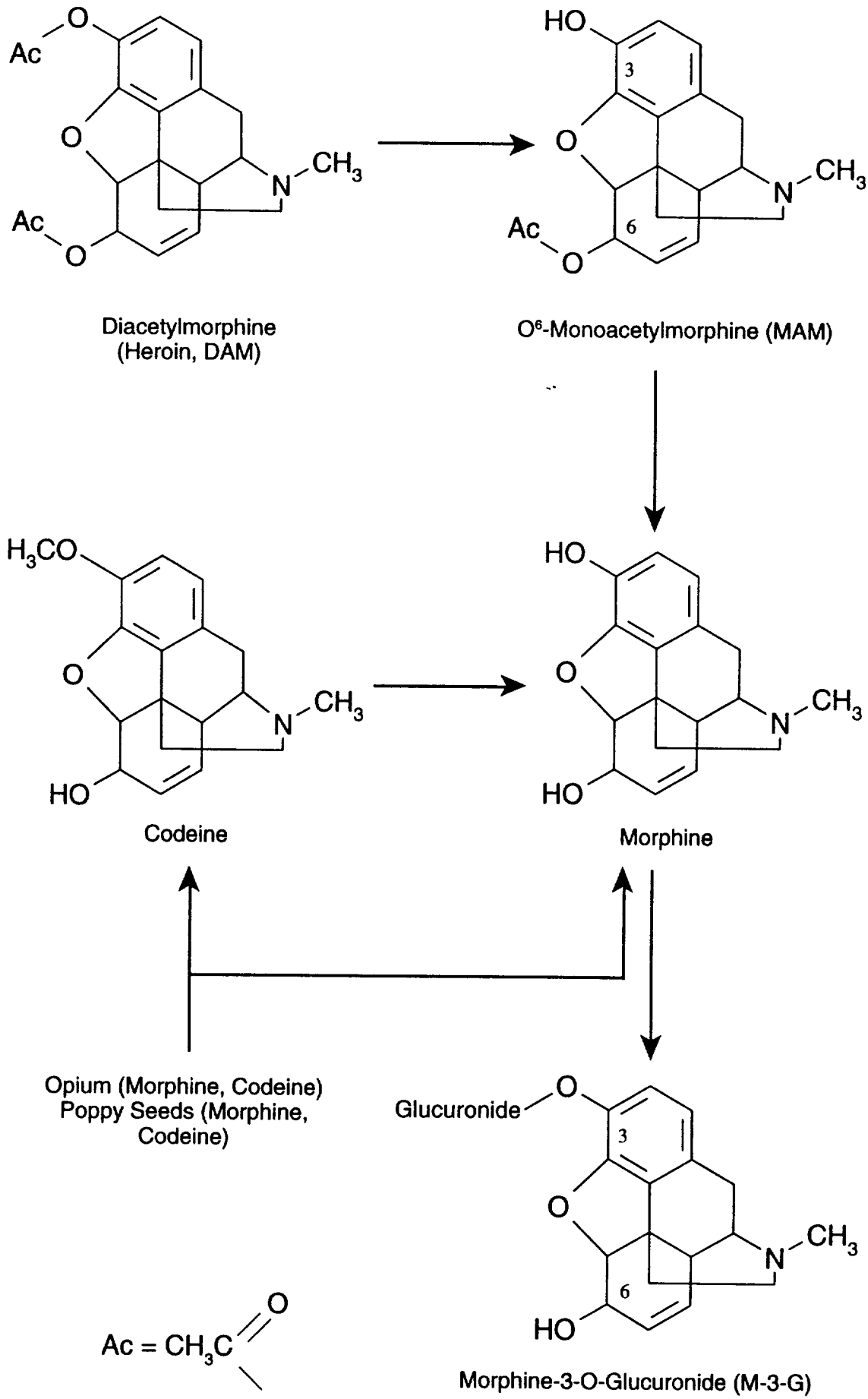
In the laboratory analysis of various biological specimens for heroin or related opiates such as morphine and codeine, the following compounds are of relevance (for structures see figure II.1):

Heroin (Diacetylmorphine, DAM)
Morphine
O^6-Monoacetylmorphine (MAM)
Codeine
Acetylcodeine.

In addition, the various morphine-O-glucuronides (see figure II.1) are of prime importance for the analyst, since the major portion of heroin is excreted in urine as conjugated morphine in the form of:

Morphine-3-O-glucuronide (M-3-G)
Morphine-6-O-glucuronide (M-6-G)
Morphine-3,6-O-diglucuronide.

Figure II.1 Metabolic Pathway of Opiates



4. *Routes of administration*

Heroin is administered in several ways. These include sniffing or snorting, smoking, subcutaneous injection and intravenous injection. When administered by injection, the powder is first dissolved in water often assisted by heating and by acidifying.

5. *Metabolism and excretion*

Following intake, heroin is rapidly deacetylated to MAM [15, 16], which is then further hydrolyzed to morphine at a slower rate. The metabolic pathways are shown in figure II.1. The major metabolites of heroin found in urine up to 20-40 h after intravenous administration are M-3-G (38.2% of dose), free morphine (4.2%), MAM (1.3%) and unchanged heroin (0.1%). Other morphine glucuronides as well as normorphine [17] may be found as minor metabolites of heroin. Codeine has often been found in the urine of persons taking illicit heroin but this is not a metabolite of heroin. Rather it is the result of deacetylation of acetylcodeine which is often found as an impurity in illicit heroin.

Morphine concentrations in urine following therapeutic administration may be up to 10 μg/ml and in heroin overdose fatalities may be considerably higher, for example, a level of 86 μg/ml has been reported in one case [18]. Morphine (and codeine) is also excreted in urine after ingestion of poppy seeds [19].

Because of its rapid metabolism, it is not practical for heroin to be determined directly in human biological fluids (half-life 2-3 min [18]).This is usually done by the assay of morphine, its major metabolite in urine. Heroin use may also be confirmed by determining MAM in urine but this specific metabolite can only be detected soon after consumption of heroin (detection time: 2-8 h [19]) as it is also further metabolized relatively rapidly to morphine (half-life 0.6 h [19]). The detection of MAM requires a modified extraction procedure and more sophisticated instrumentation. In most cases this may not be necessary.

B. Sampling and sample preparation procedures for the assay of heroin metabolites

The general sampling procedures outlined in chapter I.C and G.5 apply.

1. *Sample preparation for immunoassay*

In general, little or no sample preparation is necessary for immunoassays (see also chapter I.G.5). It is unnecessary to hydrolyze the urine samples because immunoassays measure both the free and conjugated forms of the drug and/or metabolites. It may be necessary to adjust the pH or centrifuge the urine to remove turbidity. For optimum results, the manufacturer's instructions should be followed.

2. *Sample preparation for chromatography*

The volume of urine required for analysis depends on the chromatographic technique to be used. The recommended volume for TLC and packed-column GC is 10 ml and for other chromatographic techniques it is 5 ml.

(a) Hydrolysis

Acid hydrolysis

In a 50 ml quickfit tube, add 1 ml of concentrated hydrochloric acid to 10 ml urine, loosely stopper the test tube and incubate in a water bath at 100° C for approx. 60 min.

Enzymatic hydrolysis

Adjust the urine sample (5-10 ml) to pH 7 by adding acetic acid if found to be alkaline. Then add 0.1 ml of 0.1 M sodium acetate-acetic acid buffer (pH 5.5) and 0.02 ml of β-glucuronidase (75 units/ml) per ml of urine. Incubate for 24 h at 37° C or alternatively 1 h at 55° C. Make sure that the temperature does not exceed 55° C to avoid denaturing the enzyme. Proceed with the extraction of free morphine as described below.

Precaution

Acid and enzymatic hydrolysis may lead to desacetylation of MAM to morphine.

(b) Extraction

Liquid-liquid

Adjust the pH of the urine to 8.5-9.0 and extract with twice the volume of any of the following organic solvents:

Chloroform-isopropanol (9:1, v/v)
Dichloromethane-isopropanol (9:1, v/v)
Ethyl acetate.

Care must be taken to allow the aqueous layer to separate completely from the solvent layer before drawing off the extract in order to avoid carrying over any water. If problems with emulsions occur, silicone-treated filter paper (phase separating paper) can be used to filter the extract; brake the emulsion by sonication. When a cleaner extract is required, re-extract the organic solution with 6 ml of 0.5 M hydrochloric acid. Discard the organic layer and adjust the aqueous solution to pH 8.5-9.0 then re-extract with one of the above solvents. Separate the organic layers, combine, filter the solution through a small amount of dry sodium sulfate, wash the filter with 5 ml of the organic phase. Concentrate the solution to approximately 1-2 ml and evaporate the rest of the solvent under a stream of nitrogen to dryness. Redissolve the residue in 0.1 ml methanol or methanol-chloroform (9:1) for TLC or GC analysis.

Solid-phase

Where facilities are available, the celite-supported liquid extraction procedure can be used [20]. The following SPE method using silica C-18 is also recommended [21].

Before use, prewash the columns with 5 ml of methanol, 3 ml of distilled water and 1 ml of 0.05 M borax buffer (pH 9.0). Mix the urine sample (1 ml) with 50 ml (= 50 ng) of internal standard nalorphine and 1.0 ml of buffer (pH 9.0) and

transfer it to a silica C-18 column. Wash the column with 100 ml of 80% aqueous methanol, then elute the morphine with 0.5 ml methanol.

(c) Internal standards

Internal standards should meet the criteria given in Chapter I.G.6. Nalorphine is suitable for GC. For GC/MS, the preferred internal standards are deuterated analogues of morphine or of the related compounds. If these are unavailable, one of the GC standards indicated above should be used. An internal standard suitable for HPLC is *l*-α-acetylmethadol · HCl.

(d) Calibration standards

For GC prepare separate stock solutions of morphine and nalorphine in methanol at a concentration of 1 mg/ml. From these stock solutions, prepare aqueous calibration standards containing morphine in the range 0 to 10 μg/ml morphine plus 5 μg/ml nalorphine.

For HPLC, *l*-α-acetylmethadol · HCl, DAM, MAM and morphine stock solutions at a concentration of 1 mg/ml are prepared in methanol and stored in a refrigerator. Calibration standards are prepared by diluting the stock solution with methanol-acetonitrile (20:80, v/v).

C. Screening methods

1. Immunoassay methods

It is recommended that where laboratories have access to these techniques, immunoassays should be used for initial screening. Radioimmunoassay, enzyme immunoassay, fluorescence polarisation immunoassay, and latex agglutination inhibition are recommended. Antibodies of the commercial immunoassay kits are targeted towards morphine but may cross-react with other opiates [22]. A summary of cross-reactivities of some commercial assays is given in table II.1.

Table II.1 Cross-reactivities of commercial immunoassays for morphine

	Cross-reactivity (%)			
Assay	*Morphine*	*MAM*	*M-3-G*	*Codeine*
Coat-A-Count	84 (300)[a,b]	0 (100)	1 (618)	0.1 (600)
Abuscreen-RIA	85 (300)[a,b]	15 (100)	52 (618)	198 (300)
EMIT-d.a.u.	86 (300)[a,b]	16 (100)	45 (618)	330 (300)
FPIA-TDx	90 (300)[a,b]	93 (50)	64 (185)	111 (300)
Abuscreen-Ontrak	100 (300)[c,d]	—	86 (350)	171 (175)
Abuscreen-Online	100 (300)[c,d]	97 (311)	62 (480)	134 (255)

[a]Apparent cross-reactivity calculated by dividing the apparent concentration by the target concentration and multiplying by 100 (in parenthesis concentration, at which cross-reactivity was determined, ng/ml).

[b]See Edwards [14].

[c]Cross-reactivity calculated by dividing the target concentration (300 ng/ml) by the concentration equivalent to 300 ng/ml morphine and multiplying by 100 (in parenthesis equivalents, ng/ml).

[d]Manufacturer's information.

2. *Thin-layer chromatography*

Standard TLC technique

Details of standard TLC materials and procedures are given in United Nations manual, *Recommended Methods for Testing Opium/Crude Morphine* [8] and these are applicable to the analysis of biological extracts for morphine.

TLC plates

Coating:	Activated silica gel G. Silica gel containing an additive which fluoresces under UV light, wavelength 254 nm, can also be used
Layer thickness:	0.25 mm
Size of plates:	Glass plates 20 × 20 cm, 20 × 10 cm or 10 × 5 cm. The optimum run is approximately 10 cm

Standard solutions

Morphine.
Codeine.
Make all standard solutions at a concentration of 1 mg/ml in methanol and apply 5-10 µl of each solution to the plate. Either salt or base can be used as the compounds always move on the TLC plates as the free bases.

Procedure

Spot 25 and 50 µl of the extract (see chapter II.B.d.) on the plate which is then developed in one of the solvent systems below.

Developing solvents [8, 23]

System A:	Toluene	45
	Acetone	45
	Ethanol	7
	Conc. ammonia	3
System B:	Ethyl acetate	85
	Methanol	10
	Conc. ammonia	5

Visualization

The plates must be dried prior to visualization. This can be done at room temperature, or, more quickly, in an oven of 120° C for 10 min, or by the use of a hot air blower. It is important for proper colour development that all traces of ammonia or other bases be removed from the plate. The following visualization methods are recommended [7, 8, 24]:

UV light at 254 nm.

Dragendorff's reagent.

Mix together 2 g of bismuth subnitrate (bismuth oxynitrate), 25 ml of concentrated (glacial) acetic acid and 100 ml of water to produce solution A; dissolve 40 g of potassium iodide in 100 ml of water to produce solution B. Mix 10 ml

of solution A, 10 ml of solution B, 20 ml of concentrated (glacial) acetic acid and 100 ml of water to produce Dragendorff's reagent.

Acidified potassium iodoplatinate reagent.

Dissolve 0.25 g of platinic chloride and 5 g of potassium iodide in water to 100 ml. This is potassium iodoplatinate reagent. To obtain the acidified version add 2 ml of concentrated hydrochloric acid.

Fluorescence reagent [24].

(i) AMP buffer: Add 105 mg of 2-amino-2-methyl-1,3-propanediol to 18.8 ml of concentrated hydrochloric acid and dilute with water to 1000 ml (pH 9.3 ± 0.2).

(ii) Potassium ferricyanide solution: Dissolve 58 mg of potassium ferricyanide in 100 ml of distilled water and store in refrigerator (prepare fresh solution after 1 week).

First observe the plate under ultraviolet (UV) light. Morphine gives orange spots on a yellow background with the Dragendorff reagent, blue to purple spots when sprayed with the iodoplatinate reagent and fluoresces with the fluorescence reagent under UV light.

Results

Table II.2 $R_f \times 100$ values [8]

	Developing system	
Compound	*A*	*B*
Morphine	19	20
Codeine	40	35

D. Confirmatory chromatographic methods

1. Gas chromatography

(a) Sample derivatization

Silylation

The urine extract is evaporated to dryness under a stream of nitrogen and the residue is derivatized with 20 µl *N,O-bis*-trimethylsilyltrifluoroacetamide (BSTFA) or *N,O-bis*-trimethylsilylacetamide (BSA) in a closed vial by heating at 85° C for 15 min. Alternatively, a mixture of the silylating reagent and pyridine (1:1, v/v) may be used instead of the pure silylating reagents. The mixture is injected directly into the chromatograph.

If a nitrogen-phophorus detector (NPD) is to be used, silylation may be carried out using a volatile silylating reagent such as *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) or a mixture of hexa-methyldisilasane (HMDS), trimethylchlorosilane (TMCS) and pyridine. The derivatized extracts can be evaporated to dryness and the sample reconstituted in a dry solvent such as toluene prior to injection (1-2 µl) on to the GC column.

The derivatives should be prepared shortly before analysis since silyl derivatives are not very stable.

Acylation

Add 50 µl pentafluoropropionic anhydride (PFPA) to the urine extract and heat the mixture for 30 min at 65° C in a sealed tube. Evaporate the excess PFPA reagent using a stream of nitrogen and reconstitute the residue with 50 µl ethyl acetate. The derivatives are stable in the reagent for months and after evaporation of the reagent for at least 24 h.

(b) Packed column technique [8]

Operating conditions

Detector: FID

Note: *For better sensitivity and specificity, a nitrogen-phosphorus detector (NPD) is recommended: operating parameters should be in accordance with the recommendations of the manufacturer. Sample preparation and derivatization procedures should be chosen which avoid nitrogen-containing solvents and reagents in the final solution injected into the chromatograph.*

Column:	2 m × 2-4 mm I.D.	
Packing:	*(a)* Dimethyl silicone (SE-30, OV-1)	
	(b) Phenylmethyl silicone, 50% phenyl (OV-17)	
Carrier gas:	Nitrogen at 70 ml/min	
Operating temperatures:	Injector:	275° C
	Oven:	230° C
	Detector:	275° C

Note: *Prior to use, all packed columns must be conditioned. Usually the conditioning temperature should be at least 30° C above the temperature at which the analysis is to be performed, unless this would require exceeding the upper temperature limit of the column as specified by the manufacturer. In this case, a smaller temperature differential must be used and the conditioning period substantially extended. Typically, columns are conditioned overnight, or for a minimum of 15 h.*

Conditioning is carried out with the normal carrier gas flow and with the column disconnected from the detector.

Note:

- *Silanize glass columns frequently to avoid adsorption of morphine during GC determinations.*
- *Clean injection port and detector regularly to avoid decomposition of samples and loss of detector sensitivity.*
- *Handle silylating reagents with care. Silylating reagents are very reactive and sensitive to moisture.*

(c) Megabore column technique

Operating conditions

Detector:	FID
Column:	Fused silica, 10 m × 0.53 mm I.D. with 2.6 µm chemically bonded dimethylsilicone stationary phase, e.g. OV-1

Carrier gas:	Helium at 25 ml/min	
Operating temperatures	Injector:	280° C
	Oven:	260° C
	Detector:	300° C

Note: *An appropriate capillary column procedure is also described in the United Nations manual on* Recommended methods for testing opium/crude morphine *[8]. The capillary column dimensions, stationary phase, phase thickness, carrier gas and flow rate used may be varied from those cited above depending on availability. However, a non-polar column is of general applicability for the analysis of biological samples and is recommended. The optimum operating conditions for the system should be selected according to the recommendations of the supplier.*

2. *Gas chromatography-mass spectrometry*

(a) Qualitative analysis

Operating conditions

Column:	Fused silica, 25 m × 0.31 mm I.D. with 0.17 μm cross-linked 5% phenylmethyl silicone stationary phase	
Carrier gas:	Helium at 1.8 ml/min	
Operating temperatures:	Injector:	280° C
	Oven:	230° C
Ionization:	EI mode at 75 eV	

The principal ions in the mass spectra of morphine and nalorphine trimethylsilyl derivatives, used for selected ion monitoring mass spectrometry, are given in table II.3 below.

Table II.3 Principal ions in the mass spectra of morphine, MAM, codeine and internal standards (TMS and TFA derivatives, SIM)

Compound	*Principal fragment ions m/z*
Morphine-diTMS	414, 429
Nalorphine-diTMS	441, 455
Morphine-diTFA	364, 477
d_3-Morphine-diTFA	367, 480
MAM-TFA	311, 364, 423
d_3-MAM-TFA	367, 426
Codeine-TFA	282, 395
d_3-Codeine-TFA	285, 398

(b) Quantitative analysis [25]

Operating conditions

Column:	Fused silica, 12 m × 0.2 mm I.D. with 0.33 μm cross-linked 100% dimethylpolysiloxane stationary phase

Carrier gas:	Helium at 1.9 ml/min	
Operating temperatures:	Injector:	250° C
	Oven:	150-300° C at 12° C/min
Injection technique:	Splitless	
Ionization:	EI mode at 75 eV	
Ions:	SIM, TFA derivatives (see table II.3 above)	

Quantitation

One point calibration using the ion-ratios of the analytes and its corresponding deuterated internal standards. Codeine 395/398; morphine 364/367; MAM 423/426.

Extraction

1 ml of urine is adjusted to pH 7.0 by adding 3 ml of pH 7.00 buffer. 100 µl of a 1 mg/ml solution of d_3-codeine, d_3-morphine and d_3-MAM is added and the mixture vortexed. The urine is passed through a SPE column (Bond-Elut Certify) pre-conditioned with 3 ml of methanol and 3 ml of distilled water. The column is sequentially washed with 3 ml of water, 3 ml of 0.1 M sodium acetate buffer (pH 4.5) and 3 ml of methanol. After drying the column (vacuum, 1-2 min) the analytes are eluted with 3 ml of dichloromethane-isopropanol-conc. ammonia (80:20:2) (freshly prepared).

Derivatization

TFA derivatives: The residue of the evaporated eluent (N_2, 50-60° C) is reconstituted with 200 µl of chloroform and 100 µl of trifluoroacetic anhydride (TFAA), vortexed and heated at 70° C for 15 min. After cooling and evaporating to dryness (N_2, 50-60° C) the residue is redissolved in 100 ml of chloroform and a 2 µl-aliquot injected in the GC/MS.

3. High performance liquid chromatography

Several methods for the assay of morphine using HPLC have been published. Various detection techniques such as UV absorbance and fluorescence have been used. Some of them have the disadvantage of low sensitivity or tedious sample preparation. Electrochemical detection provides a sensitive and practical alternative. Two methods are described below.

Operating conditions

Method A [16]

Column:	Silica (LiChrosorb Si-60 or equivalent), 5 µm, 30 cm × 4 mm I.D.
Mobile phase:	Acetonitrile-methanol-solution A-solution B (75:25:0.04:0.216, v/v/v/v)
	Solution A: Mix concentrated ammonia and methanol (1:2, v/v)
	Solution B: Mix glacial acetic acid and methanol (1:1, v/v)
Flow rate:	1.3 ml/min
Detection:	UV at 218 nm

Method B [26, 27]

Column:	Octadecylsilica reversed-phase, 5 μm, 25 cm × 4.6 mm I.D.
Mobile phase:	100 ml acetonitrile and 900 ml 0.2 M sodium perchlorate/ 0.005 M sodium citrate buffer (filter before use through 0.5 μm membrane filter)
Flow rate:	1.9 ml/min
Detection:	Electrochemical detection (ECD), glassy carbon electrode

Note: *For the determination of morphine-3-O-glucuronide (M-3-G) and morphine-6-O-glucuronide (M-6-G) in urine with HPLC and electrochemical or UV detection is referred to elsewhere [28, 29].*

E. GC/MS analysis of monoacetylmorphine as an indicator of heroin use

Several GC/MS methods are available for the detection and/or quantitative determination of nanogram amounts of O^6-monoacetylmorphine (MAM) in urine samples from heroin abusers. In one method [30], the detection of MAM is based on the solid-phase extraction from urine at alkaline pH, using an octadecyl column and subsequent transformation into the pentafluoropropionyl (PFP) derivative. Monoacetylmorphine pentafluoropropionate is separated and identified by GC/MS in the selected ion monitoring mode (SIM) using nalorphine or d_3-morphine as internal standards. Another GC/MS method [25] using TFA derivatives and SIM is described in chapter II.D.2. A positive identification of MAM can be used to distinguish between an intake of heroin and the abuse of morphine, codeine, opium or poppy seeds.

1. Sample preparation and derivatization

Note: *Do not hydrolyze urine samples before this analysis as both acid and enzymatic hydrolysis may result in the hydrolysis of MAM to morphine.*

Add 1 ml of buffer solution (pH 9) to 10 ml of urine in a 25 ml centrifuge tube. Check that the pH is between 8 and 9. A C-18 SPE column is used and is conditioned by washing sequentially with 5 ml of methanol and 5 ml of distilled water. Pass the urine sample through the column.

Wash the column twice with distilled water. Add a drop of concentrated ammonia then wash the column again with distilled water. Dry the column by pulling air through it (5 min). Recover MAM and morphine by eluting the column twice with 0.75 ml of dichloromethane-acetone (1:1, v/v). Evaporate the eluate in a 2 ml test tube at 60° C. Dissolve the residue in 100 μl dichloromethane-acetone (1:1, v/v) and transfer the solution to a 1.5 ml tube. Evaporate under a gentle stream of nitrogen at 65° C.

Add 50 μl of pentafluoropropionic anhydride (PFPA). Keep mixture for 30 min at 65° C in a sealed tube. Evaporate excess PFPA reagent using a stream of nitrogen. Reconstitute the residue with 50 μl of ethyl acetate. Inject 1 μl into GC/MS.

An alternative technique, using simple solvent extraction is described elsewhere [31].

2. *Operating conditions*

The conditions given earlier (chapter II.D.2) are applicable. For the detection of the PFP derivatives of morphine, codeine and MAM, the following ions are monitored: m/z 361, 414, 445, 473 and 577. The ion at m/z 603 is used for the internal standard.

F. Interpretation of results

A positive result in an initial immunoassay screen means that an opiate is present in the urine at a level above or equal to the cut-off level and should be confirmed by a method which is sensitive but more specific than the initial test. Retention of opiates in the body and the actual drug concentrations in urine depend on such factors as drug metabolism, the subject's physical condition, fluid intake and manner of ingestion. In general, by using the above approach, opiates may be detected in urine for up to three days.

Because they share common metabolic pathways, heroin, opium, codeine or morphine itself may be sources of morphine and morphine-3-glucuronide in urine. In addition, other opiates such as ethylmorphine, pholcodine and nicomorphine may also be sources of morphine [18]. Hence, the presence of morphine in urine in itself does not indicate which opiate was consumed.

Where analytical results raise doubts about the source of the morphine, detection and/or quantitation of the parent compound and the pattern of excretion of other major metabolites can give more precise information concerning the drug taken. For example, the detection of MAM can be considered as proof of heroin consumption [19, 30, 32].

In the case of codeine, although the subject remains to some extent contentious, it is generally accepted that if the total codeine to total morphine ratio is less than 0.5 and the total morphine concentration in urine is greater than 200 ng/ml, codeine may be excluded as the source of the morphine present [33, 34].

III. Recommended methods for the detection and assay of cannabinoids in biological specimens

A. Common types of cannabis products [9]

1. Herbal products (marijuana)

Cannabis *(Cannabis sativa L.)* is a plant widely distributed throughout the temperate and tropical zones of the world, and most countries have reported illegal growth and traffic of the herbal products. Large scale illicit cultivation of the cannabis plant to make herbal cannabis products occurs in North and South America, the Carribean, Africa and South East Asia. The presentation of the herbal material in the illicit traffic varies not only from region to region, but also within the countries of each region.

It is the traditional belief that only the fruiting and flowering tops and leaves of the cannabis plant contain significant quantities of the psychoactive constituents (e.g. tetrahydrocannabinol); they are known as the "drug-containing parts", and generally it is only these parts of the plant that are sold in the illicit traffic. These parts may be stripped from the plant while it remains growing. The central stem and main side stems of the plant are not removed and play no part in the production of illicit cannabis products. Alternately the entire plant may be removed by cutting the main stem at a point below the lowest leaf-bearing side stems. The separated herbal material, or whole plants, are allowed to air dry, usually by being spread out on the ground or, if in relatively small quantity, by being placed in shallow trays. Whole plants can be dried while suspended upside down and when dried, the drug-containing parts of the plant are stripped from the central and main side stems. A wide range of herbal presentations are made depending on the process subsequently used on the dried material. The separated parts may be highly compressed to make blocks of herbal materials (West African and Caribbean cannabis is frequently trafficked in this form). Alternately the cannabis may be left as a loose herbal material (samples from some Central and Southern African countries and from countries of South West and South East Asia are often in this form). A less frequently encountered presentation is produced when the herbal material is rolled into a "corn-cob" shape and wrapped in coarse vegetable fibre (Central Southern Africa).

If a high quality product is to be made for trafficking the fruiting and flowering tops alone are used. They are most often made into sticks; frequently the fruiting and flowering top is tied using twine around a central bamboo cane. Such sticks weigh about 2 grams (gross), are approximately 8 centimetres long and are known in the illicit traffic as "Buddha-sticks" (South East Asia). Often when seized from the illicit traffic these sticks are found in bundles of up to 20 sticks. Alternately the fruiting and flowering top is often in a small roll wrapped in brown paper (South

Africa). These rolls are considerably smaller than the South East Asian type. Usually there is less than 0.5 grams of cannabis per roll, and there are few, if any, seeds within the material.

A high quality product can be made by sieving herbal cannabis to remove those parts of the plant which contain relatively low levels of cannabinoids, or no cannaboids. Essentially, this removes seeds and all but the most insignificant stem material. All that passes through the sieving process has been derived from the flowering and fruiting tops of the leaves of the cannabis. The material resembles finely chopped herbal material. In the illicit traffic it is known as "Kif". It is a characteristic product of North Africa. Such material has a high cannabis resin content and can be compressed into slabs which bear *some* physical resemblence to cannabis resin slabs made in the same region. However, when subjected to microscopic examination, such slabs are found to have retained essentially herbal characteristics. This material whether loose or compressed into small blocks, has the same cannabinoid profile as cannabis resin slabs made in the same region.

An alternative high quality product is Sinsemilla. The word Sinsemilla derives from two Spanish words which mean "without seeds". Sinsemilla is produced by removal of male cannabis plants from the environment of female cannabis plants before the male plant has released its pollen. The female plants never become fertilized and therefore produce no seeds. It is claimed by those involved in the illicit cultivation of cannabis that the resin bearing parts of such plants contain a higher level of the psychoactive chemicals (e.g. THC) than ordinary female plants which have been allowed to become fertilized in the normal way. Forensic analysis would support this contention. Sinsemilla is found to contain higher levels of the cannabinoids, especially THC.

It is worth noting that the removal of male plants from the environment of female plants before fertilization has occurred, has been practiced for may years in, for example, the Indian subcontinent. It was known that if this was not done, the female plants would run to seed, and a very poor yield of "ganja" would be produced. Invariably, however, a few seed bearing flowering tops were present in such material. This may have occurred because cannabis is not entirely a *dioecious* plant. In any large field of cannabis plants, a number will be *monoecious,* that is bearing both male and female flowers.

Sinsemilla remains a product cultivated only in the Americas, although seizures of Sinsemilla have also been made outside the Americas. The seized material in these cases had, however, been cultivated within the Americas.

2. Resin products (hashish)

The production of cannabis resin is centred on two main regions of the world. The countries around the Southern and the Eastern part of the Mediterranean form one region, and the countries of the Indian subcontinent form another. A variety of processes have been used in both regions to make cannabis resin. However, in general, the countries of one region use similar techniques. This has resulted in two "families" of cannabis resin. Countries around the Southern and Eastern parts of the Mediterranean make one group of cannabis resin products, and the countries of the Indian subcontinent produce a second group of products. However, there is some similarity in the methods used to make cannabis resin in both regions, for example, there are methods in both regions in which sieving is an important part of the process.

Resin from a single country within either of these regions will show much more similarity in *physical* appearance to resin from another country of the same region, than it will to a resin from the other region. (There may be significant differences in the cannabinoid profile of resins from one region).

(a) Cannabis resin from Mediterranean countries

The herbal material is threshed, often against a wall. This process is done to separate the resin producing parts of the plant from those parts which do not produce resin, and are therefore low in psychoactive constituents. Particles of cannabis resin and of cannabis leaves, as well as cannabis seeds become detached from the more fibrous parts of the plant. The latter are discarded. The material is then sieved (seeds and minor fibrous parts are eliminated). The product remaining is now even higher in resin content. At this stage macroscopic herbal characteristics are virtually destroyed, but microscopically the material still exhibits many herbal traits. Physically it resembles a fine powder and at this stage it is compressed into slabs. In some countries (Eastern Mediterranean) the material is placed in cloth bags prior to compression, in other countries (North Africa) cellulose wrappings are added before compression. In one area (North Eastern Mediterranean) the material is trafficked occasionally as this fine powder without having been made into slabs.

(b) Cannabis resin from the Indian subcontinent

A different approach to the production of cannabis resin is used in the countries of the Indian subcontinent. The fruiting and flowering tops of the cannabis plants grown in the countries of the Indian subcontinent contain high levels of resin, to an extent that makes these parts of the plant sticky to the touch. When the fruiting and flowering tops of these plants are rubbed between the palms of the hand the resin is transferred from the plant to the palm.

Production of cannabis resin in the countries of the Indian subcontinent is, therefore, based on a rubbing or kneading process rather than a threshing process. A variety of methods may be used to achieve this. The ones described here may be taken as representative of the process.

A slow and laborious method involves the resin bearing parts of the cannabis plant being rubbed between the palms of the hand. A thin layer of cannabis resin forms on the palms of the hand as the material is rubbed. When all the resin has been transferred from the batch being rubbed, the plant is discarded (it may be used as a second class product, by for example, being made into an infusion similar to tea). The resin that has transferred to the palms of the hand is removed by scraping with the edge of a metal instrument. It may be transferred to a collecting bowl and the next batch of cannabis is subjected to the rubbing process. Gradually, separated cannabis resin builds up in the collecting bowl. A suitably quantity of the resin is then removed from the bowl and then pressed or rolled into slabs, rods, balls or whatever shape is favoured in the particular locality.

An alternative approach is to rub the flowering and fruiting tops of the cannabis against rubber sheeting. The cannabis resin is transferred to the rubber sheeting and from this it can be scraped off and collected into quantities suitable for production of slabs. This approach can be varied by the person who is harvesting the cannabis resin wearing rubber sheeting, or leather or similar fabric, while walking through a field of cannabis plants. Resin accumulates on the rubber sheeting as it

brushes against the fruiting and flowering tops of the plants and, when sufficient has been collected, the sheeting may be scraped clean. Production of slabs, etc. then follows as described above.

The flowering and fruiting tops may be collected in a similar way to that used in herbal cannabis production. These are allowed to dry and are then broken and crushed between the hands into a coarse powder. This powder is then passed through sieves so that it attains a fineness similar to that obtained in the Mediterranean. The fine powder, which is still green, is stored in leather bags for four to five months until the weather becomes hot again. The powder is then exposed to the sun for a short time—sufficient for the resin to melt. The powder is replaced in the leather bags for a few days, after which it is removed and kneaded well by means of wooden rods so that a certain amount of oily material appears on its surface. Kneading is continued until a material suitable for pressing into slabs has been produced.

Finally, a fundamentally different method is used in some localities of the Indian subcontinent. By quantity, little cannabis resin is made in this way. The plant material, apart from the main stems, is immersed in boiling water. This removes the resin from the fruiting and flowering tops (similar to the rending of meat, when meat is boiled the animal fats are removed from the flesh). The cannabis which has been extracted is discarded (it may be used for culinary purposes), and when the extracting liquid cools, a layer of solidified resin forms on its surface. This resin may be removed and formed into slabs or whatever shape is favoured. The problem with this method is that water is introduced into the resin. This results in the slabs of resin frequently turning mouldy as they age.

3. Liquid cannabis (hashish oil)

Liquid cannabis is a liquid extract of either herbal cannabis material or of cannabis resin; the extract is often concentrated prior to trafficking. The reason for making liquid cannabis is to concentrate the psychoactive ingredients (e.g. THC). This may help the trafficker evade interdiction, because more psychoactive material can be contained in a smaller concealment. Of equal value to the trafficker is the ability to insert the liquid cannabis into concealments which cannot easily accommodate herbal or resin cannabis. Furthermore, it is easy to seal hermetically the liquid cannabis, thereby overcoming the possibility of detection by the odour emitted by the material.

Liquid cannabis, whether made from herbal or resin material, is prepared by a process similar to that used to percolate coffee. Alternately the process can be considered as being similar to soxhlet extraction undertaken in chemical laboratories to extract chemicals from solid materials, with continual refluxing of the extracting solvent.

B. Description of illicit cannabis products

1. Names and synonyms for illicit cannabis products

There are so many synonyms used for the various illicit cannabis products that it is beyond the scope of this manual to list them all. The reader is referred to the United Nations publication dealing with this subject—*The Multilingual Dictionary of Narcotic Drugs and Psychotropic Substances under International Control,* (ST/NAR/1).

2. *Physical appearance and chemical characteristics of illicit cannabis products*

It must be stressed that no two cannabis products have exactly similar physical appearances. Produced from a highly variable natural product, by a batch process capable of wide variation, and subsequently subjected to processing and transformation for trafficking purposes, it is not surprising that cannabis products occur in such a multitude of forms. Those described here are just a selection, albeit the most common. Because material submitted for forensic examination bears no physical relationship to any type described here, that does not mean, of course, that it is not cannabis or a cannabis containing product.

(a) Herbal products (marijuana)

Cannabis grown in a temperate climate

Cannabis cultivated in Europe, the North Americas, and the southern parts of the Southern Hemisphere is bright green when growing; after harvesting some samples lose their green colour and turn yellow, but rarely brown coloured. Generally the fruiting and flowering tops are devoid of resin—unlike herbal cannabis from the Indian subcontinent they are not sticky when compressed in the palm of the hand. For the same reason it is difficult to compress this material into slabs as can be easily done with, for example, West African cannabis. Seeds are invariably present. European cannabis will contain a higher leaf content than North American cannabis, in which fruiting and flowering tops predominate.

Chemical characteristics: very variable, because the seeds have been imported, often illicitly, from many different regions where cannabis grows wild. Different cannabinoid profiles, with and without both CBD and THV, are encountered.

Cannabis grown in tropical climates

North African cannabis

Rarely trafficked out of the region; a finely chopped light green or yellow green herb which contains no seeds or fibrous material.

Chemical characteristics: identical to the resin produced in the region, i.e. both THV and CBD are low relative to THC.

West African and Caribbean cannabis

When growing, the material is green; on harvesting and drying, it turns brown. Some samples retain their green colour. Generally, Caribbean cannabis retains its green colour more than West African. It is rare to find a dried sample of West African cannabis which is not brown. Colour apart, these two types of cannabis are physically and chemically very similar. In some samples of West African cannabis the fruiting and flowering tops have been destroyed in processing; many dark brown seeds are visible within the compressed mass of herbal material.

Until recent years Caribbean cannabis was of a low quality, containing many stems and stalks which are low in or completely devoid of the psychoactive constituents of cannabis. A recent trend has been the attempt to produce Sinsemilla; no samples completely free of seeds have yet been detected, but the amount of non-psychoactive containing material in these seizures is greatly reduced, and the

fruiting and flowering tops of some seizures are comparable to those found in North American Sinsemilla.

Chemical characteristics: Both types lack CBD and have low THV:THC ratios.

Cannabis from Central Africa

Most samples are similar to West African cannabis, but a few are similar to those produced in the southern part of Africa.

Chemical characteristics: Brown samples similar to West African cannabis in cannabinoid profile; green samples similar to Southern African cannabis in cannabinoid profile.

Cannabis from Southern Africa

When dried, and prepared for trafficking, this material generally resembles cannabis grown in temperate areas. It is both much greener and contains a higher proportion of leaves than West African cannabis.

Chemical characteristics: No CBD. THV and THC in roughly equal amounts.

Cannabis from South America

Similar to Caribbean cannabis; samples vary enormously in quality from products containing high proportion of fibrous, non psychoactive containing material, to Sinsemilla type products consisting of only fruiting and flowering tops.

Chemical characteristics: Similar to Caribbean. The occasional sample contains a small amount of CBD.

Cannabis from the Indian subcontinent

Three types may be trafficked: (1) brown fruiting and flowering tops which are high in resin and sticky to the palm of the hand; (2) dark green-brown material similar to some samples from West Africa; (3) green, largely leafy material devoid of fruiting and flowering tops.

Chemical characteristics: (1) CBD present, THC and THV approximately equal; (2) resembles West African cannabis; (3) similar to type (1) but low levels of cannabinoids.

Cannabis from South East Asia

"Buddha Sticks"—see chapter III.A.1

Chemical characteristics: Normally only THC, no CBD and negligible THV.

(b) Cannabis resin products

North African cannabis resin

Yellow brown, thin rectangular slabs wrapped in cellophane which rarely bears a mark. Coin imprints occur from time to time.

A recently introduced product is superficially similar to cannabis resin from the Indian subcontinent—it is almost black on the surface, and internally is much darker than the yellow brown slabs. This type is in the shape of blocks of toilet soap, and is wrapped in cellophane. No markings but coin imprints on some samples.

Chemical characteristics: CBC generally low relative to THC, and THV very low. Cannabinoid acids present in variable amounts from seizure to seizure.

East Mediterranean cannabis resin

Red-brown and powdery. Invariably trafficked inside cloth bags, which, until a few years ago were always white, but which occasionally bore an ink stamp. Nowadays the cloth bags are sometimes brightly coloured, with or without ink stamps. Slabs up to 0.5 kg in weight, occasionally 1 kg. The resin bears the imprint of the cloth when unwrapped.

Chemical characteristics: CBD present to greater extent than in any other cannabis resin product. THV very low. Acids mostly CBDA, are also present to a greater extent than in any other cannabis resin product.

North Eastern Mediterranean cannabis resin

Greenish-brown powder or (rarely) as small thin wafers of brittle material wrapped in cellophane.

Chemical characteristics: CBD much less than THC. THV low. Acids present in high amounts.

Cannabis resin from the Indian subcontinent

A great variety of products are made. In quantity the rectangular slabs, black on the surface, and dark green within, which originate from the north west part of the subcontinent, predominate over all other types. These slabs, which frequently bear an embossed mark on the surface, are often wrapped in dark cellophane prior to trafficking. A few slabs are square. The slabs vary in thickness from 5 mm to 20 mm and are odorous and pliable when freshly made. On ageing they lose their odour and become brittle. Typically the slabs weigh 0.25, 0.5 or 1 kg, but higher weights are occasionally encountered. Slabs from the northern part of the Indian subcontinent are often mouldy, and crumble readily.

Other cannabis resin products from the Indian subcontinent include sticks, often in bundles, small balls (1 cm in diameter), large balls (8 cm in diameter), and irregular shaped pieces of resin. All of these products are dark brown or black on the surface and dark green or dark brown internally.

Chemical characteristics: Varies as greatly as the physical variation. Generally, cannabinoid acid content is lower than for the Mediterranean cannabis resin. The cannabidiol content of the slab variety is less than that of the Eastern Mediterranean resin, but greater than that of the North African resin; it can be very low or absent in some othery types. Generally THV is low, but some types contain more THC than any other cannabis resin, and accordingly reach a higher value when sold in the illicit traffic.

(c) Liquid cannabis (hashish oil)

Liquid cannabis is a dark viscous oil with a characteristic odour. When diluted with organic solvents, it becomes either a green coloured or brown coloured solution. The colour is not necessarily an indication of origin because the maturity of the plant material and the solvent used to prepare the liquid cannabis may influence its colour. Generally, liquid cannabis, which on dilution produces a green coloured

solution, has been made from herbal cannabis, and liquid cannabis, which on dilution produces a brown solution, has been made from cannabis resin. Liquid cannabis cannot be diluted with water; if water is added to liquid cannabis which has been diluted with, for example, ethanol, an emulsion is formed.

Some liquid cannabis is not concentrated before being trafficked; this product has the consistency (and often the odour) of an organic solvent, and may be green or brown coloured.

Chemical characteristics: The cannabinoid profile is, with one important difference, similar to that of the cannabis or cannabis resin from which the liquid cannabis has been made. The difference is that liquid cannabis is devoid of cannabinoid acids. The major producing regions of liquid cannabis are the resin producing countries of the Mediterranean and of the Indian subcontinent, and the herbal cannabis producing Caribbean. The neutral cannabinoid profiles of the liquid cannabis from these regions are similar to those of the resin or herbal products produced in these regions. However, the cannabinoids form a much higher proportion of the material.

Typical THC levels in the three illicit cannabis products:

Herbal cannabis:	0.5— 5%
Resin cannabis:	2—10%
Liquid cannabis:	10—30%

It should be noted that these values are only a guide to levels likely to be encountered by the forensic analyst. Many samples of herbal, resin or liquid cannabis will have a THC content outside these limits.

In addition to the neutral cannabinoids, seized cannabis material may also contain, in greatly varying levels, the corresponding cannabinoid acids as well. Although there does not seem to be a consistent relationship between the origin of the material and the actual cannabinoid acid content and composition, the forensic chemist may be called upon, depending on national legislation, to demonstrate the presence and/or determine the content of these acids separately in the sample under examination.

In addition, reference is made to various handbooks and review papers which deal with cannabinoid chemistry in full detail [35-37].

Cannabis contains a complex mixture of many specific individual chemicals called cannabinoids. Four major constituents are:

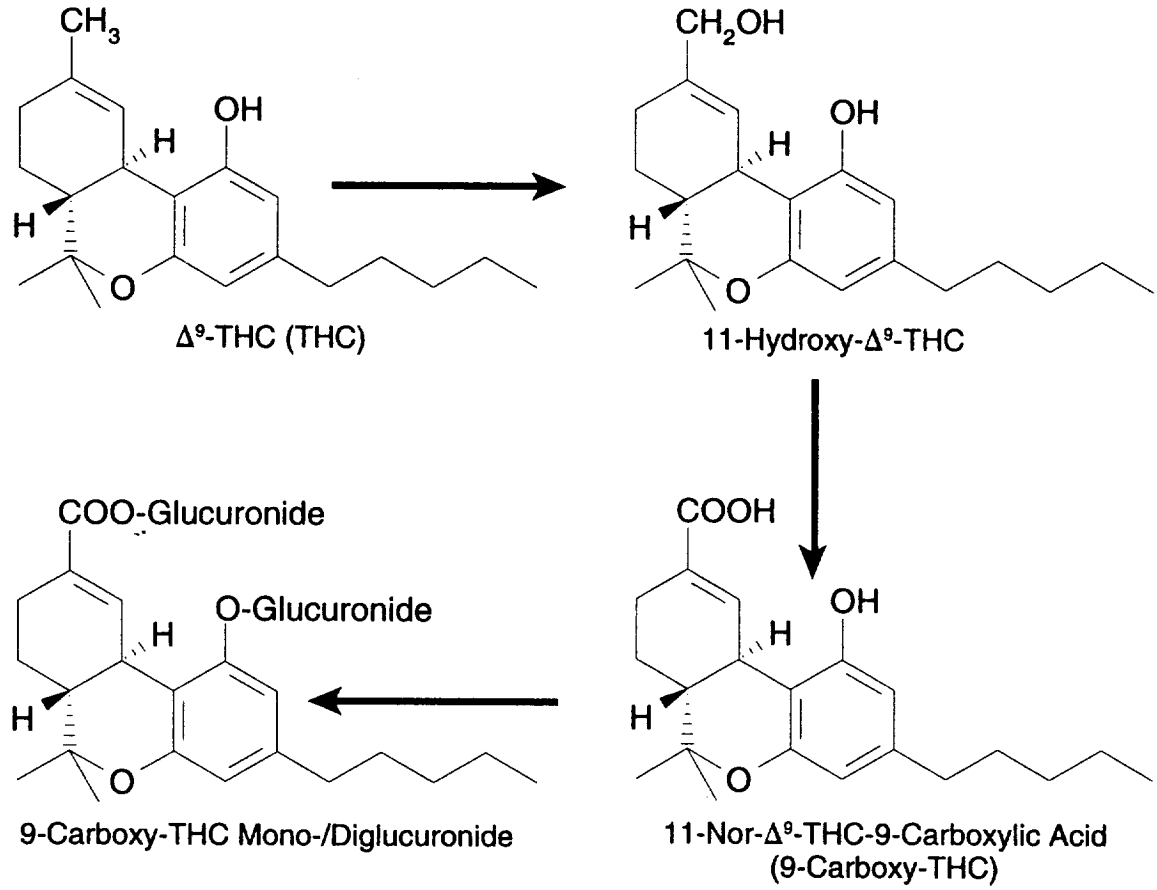
Δ-9-tetrahydrocannabinol (THC)
Cannabinol (CBN)
Cannabidiol (CBD)
Cannabichromene (CBCh).

THC is the principal cannabinoid responsible for most of the characteristic psychological effects of the cannabis products. It is therefore the only cannabinoid of relevance to the present context.

3. Routes of administration, metabolism and excretion of THC [38, 39]

The most widespread abuse of cannabis is by smoking. It may occasionally be abused orally. THC is extensively metabolized in humans and less than 1% of unchanged THC is recovered in the urine. When smoked, initial metabolism occurs in the lung, whereas this takes place in the liver when marijuana is taken orally. The metabolic pathway of THC is summarized in figure III.1.

Figure III.1 Metabolic pathway of THC



Seventy-two hours after smoking, approximately 50% of the inhaled THC is excreted as metabolites and the remaining 50% is distributed throughout the body where it is mostly absorbed by the fatty tissue and excreted slowly over the next few days. Excretion is mainly via urine (25%) and faeces (65%).

Although over 20 metabolites of THC have so far been identified, oxidation at the C-11 position and glucuronidation account for the major compounds appearing in urine. The major acidic metabolite is 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid (9-carboxy-THC) which is converted to mono- and di-glucuronide conjugates and these are the major forms of metabolites excreted in urine. Thus, the identification of 9-carboxy-THC in urine is considered the best indication of previous cannabis consumption.

The concentration of THC in plasma diminishes rapidly due to metabolism and tissue storage. However, the terminal half-life is long and usually greater than 20 h. This results in THC being present in the body for many hours, and possibly days, after the last consumption. Consequently, the excretion of 9-carboxy-THC is prolonged. The pattern of urinary excretion in the occasional user is different from that of chronic users. In the occasional user, the metabolite is detectable in urine for 1-3 days depending on the analytical method, whereas the urine of chronic smokers contains detectable levels one week or more after the last dose.

C. Sampling and sample preparation procedures for the assay of 9-carboxy-THC

The sampling procedures outlined in chapter I.C and G.5 apply.

Precautions

Certain precautions must also be observed when dealing with urine samples for 9-carboxy-THC assay. Thermal degradation of cannabinoid metabolites occurs relatively rapidly and the major metabolite, 9-carboxy-THC, can decrease significantly even at room temperature after 1 week and by as much as 45% after 6 months [40, 41]. The decrease depends on such factors as oxidation, the amount of urine in the container and the type of container used. It has also been reported that 9-carboxy-THC irreversibly absorbs to various container types resulting in considerable losses.

1. Sample preparation for immunoassay

In general, little or no sample preparation is necessary for immunoassay (see also chapter I.G.5).

2. Sample preparation for chromatography

(a) Hydrolysis

Over 80% of the excreted cannabinoid metabolite, 9-carboxy-THC is present in urine in the form of glucuronide conjugate. To free the metabolite, it is necessary to hydrolyze the urine and this can be performed either by alkaline or enzymatic hydrolysis. Alkaline hydrolysis is considered more efficient and reproducible than either acid or enzyme hydrolyses [42, 43].

Pipette 10 ml urine from the original container into a glass-stoppered tube. For methods requiring an internal standard (GC, GC/MS, HPLC), this should also be added to the tube. Add 2 ml 10 N potassium hydroxide, close the tube and incubate for 20 min at 50° C with occasional stirring.

At this stage, a single extraction with 20 ml cyclohexane-ethyl acetate (7:1, v/v) as a clean-up step may be performed, especially before GC, GC/MS and HPLC analysis, to remove neutral and basic impurities.

(b) Extraction

The extraction procedure should be efficient and selective. A good recovery is important since the total amount of cannabinoids present is very low. Selectivity is important to ensure that interfering substances present in the urine are removed.

Extraction of 9-carboxy-THC from hydrolyzed urine is carried out at acidic pH, to ensure that the metabolite is soluble in the organic solvents used. The commonly used solvents or solvent mixtures in liquid-liquid extraction that have been published are petroleum ether, hexane, diethyl ether, chloroform and combinations of hexane and ethylacetate. Several solid-phase extraction methods have also been proposed in the literature.

Liquid-liquid

After cooling the sample following hydrolysis, adjust its pH to 2 with 2 N HCl or 2 N H_2SO_4. Add 15 ml cyclohexane-ethyl acetate (7:1, v/v) and extract the solution by mechanical shaking for 10 min. Remove the organic layer and filter it through a small amount of dry sodium sulphate into a tapered tube, wash the filter

with 5 ml solvent and evaporate to dryness at room temperature with a stream of air or nitrogen. Redissolve the residue in 0.2 ml methanol or acetonitrile-methanol (3:1, v/v) by agitation or sonication.

Solid-phase

As an alternative to liquid-liquid extraction, SPE may be used. A chemically-bonded reversed-phase (modified silica) absorbent is recommended and this should be used according to the manufacturer's instructions. A number of methods published in the relevant literature have reportedly shown high recoveries [44, 45] and one of these is given below.

Condition the columns by rinsing slowly with 3 ml portions of methanol, water, methanol and water in turn. A 10 ml plastic syringe body attached to the column provides a reservoir. Apply weak vacuum to increase flow. Draw the hydrolyzed urine (2 ml) through the column and wash with 10 ml of 0.1 N HCl and 25 ml of 50 mM phosphoric acid in 10% acetonitrile. Elute 9-carboxy-THC with 1 ml of acetone. Evaporate the solvent under a stream of nitrogen and redissolve the residue in 0.1 ml methanol.

A new, simple and fast sample preparation and clean-up procedure for GC and GC/MS using SPE extraction disc (microcolumn) and on-disc derivatization (ODD) is described elsewhere [46].

(c) Internal standards

Internal standards should meet the criteria given in chapter II.G.6. Cannabinol (CBN), oxyphenbutazone or ketoprofen are suitable for most GC methods. Deuterated 9-carboxy-THC analogues (d_3 or d_6), if available, are recommended for GC/MS. Cannabinol or n-octyl-p-hydroxybenzoate are suitable for HPLC.

Preparation of internal standard solutions:

Prepare a stock solution in absolute ethanol containing 1 mg/ml of cannabinol. Transfer 1 ml of the solution into a 200 ml standard flask and make up to the mark with absolute ethanol (1 ml of internal standard solution = 5 μg of cannabinol).

(d) Standard solution

Since usually there is no need for quantitation, the available 9-carboxy-THC solution is used for the identification of the urine metabolite.

D. Screening methods

1. Immunoassay methods

It is recommended that where laboratories have access to these techniques, immunoassays should be used for initial screening. Several immunoassays are commercially available to screen for cannabinoids in urine [47, 48]. All immunoassays detect 9-carboxy-THC and with middle to high cross-reactivity towards other urinary THC metabolites with the dibenzopyran ring structure (such as 11-hydroxy-THC) [49, 50]. All positive results obtained by initial screening tests should be confirmed by a second assay of the original specimen using methods based on techniques and chemical principles different from the initial screening test. These assays should be more specific and at least equally sensitive. A summary of cross-reactivities of some commercial assays is given in table III.1.

Table III.1 Cross-reactivities of commercial immunoassays for cannabinoids

Assay	Cross-reactivity (%)			
	9-Carboxy-THC	*11-Hydroxy-THC*	*THC*	*CBN*
EMIT-d.a.u	100 (50)[a,d]	56 (90)	---	---
FPIA-TDx	100 (100)[b,c]	36 (277)	15 (655)	11 (899)
Abuscreen-Ontrak	100 (100)[b,d]	40 (250)	14 (714)	1 (10 640)
Abuscreen-Online	100 (100)[b,d]	>100 (50)	3 (3 000)	5 (2 000)

[a,b]Cross-reactivity calculated by dividing the target concentration (50[a]/100[b] ng/ml) by the concentration equivalent to 50[a]/100[b] ng/ml 9-carboxy-THC and multiplying by 100 (in parenthesis equivalents, ng/ml).

[c]M. A. ElSohly [50].

[d]Manufacturer's information.

2. *Thin-layer chromatography*

Standard TLC technique

Details of standard TLC materials and procedures are given in the United Nations manual, *Recommended Methods for Testing Cannabis* [9] and these are applicable to the analysis of biological extracts for 9-carboxy-THC.

TLC plates

Coating: Activated silica gel G. Silica gel containing an additive which fluoresces under UV light, wavelength 254 nm, can also be used

Layer thickness: 0.25 mm

Size of plates: Glass plates 20 × 20 cm, 20 × 10 cm or 10 × 5 cm. The optimum run is approximately 10 cm

Procedure

25-50 µl reconstituted solution is applied to the TLC plate. A standard solution of 9-carboxy-THC is also applied to the plate which is then developed using one of the solvent systems below.

Developing solvents

System A [51]:	Ethyl acetate	12
	Methanol	5
	Conc. ammonia	1
	Water	0.5
System B [52]:	Chloroform	70
	Methanol	30
	Conc. ammonia	2

Visualization

The plates must be dried prior to visualization. This can be done at room temperature, or, more quickly, in an oven at 120° C for 10 min, or by the use of a hot air blower. It is important for proper colour development that all traces of

ammonia or other bases be removed from the plate. The following visualization method is recommended:

Spray reagent:

0.1% aqueous solution of Fast Blue B salt. The solution must be freshly prepared. An acceptable frequency is once per day.

It is important for colour development that the TLC plate is made alkaline. This can be done by exposing the plate to vapours of ammonia or diethylamine after spraying. The plate is then dried using a warm air blower. 9-carboxy-THC appears as a pink or rosy red spot at the same Rf value as the standard 9-carboxy-THC spot.

Note: *Fast Blue B salt is claimed by some authorities to be a potential carcinogen. The same authorities assert that the dye Fast Blue BB is less suspect as a potential carcinogen [53]. Therefore, as an alternative, the plate may be sprayed with a freshly prepared solution of Fast Blue BB salt in 0.1 M sodium hydroxide (0.75 mg/10 ml) and then dried to ensure proper colour development and stability.*

Results

Table III.2 R_f 3 100 values

	Developing system	
Compound	*A*	*B*
9-Carboxy-THC	35-40	25-38

These values are subject to variation depending on laboratory conditions (humidity, temperature, drafts) and other parameters (e.g. quality of material used).

E. Confirmatory chromatographic methods

1. Gas chromatography

(a) Sample derivatization

The urine extract is evaporated to dryness under a stream of nitrogen and 50 µl N,O-*bis*-trimethylsilyl-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) are added to the tube, which is vortexed and heated at 60° C for 10 min. Alternatively MSTFA/TMCS [39, 54] or MTBSTFA/ TBDMS [55, 56] can be used for derivatization. *t*-Butyldimethylsilyl-trifluoroacetamide (MTBSTFA) is more reactive and the resulting TBDMS derivatives are more stable and show greater sensitivity in the GC/MS.

An alternative derivatization method, giving the dimethyl derivative of 9-carboxy-THC, can be carried out using tetramethylammonium hydroxide (TMAH) [57, 58]. In this case, 70 µl of 10% TMAH-dimethylsulfoxide (1:20, v/v) is added to the dry residue followed after 2 min by 5 µl of methyl iodide. After a further 10 min, add 200 µl of 0.1 N hydrochloric acid and extract the solution with 2 ml of isooctane. The isooctane layer is separated and evaporated under a stream of nitrogen. The residue is reconstituted in 50 µl of solvent. 1-2 µl of the derivatized solution is injected.

(b) Packed column technique [57]

Operating conditions

Detector:	FID	
Column:	2 m × 2 mm I.D.	
Packing:	*(a)* 3% Dimethyl silicone (OV-1)	
	(b) 3% Phenylmethyl silicone, 50% phenyl (OV-17)	
Carrier gas:	Nitrogen or helium at 30 ml/min	
Operating temperatures:	Injector:	260° C
	Oven:	255° C
	Detector:	275° C

Note: *Prior to use, all packed columns must be conditioned. Usually the conditioning temperature should be at least 30° C above the temperature at which the analysis is to be performed, unless this would require exceeding the upper temperature limit of the column as specified by the manufacturer. In this case, a smaller temperature differential must be used and the conditioning period substantially extended. Typically, columns are conditioned overnight, or for a minimum of 15 h.*

Conditioning is carried out with the normal carrier gas flow and with the column disconnected from the detector.

Note:

- *Silanize glass columns frequently to avoid adsorption of morphine during GC determinations.*
- *Clean injection port and detector regularly to avoid decomposition of samples and loss of detector sensitivity.*
- *Handle silylating reagents with care. Silylating reagents are very reactive and sensitive to moisture.*

(c) Megabore column technique [58]

Operating conditions

Detector :	FID	
Column :	Fused silica, 10 m × 0.52 mm I.D. with 2.6 μm chemically bonded dimethylsilicone stationary phase, e.g. OV-1	
Carrier gas :	Helium at 2 ml/min	
Injection technique:	Split/splitless	
Operating temperatures:	Injector:	290° C
	Oven:	240° C
	Detector:	290° C

2. Gas chromatography-mass spectrometry

Operating conditions [46, 56, 59, 60]

Column:	Fused silica, 10-30 m × 0.18-0.25 mm I.D. with 0.25 μm chemically bonded phenylmethyl or dimethyl-polysiloxane stationary phase

Carrier gas:	Helium at 2 ml/min
Column temperature:	150-220° C to 270-290° C at 5-25° C/min
Injector temperature:	250-260° C, operated in the splitless mode
Ionization:	Electron impact (EI) or chemical ionization (CI) mode

Internal standards

Deuterated 9-carboxy-THC analogues (d_3 or d_6) or non-isotopic standards (e.g. meclofenamic acid)

Extraction

Solid-phase extraction (SPE) [46, 59, 60] or liquid/liquid extraction [56]

Derivatization

With BSTFA or MSTFA (TMS derivatives) [46, 59], MTBSTFA (TBDMS derivatives) [56] or trimethylammonium hydroxide (methyl derivatives) [60].

Table III.3 Principal ions in the mass spectra of 9-carboxy-THC derivatives (SIM)

Compound	*Principal fragment ions m/z*
9-Carboxy-THC dimethyl derivative	372 (M^+), 357, 313
9-Carboxy-THC-diTMS	488 (M^+), 473, 371
9-Carboxy-THC-diTMS[a]	489 (M^+), 399, 371
9-Carboxy-THC-diTBDMS	572 (M^+), 557, 515, 413

[a](CI mode, isobutane as reagent gas).

3. *High performance liquid chromatography*

High performance liquid chromatography on reversed phase columns with ultraviolet [61] or electrochemical detection [55, 62] offers high sensitivity and sufficient specificity for the confirmation of positive results obtained in an immunoassay screening. It allows for the rapid detection of 9-carboxy-THC at the low ng/ml level without prior derivatization. For reproducible quantitation, the use of an internal standard (e.g. cannabinol [55, 61]) is recommended.

Operating conditions

Method A [61]

Column:	Octylsilica (Spherisorb C-8 or equivalent), 5 μm, 25 cm × 4.6 mm I.D.
Mobile phase:	Acetonitrile-50 mM phosphoric acid (65:35, v/v)
Flow rate:	1.5 ml/min
Detection:	UV at 211 nm (200-350 nm scan range if a diode array detector is used)
Injection volume:	10-15 μl
Internal standard:	Cannabinol

Method B [62]

Column:	Octylsilica (Zorbax C-8, or equivalent), 5 μm, 25 cm × 4.6 mm I.D.
Mobile phase:	Acetonitrile-methanol-0.02 N sulphuric acid (35:15:50, v/v/v)
Flow rate:	1.1 ml/min
Detection:	ECD, 110 mV (Ag/AgCl) (working electrode glassy carbon)
Injection volume:	5-10 μl
Internal standard:	n-Octyl-p-hydroxybenzoate

For alternative HPLC techniques see Dixit and Dixit [63].

F. Interpretation of results

1. Time course of detection

Depending on the immunoassay methods and the cut-off level used in the initial screening, the length of time that the metabolite can be detected varies. Generally, acute marijuana use (less than twice a week) can be detected by urine analysis for 1-3 days when using a cut-off level about 100 ng/ml (or less). When the individual has used marijuana chronically for an extended period, this detection time can be appreciably lengthened due to the tendency of THC to be absorbed and accumulated into fatty tissues. Under these circumstances, detection of 9-carboxy-THC for a week or more is possible [39, 64].

2. Passive inhalation

The issue of passive or inadvertent exposure to marijuana smoke is sometimes raised as an explanation of a positive urine assay result. While this has been demonstrated to occur, achieving a sufficient dose of THC by this route is difficult and unlikely in most instances. If screening assays with a cut-off 20 ng/ml are used, positive results can occur but very infrequently. With cut-off levels of 100 ng/ml, the possibility of a passive positive is virtually eliminated [43, 65-68].

3. Concentration variation

Urine drug concentrations can be influenced by a number of different factors, primarily liquid intake. The urine concentration of a drug may change 10-fold in a matter of hours. This means that caution must be exercised when interpreting a positive result occurring, for instance, after a negative one in a daily sampling regime. This is particularly problematic in the case of a drug such as THC with a long half-life where detectability is probable over several days but with the concentration varying significantly around the cut-off level. Positive samples following negative ones do not necessarily indicate additional use of marijuana.

IV. Recommended methods for the detection and assay of cocaine in biological specimens

A. Common types of illicit coca products [10]

1. Coca leaf

Different *Erythroxylon* species produce leaves varying in size and appearance. In all species the upper side of the leaf is darker than the underside which may be grey-green in colour. On the underside of the leaves are found two lines parallel to the midrib which are considered to be characteristic of coca leaf.

2. Cocaine

Although produced from a somewhat variable natural product by a batch process capable of wide variation, cocaine varies comparatively little when compared, for example, with heroin products. Nevertheless, no two illicit samples of cocaine are exactly identical. For the most part it is a white or off-white powder which is often fine, and rarely damp. It has a characteristic odour.

Coca paste is an off-white, creamy or beige-coloured powder. Rarely fine, it often contains aggregates and is generally damp. Unless the aggregates are crystalline (which is rare) they usually break down under slight pressure. It has a characteristic odour.

Occasionally cocaine is encountered as material containing large, sometimes colourless crystals ("rock cocaine"). These crystals can be quite hard. Usually some, if not the major part, of such samples consist of material similar to ordinary "powder" cocaine.

Adulteration is comparatively rare within the producing countries, the material being internationally trafficked with a purity often of 80-90% (as cocaine hydrochloride). Subsequent adulteration and transformation for trafficking purposes within the economically developed countries usually involves the addition of either an uncontrolled synthetic local anaesthetic (e.g. lidocaine, procaine or benzocaine) or a carbohydrate (e.g. mannitol, lactose or glucose). In either case the physical appearance is changed only slightly, for virtually all adulterants are themselves fine dry white powders.

The typical purity for trafficking in cocaine within the economically developed countries is about 30%. The internationally trafficked material is subject to adulteration with about three times its own weight of the diluent.

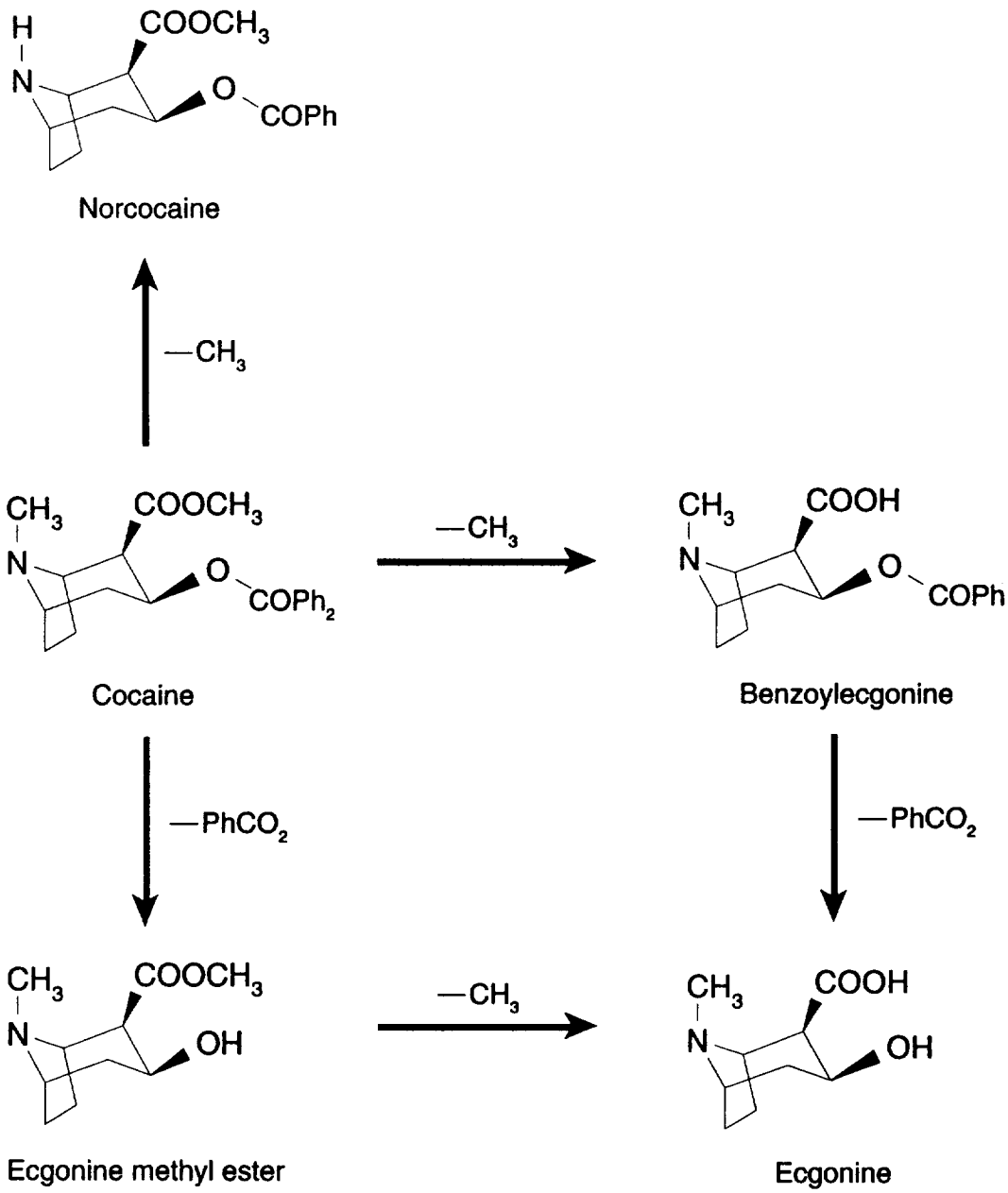
In addition to the adulterants and impurities mentioned above, cocaine hydrochloride may also contain a range of substances including starch, boric acid, sodium hydrogen carbonate and dipyrone.

Newer forms of cocaine have become available, especially the free base forms such as extracted cocaine base and "crack". In these forms, solvent residues may be present as well as local anaesthetics which are used as adulterants. A frequent combination is the so-called "speed-ball" which consists of cocaine plus heroin.

In addition, reference is made to review papers which deal with the chemistry of coca alkaloids in full detail [69-72]. In the laboratory analysis of various biological specimens for cocaine the following compounds are of relevance (for structures see figure IV.1):

Cocaine
Benzoylecgonine
Ecgonine methyl ester
Ecgonine

Figure IV.1 Metabolic pathway of cocaine



3. *Routes of administration*

Cocaine can be administered intranasally (i.e. snorted or sniffed, the most common route) or by intravenous and intramuscular injection. It can also be taken orally, sublingually, vaginally or rectally and it can be smoked. When snorted, cocaine powder is formed into a narrow line on a smooth surface prior to insufflation through a straw or tube formed from rolled-up paper. A "line" of cocaine is normally 3-5 cm long and contains 10-100 mg of powder. Cocaine free base and crack are administered by inhalation, i.e. by smoking in special devices. Coca leaves are traditionally chewed together with alkaline earth in some geographic areas.

The bioavailability of cocaine varies according to the route of administration (see table IV.1) [73].

Table IV.1 Bioavailability of cocaine administered by different routes

Route of administration	*Bioavailability (%)*
Oral	20-30
Intranasal	20-30
Smoking	6-32
Intravenous	100

4. *Metabolism and excretion*

Cocaine is transformed into two major metabolites i.e. benzoylecgonine and ecgonine methyl ester [74]. Some other minor metabolites including norcocaine have been identified recently in human urine [75]. The metabolic pathways are summarized in figure IV.1. Cocaine is eliminated in the urine as unchanged drug (1-9% of the dose), benzoylecgonine (35-54%) and ecgonine methyl ester (32-49%) [76]. The recommended target analytes are cocaine and its metabolites benzoylecgonine and ecgonine methyl ester.

The peak plasma level of cocaine is reached shortly after intranasal, intrapulmonary or intravenous administration. The time to maximum psychotropic and physiological effects is also short; thereafter the euphoric effects diminish within 30-60 min (20 min if smoked). The plasma elimination half life of cocaine following intravenous or intrapulmonary administration is 38-39 min [77].

B. Sampling and sample preparation procedures for the assay of cocaine and its metabolites

The general sampling procedures outlined in chapter I.C and G.5 apply.

Precautions

Certain precautions must be observed when dealing with urine samples for assay of cocaine and its metabolites. The target analytes show poor hydrolytic stability, particularly under alkaline conditions [78, 79]. Samples should be kept cool and in the dark as much as possible after collection. At pH 8, cocaine concentrations in urine samples have been found to decrease by 40-70% during storage at 4° C for 21 days. It is therefore advisable to adjust the sample to pH 5 with dilute acetic acid. Ecgonine methyl ester for example is stable up to three years

in urine between pH 3 and 5 and stored at 4-5° C [80]. It should also be noted that blood samples to be used for assay of cocaine and its metabolites are best preserved with fluoride and at pH 5.

1. Sample preparation for immunoassay

If necessary, the urine should be centrifuged to remove turbidity. The pH of the urine samples should be adjusted between 6.5 and 8.0 as necessary. The manufacturer's instructions should be followed regarding further procedures.

2. Sample preparation for chromatography

(a) Hydrolysis

There is no need for hydrolysis.

(b) Extraction

Liquid-liquid

A urine aliquot of 1 to 20 ml is extracted according to the methodology to be used subsequently. The sample is adjusted to pH 9 (range 8-9.5) with appropriate buffer. Any of the following buffers is suitable [89]:

Borax (pH 9-9.6): A solution containing 19.07 g of sodium tetraborate ($Na_2B_4O_7 \cdot 10\ H_2O$) in 1 litre of water.

Ammonia buffer (pH 9.5): Ammonium chloride (10.7 g) is dissolved in aqueous ammonia (5 M; 40 ml) and the solution is made up to 1 litre with water.

The sample is then extracted at least two times with equal volumes of extraction solvent. A mixture of dichloromethane-isopropanol (85:15, v/v) or chloroform-isopropanol (50:50, v/v) is suitable. Care must be taken to allow the aqueous (upper) layer to separate completely from the solvent (lower) layer before drawing off the extract, in order to avoid carrying over any water. If problems with emulsions occur, silicone-treated filter paper (phase separating paper) can be used to filter the extract. The organic layers are filtered through a small amount of dry sodium sulfate (on the filter), the filter is rinsed with 5 ml of solvent. The extract is evaporated to dryness under vacuum or under a stream of nitrogen.

Solid-phase

Diatomaceous earth

Extraction procedures utilizing a diatomaceous earth should observe the recommendations of the manufacturer (e.g. Extrelut®). The following procedure is typical [90-92].

A sample of urine (20 ml) is adjusted to pH 9 with saturated NH_4Cl/NH_3 buffer. The sample is then transferred to a liquid chromatography column containing 20 g of diatomaceous earth and allowed to soak for 10-15 min. The column is eluted with 40 ml of dichloromethane-isopropanol (85:15, v/v). About 25 ml eluate is recovered. The organic eluate is collected and evaporated to dryness.

Modified silica

Solid-phase extraction methods using modified silica cartridges are also recommended [82, 93, 94]. This type of method offers advantages in the saving of time, reduction of the volume of solvents required and avoidance of the problems caused by emulsion formation which sometimes arise during liquid-liquid extraction. These advantages are offset by the cost of the cartridges used. Two representative SPE procedures are described below, each being typical of this type of method. Other SPE materials are available and the procedures recommended by the manufacturers should be followed carefully.

Procedure A The procedure uses a modified silica cartridge (e.g. Bond Elut Certify®) which allows both ionic and nonpolar interactions between the analytes and the adsorbent [85].

Extraction cartridges are inserted into a vacuum work station and conditioned by washing with methanol (2 ml) and then phosphate buffer (0.1 M; pH 7; 2 ml). Care is taken not to dry the cartridges after conditioning.

Urine samples are centrifuged and aliquots (2.5 ml) are mixed with internal standard solution, if required, and phosphate buffer (0.1 M; pH 7; 1 ml). The pH is checked and adjusted if necessary to pH 7.

The urine samples are then transferred to the cartridges and slowly drawn through under vacuum.

The cartridges are washed with deionized water (3 ml), aqueous hydrochloric acid (0.1 M; 3 ml) and methanol (9 ml).

Analytes are eluted with a mixture of chloroform-isopropanol-conc. ammonia (80:20:2, v/v/v; 2 ml).

The eluates are evaporated to dryness.

Procedure B The procedure uses a cyclodextran-based column (e.g. Cyclobond I) which was found to give a clean extract of benzoylecgonine with a recovery efficiency of 50% [85].

Cyclodextran columns (500 mg/3 ml) are used without conditioning.

Urine (5 ml) is added to a column and slowly drawn through under vacuum.

The column is washed with water (10 ml) and dried by centrifugation or by drawing air through the column for 10 min.

The column is then washed with acetone (2.5 ml) and dried under vacuum.

Benzoylecgonine is eluted with chloroform-ethanol (8:2, v/v; 2 ml) by application of slight positive pressure to the top of the column with a rubber bulb.

The eluate is evaporated to dryness.

An improved, rapid and efficient SPE method for benzoylecgonine with a recovery of 90-100% is described by Anderson [95]. A new simple and fast sample preparation and clean-up procedure for GC and GC/MS using SPE disc (microcolumn) and on-disc derivatization (ODD) is described elsewhere [96].

(c) Internal standards

Selection of a suitable internal standard should observe the general criteria given in chapter II.G.6 if possible. Internal standards for analysis of cocaine and its metabolites by gas chromatography fall into three groups:

An analogue of benzoylecgonine, e.g. propyl benzoylecgonine [81].

An opiate alkaloid, e.g. levallorphan [82], nalorphine [83], ethylmorphine [84] or codeine [85].

Miscellaneous substances, e.g. *n*-tetracosane, tetraphenylethylene (FID only) or butylanthraquinone [86].

For GC/MS, the preferred internal standards are deuterated analogues of cocaine and its metabolites, but if these are unavailable, one of the standards listed above for GC should be used. Internal standards suitable for HPLC are lidocaine [87] and tropacocaine (benzoyltropine) [88].

(d) Calibration standards

Prepare stock solutions in methanol containing 1 mg/ml of cocaine, benzoylecgonine, ecgonine methyl ester and internal standard. From these stock solutions prepare urine standards containing cocaine in the range of 0-5 μg/ml, benzoylecgonine and ecgonine methyl ester in the range of 0-25 μg/ml and internal standard at a concentration of 25 μg/ml. A set of calibration urine standards should be processed simultaneously with the test samples.

C. Screening methods

1. Immunoassay methods

Radioimmunoassay, fluorescence polarisation immunoassay, enzyme multiplied immunoassay technique and latex agglutination inhibition are recommended where laboratories have the necessary facilities to perform these techniques (see chapter I.G.1). The limits of detection of radioimmunoassay and fluorescence polarisation immunoassay are generally 50 ng/ml or less while for EMIT it is 300 ng/ml. Antibodies of the commercial immunoassay kits are targeted towards benzoylecgonine, but may cross-react in different ways with cocaine and its other metabolites [97]. A summary of cross-reactivities of some commercial assays is given in table IV.2.

Table IV.2 Cross-reactivities of commercial immunoassays for cocaine and cocaine metabolites

	Cross-reactivity (%)			
Assay	*Benzoylecgonine*	*Cocaine*	*Ecgonine methyl ester*	*Ecgonine*
Coat-A-Count	104 (300)[a, b]	7 259 (50)	1.3 (5 000)	--
Abuscreen-RIA	108 (300)[a, b]	215 (300)	0.6 (5 000)	--
EMIT-d.a.u.	100 (300)[c, d]	0.15 (200 000)	--	1.5 (20 000)
EMIT-s.t.	pos. (300)[b]	neg. (5 000)	neg. (5 000)	--
FPIA-TDx	95.7 (300)[a, b]	1.2 (5 000)	0.1 (5 000)	--
Abuscreen-Ontrak	100 (300)[c, d]	10 (3 000)	< 0.01 (> 100 000)	0.75 (40 000)
Abuscreen-Online	100 (300)[c, d]	0.97 (30 928)	0.31 (96 774)	1.2 (25 000)

[a]Apparent cross-reactivity calculated by dividing the apparent concentration by the target concentration and multiplying by 100 (in parenthesis concentration, at which cross-reactivity was determined, ng/ml).

[b]J. E. Wallace [86].

[c]Cross-reactivity calculated by dividing the target concentration (300 ng/ml) by the concentration equivalent to 300 ng/ml benzoylecgonine and multiplying by 100 (in parenthesis equivalents, ng/ml).

[d]Manufacturer's information.

2. *Thin-layer chromatography*

A volume of 5 to 20 ml of urine must be extracted. Conventional TLC has a detection limit for cocaine and benzoylecgonine of 1 mg/ml urine. High performance thin-layer chromatography (HPTLC) has a detection limit for benzoylecgonine of about 0.3 mg/ml urine and is to be preferred if the equipment is available.

Standard TLC technique

Details of standard TLC materials and procedures are given elsewhere [10] and these are applicable to the analysis of biological extracts.

TLC plates

Coating:	Activated silica gel G containing an additive which fluoresces when irradiated with UV light, wavelength 254 nm
Layer thickness:	0.25 mm
Size of plates:	Glass plates 20 × 20 cm, 20 × 10 cm or 10 × 5 cm. The optimum run is approximately 10 cm

HPTLC plates

Commercially prepared HPTLC plates, 10 × 10 cm, normally developed over 5 cm.

Standard solutions

Cocaine
Benzoylecgonine
Ecgonine methyl ester.

Prepare standard solutions at a concentration of 1 mg/ml in methanol and apply 5 µl of each solution onto the plate.

Procedure

Urine extracts (see chapter IV.B.c) are evaporated to dryness in a test tube and redissolved in methanol (50 µl). The entire extract is spotted on the plate.

Developing solvents

System A (TLC) [10]:	Methanol	100
	Conc. ammonia	1.5
System B (TLC) [98]:	Chloroform	50
	Methanol	50
System C (HPTLC) [99]:	Ethyl acetate	15
	Methanol	15
	Dichloromethane	5
	Conc. ammonia	3

Visualization

The plates must be dried prior to visualization. This can be done at room temperature, or, more quickly, by the use of a hot air blower, or in an oven at 120° C for 10 min. It is important for proper colour development that all traces of ammonia or other bases are removed from the plate. The following visualization methods are recommended:

UV light at 254 nm.

Acidified potassium iodoplatinate reagent. (For preparation see chapter II.C.2)

Dragendorff's reagent. (For preparation see chapter II.C.2)

Subsequent overspraying with acidified ferric chloride may give an enhanced response [100].
Overspray reagent: Concentrated sulphuric acid (1 ml) is carefully added to aqueous ferric chloride solution (5%, w/v; 10 ml) and mixed.

Results

Table IV.3 $R_f \times 100$ values

	Developing system	
Compound	*A*	*B*
Cocaine	59	61
Benzoylecgonine	25	28
Ecgonine methyl ester	65	—

Table IV.4 Appearance of spots with each visualization method

	Detection method		
Compound	*UV*	*Iodoplatinate*	*Dragendorff*
Cocaine	Dark	Violet	Orange[a]
Benzoylecgonine	Dark	Negative[b]	Orange[a]
Ecgonine methyl ester	Negative	Blue	Orange[a]

[a]Colour is the same before and after overspraying with acidified ferric chloride solution.
[b]Spots of > 1 μg give a purple spot on a slightly lighter purple background [85].

D. Confirmatory chromatographic methods

1. *Gas chromatography*

(a) *Sample derivatization*

In order to detect the metabolites of cocaine, the urine extract should be derivatized (see also the notes in chapter I.G.5).

Alkylation/acylation (PFP derivatives, suitable for FID and NPD) [83]

The urine extract is evaporated to dryness and pentafluoropropionic anhydride (50 μl) and pentafluoropropanol (25 μl) are added. The mixture is heated at 90° C

for 15 min. The derivatizing reagents are evaporated and the residue redissolved in ethylacetate (25 µl). The PFP derivatives formed are stable in the reagent for months and after evaporation of the reagent for at least 24 hours.

Silylation (TMS or TBDMS derivatives, suitable for FID)

The urine extract is evaporated to dryness and reconstituted in acetonitrile (25 µl). Silylating reagent, e.g. 25 µl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) for TMS derivatives or *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) [88] for TBDMS derivatives, are added and the reaction is allowed to proceed at 50-60° C for 30 min. The mixture is injected directly into the chromatograph. The derivatives should be prepared shortly before analysis by GC or GC/MS. They are not stable in the reagent for more than a few days.

(b) Packed column technique [10]

Operating conditions

Detector: FID, hydrogen at 30 ml/min, air at 450 ml/min.

Note: *For better sensitivity and specificity, a nitrogen-phosphorus detector (NPD) is recommended: operating parameters should be in accordance with the recommendations of the manufacturer. Sample preparation and derivatization procedures should be chosen which avoid nitrogen-containing solvents and reagents in the final solution injected into the chromatograph.*

Column:	2 m × 2-4 mm I.D.	
Packing:	*(a)* Dimethyl silicone (SE 30, OV-1)	
	(b) Phenylmethyl silicone, 50% phenyl (OV-17)	
Carrier gas:	Nitrogen at 30 ml/min	
Operating temperatures:	Injector:	220° C
	Oven:	220° C
	Detector:	300°C

Conditioning of packed columns:

Note: *Prior to use, all packed columns must be conditioned. Usually the conditioning temperature should be at least 30° C above the temperature at which the analysis is to be performed, unless this would require exceeding the upper temperature limit of the column as specified by the manufacturer. In this case, a smaller temperature differential must be used and the conditioning period substantially extended. Typically, columns are conditioned overnight, or for a minimum of 15 hours.*

Conditioning is carried out with the normal carrier gas flow and with the column disconnected from the detector

Note:

- *Silanize glass columns frequently to avoid adsorption of morphine during GC determinations.*
- *Clean injection port and detector regularly to avoid decomposition of samples and loss of detector sensitivity.*
- *Handle silylating reagents with care. Silylating reagents are very reactive and sensitive to moisture.*

(c) Capillary column technique

Operating conditions

Detector:	FID or NPD (see note above under Detector)	
Column:	Fused silica, 25 m × 0.32 mm I.D. with 0.15 μm non-polar (methylsilicone) chemically bonded stationary phase	
Carrier gas:	Helium or hydrogen at 2 ml/min	
Operating temperatures:	Injector:	250° C
	Oven:	150° C to 280° C, at 9° C/min
	Detector:	280° C

Note: *The capillary column dimension, stationary phase, phase thickness, carrier gas and flow rate used may be varied from those cited above depending on availability. However, a non-polar column is of general applicability for the analysis of biological samples and is recommended. The optimum operating conditions for the system should be selected according to the recommendations of the supplier.*

2. *Gas chromatography-mass spectrometry*

When a mass spectrometer is used as a detector, the principal ions (m/z) in the EI^+ spectra are as given in table IV.5. For detailed GC/MS procedures using selected ion monitoring (SIM) is refered to elsewhere [95, 101].

Table IV.5 Principal ions in the mass spectra for cocaine and its metabolites

	Principal fragment ions (m/z)			
Compound	*Underivatized*	*PFP Deriv.*	*TMS Deriv.*	*TBDMS Deriv.*
Cocaine	82,105,182,303	(not formed)	(not formed)	(not formed)
Benzoylecgonine	82,93,124,168	272,300,316,421	82,240,361	282,346,403
Ecgonine methyl ester	82,96,168,199	119,182,345	82,83,96,98,182	182,256,313

3. *High performance liquid chromatography*

Procedure

Urine extracts are evaporated to dryness, reconstituted in acetonitrile or mobile phase (50-100 μl) and 10-20 μl injected.

The following HPLC systems are suitable:

Operating conditions

Method A [87]

Column:	Octadecylsilica reversed-phase column, 3 μm, 10 cm × 3.2 mm I.D.
Mobile phase:	Phosphate buffer (0.01 M; pH 2.1) containing tetrabutylammonium hydroxide (0.2 mM) and acetonitrile (6%, v/v)
Flow rate:	1.5 ml/min
Detection:	UV at 233 nm

Method B [88]

Column:	Octadecylsilica reversed-phase column, 3 μm, 15 cm × 4.6 mm I.D. + precolumn.
Mobile phase:	Acetonitrile-water (17.5:82.5, v/v) containing phosphoric acid (8.5 g/l) and hexylamine (0.28 ml/l), pH 3
Flow rate:	1.2 ml/min
Detection:	UV at 230 nm

Method C [94]

Column:	Octadecylsilica reversed-phase column, 25 cm × 4.6 mm I.D. + precolumn
Mobile phase:	Initial: 10% acetonitrile in potassium phosphate buffer (0.05 M; pH 3.2). This is increased to 50% acetonitrile over 15 min and the final composition is maintained for 5 min. A re-equilibration time of 5 min is required between injections
Flow rate:	1.5 ml/min
Detection:	UV at 200 nm

E. Interpretation of results

1. Time course of detection

After a single dose of cocaine, the unchanged drug can be detected for up to 24 h and the metabolites benzoylecgonine and ecgonine methyl ester for up to 48 h [102-104]. After chronic use, the detection time can be longer, up to 5 days or more [105, 106].

Minimal differences are found in the relative amounts of metabolites excreted following administration of cocaine intranasally, intravenously or by smoking [107].

It is generally not possible to draw conclusions from urinary concentrations of cocaine and its metabolites concerning the amount of drug administered, the time since the last dose, or the level of impairment.

2. Precautions

Ecgonine methyl ester is formed by the action of the enzyme pseudocholinesterase. Abnormalities of pseudocholinesterase activity, resulting from genetic causes [108] or from the intentional simultaneous ingestion of cholinesterase inhibitors such as organo-phosphorus pesticides, could alter the pattern of metabolite excretion.

Benzoylecgonine ethyl ester (cocaethylene), a cocaine homologue, and other minor transformation products may be observed following simultaneous administration of cocaine and ethanol [99, 109].

Consumption of beverages prepared from tea containing coca leaves ("Health Inca Tea") may result in the ingestion of cocaine and subsequent urinary excretion of benzoylecgonine at concentrations of several milligrams per litre of urine [110].

Anhydroecgonine methyl ester can be detected after smoking of cocaine free base ("crack") [111].

F. Analysis and interpretation in other biological matrices

Plasma concentrations of cocaine and benzoylecgonine following therapeutic administration of cocaine are normally less than 0.5 and 0.1 μg/ml, respectively. In overdose cases, levels in autopsy blood are in the range of 1-20 and 1-10 μg/ml, respectively [76, 112].

As noted earlier (chapter IV.B), cocaine and its metabolites show poor stability with respect to hydrolysis (both enzymatic and non-enzymatic). Blood and plasma samples should be collected in tubes containing sodium fluoride and the pH adjusted to pH 5 with acetic acid (10%, v/v). They can then be kept, in a refrigerator at 4° C or frozen if possible for some months [78, 79, 113].

Both hair and saliva samples from cocaine users have been found to contain cocaine [114-118]. While the quantitative interpretation of cocaine concentrations in hair is not yet fully developed, saliva cocaine levels appear to correlate well with plasma concentrations.

V. Recommended methods for the detection and assay of amphetamine and methamphetamine in biological specimens

A. Introduction

Illicit amphetamine and methamphetamine are largely derived by synthesis in clandestine laboratories, with substantial though relatively smaller amounts coming from diversion or improper use of legally produced materials. There is a marked variation in the occurrence of amphetamine and methamphetamine in different parts of the world: amphetamine is more prevalent in Europe whereas methamphetamine is the more common drug in the United States of America, Japan and South-East Asia.

The methods of production, chemical characteristics and physical appearance of illicit amphetamine and methamphetamine are described in some detail in the United Nations manual *Recommended Methods for Testing Amphetamine and Methamphetamine* [119] and will not be repeated here. However, since the publication of the manual, there has appeared a pure form of (+)- methamphetamine hydrochloride, which, because of its transparent sheet-like crystals, is called "ice" [120].

1. Routes of administration

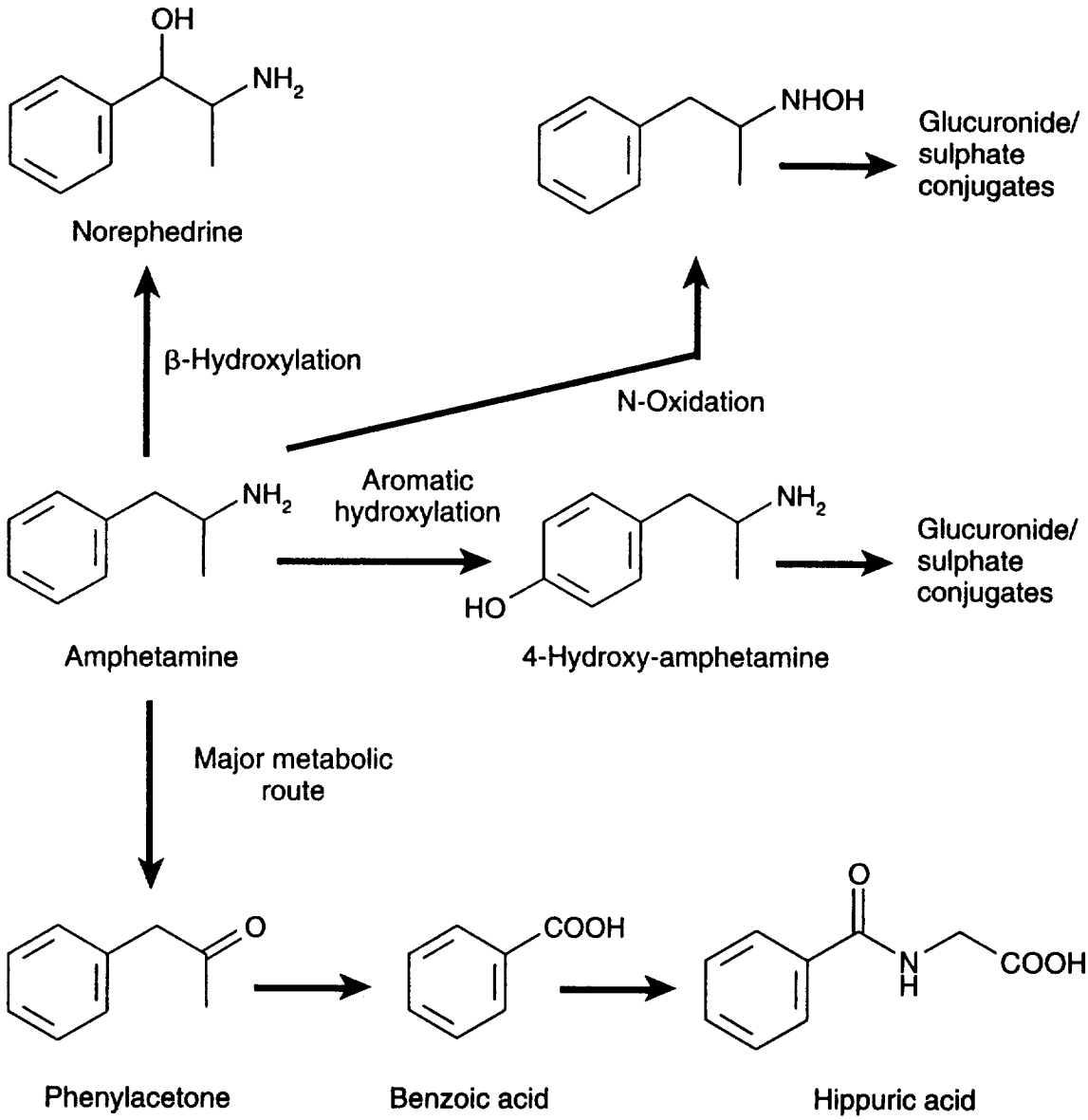
Amphetamine is most frequently taken orally or intranasally (snorting) as the sulphate or phosphate salt in doses ranging from 5-15 mg in occasional users to 100-2,000 mg per day in habitual users [121]. Methamphetamine, as the hydrochloride salt, is most frequently prepared for injection or for smoking ("ice") [120] but is also available in tablet form.

2. Metabolism and excretion

Following oral doses of 2.5-15 mg of amphetamine, peak plasma levels of 30-170 μg/ml are reached in 2 h and plasma elimination half-lives range from 8 to 12 h. Blood concentrations in fatalities are normally above 500 μg/ml [106].

Amphetamine and methamphetamine begin to appear in the urine within 20 min of administration. Amphetamine is excreted as the unchanged drug, typically 20-30% of the dose, and as deaminated (hippuric acid and benzoic acid) and hydroxylated metabolites, partly as conjugates, typically adding up to 25% of the dose. The rate of excretion and the fraction of the dose excreted as unchanged drug vary according to the pH of the urine. In alkaline urine about 45% of the dose is excreted in 24 h, 2% of the dose as the unchanged drug, while in acid urine, up to 78% of the dose may be excreted in 24 h, 68% as the unchanged drug [122]. The recommended target analytes are, therefore, the unchanged drugs. The major and minor metabolic pathways of amphetamine are summarized in figure V.1.

Figure V.1 Metabolic pathway of amphetamine



Methamphetamine is excreted as the unchanged drug (44%) and as its major metabolites amphetamine (6-20%) and 4-hydroxymethamphetamine (10%) [123]. The metabolic pathway of methamphetamine is summarized in figure V.2. As with amphetamine, acidic urine increases both the rate of excretion and the percentage of unchanged drug excreted.

After chronic administration, abusers have shown amphetamine concentrations in urine of 1-90 μg/ml and methamphetamine concentrations of 25-300 μg/ml [124].

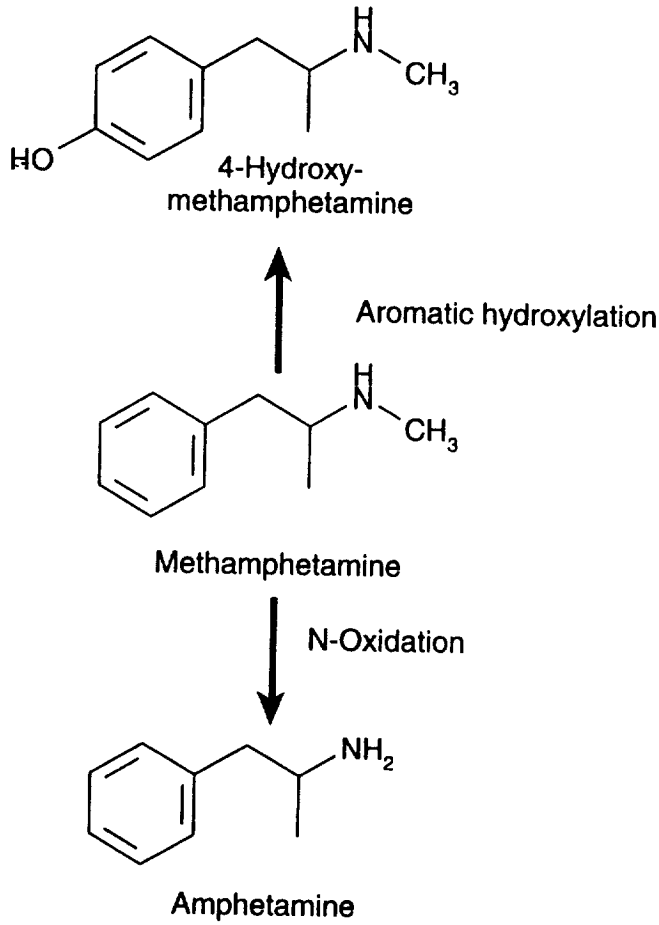
B. Sampling and sample preparation procedure for the assay of amphetamine and methamphetamine and their metabolites

The general sampling procedures and precautions given in chapter I.C and G.5. are applicable to samples used for assay of amphetamine and methamphetamine.

Precautions

Care must be taken when concentrating extracts containing amphetamine and methamphetamine by evaporation as the free bases may be lost during evaporation of the solvent. These losses may be avoided by adding methanolic hydrochloric acid (methanol : concentrated hydrochloric acid (9:1, v/v; 50 μl)) to the extract to form the corresponding hydrochloride salts of the drugs before the solvent is evaporated.

Figure V.2 Metabolic pathway of methamphetamine



1. Sample preparation for immunoassay

In general, very little sample preparation is required for initial immunoassay tests. It is unnecessary to hydrolyse the urine samples because immunoassays measure both the free and conjugated forms of the drug and/or metabolites. It may be necessary to adjust the pH or centrifuge the urine to remove turbidity. For optimum results, the manufacturer's instructions should be followed.

2. Sample preparation for chromatography

(a) Hydrolysis

There is no need for hydrolysis.

(b) Extraction

Liquid-liquid

Amphetamine and methamphetamine are extracted from urine at alkaline pH, when the amino group is in the uncharged state. The pK_a values of the two drugs are 9.9 and 10.1, respectively, and the pH to which the urine should be adjusted for optimum recovery is pH 11. The following procedure may be used [125]:

Urine (2 ml) is pipetted into a 50 ml conical-bottom tube and internal standard solution (a solution of 2-methyl-phenylethylamine; 8 μg/ml; 0.25 ml), sodium hydroxide solution (1 M; 2 ml), water (5 ml) and dichloromethane (20 ml) are added. The tube is capped, shaken, centrifuged at low speed for 5 min, and the upper layer is discarded.

If further purification of the extract is required, an additional clean-up step may be used which is based on back-extraction into acid.

To the extract sulphuric acid (0.15 M; 2 ml) is added, and the tube is capped, shaken and centrifuged as before. The upper aqueous phase is transferred to a 15 ml round-bottom tube into which sodium hydroxide solution (1 M; 1 ml) and 1-chlorobutane or dichloromethane (2.5 ml) are added. The tube is capped, vigorously vortexed and centrifuged. The organic solvent is transferred to a clean tube. Methanolic hydrochloric acid (9:1, v/v; 50 μl) is added and the extract is evaporated to dryness.

Solid-phase

This type of method offers advantages in the saving of time, reduction of the volume of solvents required and avoidance of the problems caused by emulsion formation which sometimes arise during liquid-liquid extraction. These advantages are offset by the cost of the cartridges used.

Suitable cartridges contain diatomaceous earth or silica substituted with non-polar groups (octadecyl silica) [126], cation exchange groups, or with mixed non-polar and ion-exchange substituents [127]. The manufacturer's instructions should be followed according to the cartridges used.

The following procedure is representative:

Strong cation-exchange cartridges (benzenesulphonylpropyl silica, 1 ml capacity) are conditioned under vacuum on a vacuum work station with methanol (2 ml), water (1 ml) and phosphoric acid 10 mM; 0.5 ml). Urine (1 ml) and phosphoric acid (10 mM; 0.5 ml) are mixed thoroughly in a tube and applied to the cartridge. The cartridge is air-dried for approximately 30 sec and then washed with phosphoric acid (10 mM; 1 ml), acetic acid (0.1 M; 0.5 ml) and methanol (1 ml). The column is again air-dried for approximately 30 sec and the analytes are eluted with ammoniacal methanol (3%, v/v; 2 ml). The extract is evaporated to dryness under vacuum or using a stream of nitrogen noting the precaution given in chapter V.B.2.b.

(c) Internal standards

Selection of a suitable internal standard should observe the general criteria given in chapter I.G.6 if possible. Suggested internal standards for GC or HPLC analysis of amphetamines in urine are phentermine, propylamphetamine and other amphetamine analogues. For GC/MS, the preferred internal standards are deuterated analogues of amphetamine and methamphetamine, but if these are unavailable, one of the standards listed above for GC should be used.

(d) Calibration standards

Prepare separate stock solutions in methanol containing 1 mg/ml of amphetamine, methamphetamine and internal standard. From these stock solutions prepare urine standards containing amphetamine and methamphetamine in the range 0-5 μg/ml and internal standard at a concentration of 5 μg/ml. A set of calibration urine standards should be processed simultaneously with the test samples.

C. Screening methods

1. Immunoassay methods

As noted in chapter I.G.1, immunoassay techniques may be used for screening purposes and positive findings must be confirmed by a different, more specific method. This is particularly important in the analysis of amphetamine and methamphetamine because there is a large number of amphetamine-like drugs, some of which may cross-react with antibodies targeted towards amphetamine and methamphetamine (see also chapter VI.C.1). Immunoassay kits available from commercial sources for amphetamine and methamphetamine involve enzyme immunoassay, fluorescence polarisation immunoassay, latex agglutination inhibition and radioimmunoassay. The specificities of these immunoassays vary widely, depending on the particular antibody used. Some characteristics of the available immunoassays are summarized in table V.1.

Table V.1 Cross-reactivities of commercial immunoassays for amphetamine (A) and methamphetamine (MA)

	Cross-reactivity (%)				
Assay	*d-A*	*l-A*	*d,l-A*	*d-MA*	l-MA
EMIT-d.a.u. monoclonal	100 (300)[a,b]	10 (2 900)	60 (500)	100 (1 000)	14 (7 000)
FPIA-TDx amphetamine	91 (1 000)[c,d]	61 (1 000)	100 (1 000)	123 (1 000)	115 (1 000)
FPIA-TDx amphetamine/ methamphetamine II	100 (1 000)[c,d]	57 (1 000)	165 (1 000)	98 (1 000)	7 (1 000)
Abuscreen-Online	100 (1 000)[a,b]	--	66 (1 517)	0.5 (219 298)	--

[a]Cross-reactivity calculated by dividing the target concentration (300 or 1 000 ng/ml) by the concentration equivalent to 300 or 1 000 ng/ml *d*-amphetamine or 1 000 ng/ml *d*-methamphetamine and multiplying by 100 (in parenthesis equivalents, ng/ml).

[b]Manufacturer's information.

[c]Cross-reactivity calculated by dividing the apparent (measured) concentration by the actual concentration and multiplying by 100 (in parenthesis concentration, at which cross-reactivity was determined, ng/ml).

[d]J. T. Cody [128].

2. Thin-Layer chromatography

Standard TLC technique

General comments concerning the application of thin-layer chromatography as a screening technique are given in chapter I.G.2. Details of standard TLC materials and procedures are given elsewhere [10] and these are applicable to the analysis of biological extracts.

TLC plates

Coating:	Activated silica gel G containing an additive which fluoresces when irradiated with UV light, wavelength 254 nm
Layer thickness:	0.25 mm
Size of plates:	20 × 20 cm, 20 × 10 cm or 10 × 5 cm; the optimum run is approximately 10 cm

Standard solutions

Amphetamine
Methamphetamine.

Make all standard solutions at a concentration of 5 mg/ml in methanol and apply 1 µl of each solution to the plate.

Procedure

Urine extracts are evaporated to dryness in a test tube, noting the precaution given in chapter V.B.2.b, and redissolved in methanol (50 µl). The entire extract is spotted on the plate with a glass capillary.

Developing solvents [129]

System A:	Methanol	100
	Conc. ammonia	1.5
System B:	Ethyl acetate	85
	Methanol	10
	Conc. ammonia	5

Visualization

The plates must be dried prior to visualization. This can be done at room temperature, in an oven at 120° C for 10 min, or, more quickly, by use of a hot air blower. It is important for proper colour development, however, that all traces of ammonia be removed from the plate. The following visualization methods are recommended:

Fast Black K reagent [130-132].

Solution A: 1% Fast Black K salt in water.
Solution B: 1 M sodium hydroxide.

Spray the plates with solution A and observe any coloured spots. Secondary amines such as methamphetamine produce spots immediately. Overspraying with solution B produces a coloured spot for amphetamine (and for any other substituted amphetamine). Air dry the plates and spray once more with solution A. This produces more intensely coloured spots. The colours vary from violet for primary amines to almost pink for secondary amines such as methamphetamine. The limits of detection for amphetamine and methamphetamine are 0.1 and 0.05 µg, respectively [130].

Ninhydrin reagent.

Prepare a 10% solution in ethanol.

Spray with the ninhydrin reagent and heat in an oven at 120° C for at least 15 min. Violet or pink spots are given by primary amines such as amphetamine and more intense spots for secondary amines such as methamphetamine.

Fluorescamine reagent (Fluram).

Prepare a solution of 10 mg fluorescamine in 50 ml acetone.

Spray with the fluorescamine reagent. Air dry the plate with a hot air blower. Observe the plate under a UV light at 365 nm. Amphetamine gives a bright yellow fluorescent spot. The detection limit for amphetamine and other primary amines is about 10 ng. Methamphetamine is not detected.

Simons reagent.

Solution A: 20% aqueous sodium carbonate.
Solution B: 1% aqueous sodium nitroprusside.

Spray the plate with solution A then overspray with solution B. Place the plate in an empty developing tank along with a beaker containing acetaldehyde. Cover the tank. The acetaldehyde vapour will cause a methamphetamine spot to become an intense blue colour. The limit of detection for methamphetamine in urine is about 0.1 μg/ml. Amphetamine and other primary amines give pale pink to red coloured spots and the reaction is less sensitive.

Results

Table V.2 $R_f \times 100$ values

Compound	*Developing system*	
	A	*B*
Amphetamine	44	66
Methamphetamine	33	63

3. Colour test

A sensitive and specific screening test for methamphetamine in urine has been reported [133].

Preparation of adsorption cartridges

Octadecylsilyl silica (ODS-silica; 0.13 g) is packed by suction with an aspirator into a polyethylene tube (35 mm × 4 mm I.D.) which is drawn out into a fine tip (approximately 2 mm I.D.).

Preparation of modified Simons reagent

Acetaldehyde (25 ml) containing 1.5% acetic acid is mixed with methanol (25 ml) and kept in a refrigerator. Before use, one volume of the acetaldehyde solution was mixed with 1 volume of 1% aqueous sodium nitroprusside solution.

Procedure

Each adsorption cartridge is first activated by passing through it methanol-0.1 M hydrochloric acid (9:1, v/v; 2 ml) and then distilled water (3 ml). An aliquot of the urine sample (5 ml) is mixed with buffer (0.5 M, pH 8.0; 2.5 ml) and the solution is passed through the adsorption cartridge slowly with a disposable syringe. The cartridge is washed with aqueous acetone (20%, v/v; 4 ml) and then modified Simons reagent (0.4 ml) is passed through the adsorbent. The eluent is collected in four fractions of three drops and one drop of sodium carbonate solution (1%, w/v) is added to each fraction. The presence of methamphetamine is indicated by the development of a blue colour, mostly with the third fraction, but also with the second and fourth fractions if there is a high concentration of methamphetamine in the urine. If methamphetamine is absent, a pale orange colour is obtained. The detection limit is 1 μg/ml of methamphetamine in urine and the test can be completed within 3 min.

D. Confirmatory chromatographic methods

1. Gas chromatography

(a) Sample derivatization

Heptafluorobutyric anhydride (HFBA) [134]:

HFBA (50 μl) is added to the dry residue. The tube is capped, vortexed and incubated at 75° C for 20 min. The tube is uncapped and dried under air or nitrogen at 30° C. The contents are dissolved in 50 μl of ethyl acetate and 1-2 μl are injected on to the GC column.

Alternative procedure [135]:

Potassium hydroxide (0.5 M; 50 μl) is added to the dry residue followed by 500 μl of toluene. After mixing and centrifugation, the organic layer is transferred to a clean test tube and 5 μl of HFBA is added. The solution is mixed thoroughly and sodium bicarbonate (10%, w/v; 500 μl) is added immediately with continuous mixing. The tube is centrifuged and 1 μl of the toluene (upper) layer is injected onto the GC column.

Trifluoroacetic anhydride (TFAA) [125, 136]:

Ethyl acetate (100 μl) and TFAA (50 μl) are added to the dry residue. The tube is shaken and incubated at 60° C for 20 min. The mixture is evaporated carefully to 50 μl final volume under a gentle stream of air or nitrogen at room temperature and 1-2 μl are injected onto the GC column.

N-Methyl-N-tert-butyldimethylsilyl trifluoroacetamide (MTBSTFA) [137]:

100 μl of acetonitrile and 150 μl of MTBSTFA are added to the dry residue. The vial is capped and heated at 90° C for 15 min and then left at ambient temperature for 2 h or more. 500 μl of acetonitrile are added and the content mixed and used for GC or GC/MS.

(b) Method without derivatization

Operating conditions

Detector: FID, NPD

Columns: Glass packed columns (2 m × 3-4 mm I.D.) with dimethylsilicone (e.g. OV-1, SE-30) or methylphenyl silicone (e.g. OV-17) liquid stationary phases

Carrier gas: Nitrogen at 30 ml/min.

Fused silica capillary columns with chemically bonded nonpolar stationary phases (e.g. SE-54) are a useful alternative to the packed columns described above. Helium carrier gas is used at a flow rate of 1 ml/min

Operating temperatures:

Injector: 250-280° C

Oven: 90-280° C (programmed, depending on the column used)

Detector: 280-300°C

(c) Method with derivatization

Operating conditions

Detector: FID, NPD, ECD or MS with EI ionization, operated in the selected ion monitoring (SIM) mode.

Columns and temperatures: See method without derivatization above.

Results

Table V.3 Retention data for amphetamine and methamphetamine and their derivatives

Compound	Column		
	SE-30 *Underivatized*[a]	*3% OV-17* *TFA derivatized*[a]	*SE-54* *HFBA derivatized*[b]
Amphetamine	1 129	1 536	2.74
Methamphetamine	1 176	1 722	3.61

[a]Retention indices.

[b]Retention times (min) on a SE-54 capillary column, 25 m × 0.3 mm I.D., film thickness 0.17 μm, temperature programmed from 120° C (1 min) to 280° C at 10°/min.

2. Gas chromatography-mass spectrometry [125, 134, 136, 137]

When a mass spectrometer is used as a detector, the principal ions (m/z) in the EI^+ ionization, operated in the selected ion monitoring (SIM) mode spectra are as given in table V.4.

Table V.4 Principal ions in the mass spectra of amphetamine and methamphetamine and their derivatives

Compound	Principal fragment ions (m/z)			
	Underivatized	HFBA Underivatized[a]	TFA derivatized[a]	TBDMS derivatized[b]
Amphetamine	44, 65, 91	91, 118, 240	91, 118, 140	73, 100, 158, 192
Methamphetamine	58, 91, 134	91, 118, 254	110, 118, 154	73, 172, 173, 206

3. *High performance liquid chromatography*

Two methods are given below for the assay of amphetamine and methamphetamine in urine by HPLC [138-141].

(a) Method without derivatization [138]

Sample preparation

Extraction of samples is carried out as described earlier (chapter V.B) using phentermine as internal standard. The extracts are evaporated to dryness under a stream of nitrogen, noting the precautions given in chapter V.B.2, and then redissolved in 50-100 µl of acetonitrile or mobile phase.

Standard solutions

Standard solutions are prepared by dissolving reference material in acetonitrile to give a concentration of 0.01 mg/ml.

Operating conditions

Column:	Octadecylsilica (Spherisorb ODS-1 or equivalent), 3 or 5 µm, 12.5 cm × 4.0 mm I.D. + precolumn
Mobile phase:	Acetonitrile-water (57:943, w/w) + phosphoric acid (8.5 g/l) + hexylamine (0.28 ml/l). Depending on the type of column used, k' values can be optimized by variation of the acetonitrile-water ratio
Flow rate:	0.8 ml/min
Detection:	UV at 190 nm
Injection volume:	10 µl
Quantitation:	By peak areas, using the internal standard method

Results

Retention times relative to phentermine (I.S.):

Amphetamine	0.64
Methamphetamine	0.93
Phentermine	1.00 (8.1 min)

(b) Method with derivatization [141]

Sample preparation

Extraction of samples is carried out as described earlier (chapter V.B) using phentermine as internal standard. Standard solutions are prepared by dissolving reference material in blank urine to give a concentration of 5 μg/ml and are processed in the same manner as the urine test samples. The extracts are evaporated to dryness under a stream of nitrogen, noting the precautions given in chapter V.B.2, and then redissolved in sodium bicarbonate (2%, w/v; 200 μl) and an equal volume of sodium ß-naphthoquinone-4-sulphonate is added. After heating in an oven at 60° C for 30 min, the aqueous solution is extracted with hexane-diethylether (2:1, v/v) using a vortex mixer for 1 min. The organic layer is transferred to a clean tube and evaporated to dryness under a stream of nitrogen. The residue is dissolved in acetonitrile (100 μl).

Operating conditions

Column:	Octadecylsilica (μ-Bondapack C-18 or equivalent), 3 or 5 μm, 15 cm × 3.9 mm I.D.
Mobile phase:	Acetonitrile-methanol-0.01 M sulphuric acid (20:20:60, v/v/v)
Flow rate:	0.8 ml/min
Temperature:	40° C
Detector:	UV at 248 nm or electrochemical detector at 0.0 V versus Ag/AgCl
Injection volume:	5-10 μl
Quantitation:	By peak areas using the internal standard method

Results

Retention times relative to phentermine (I.S.) are as follows:

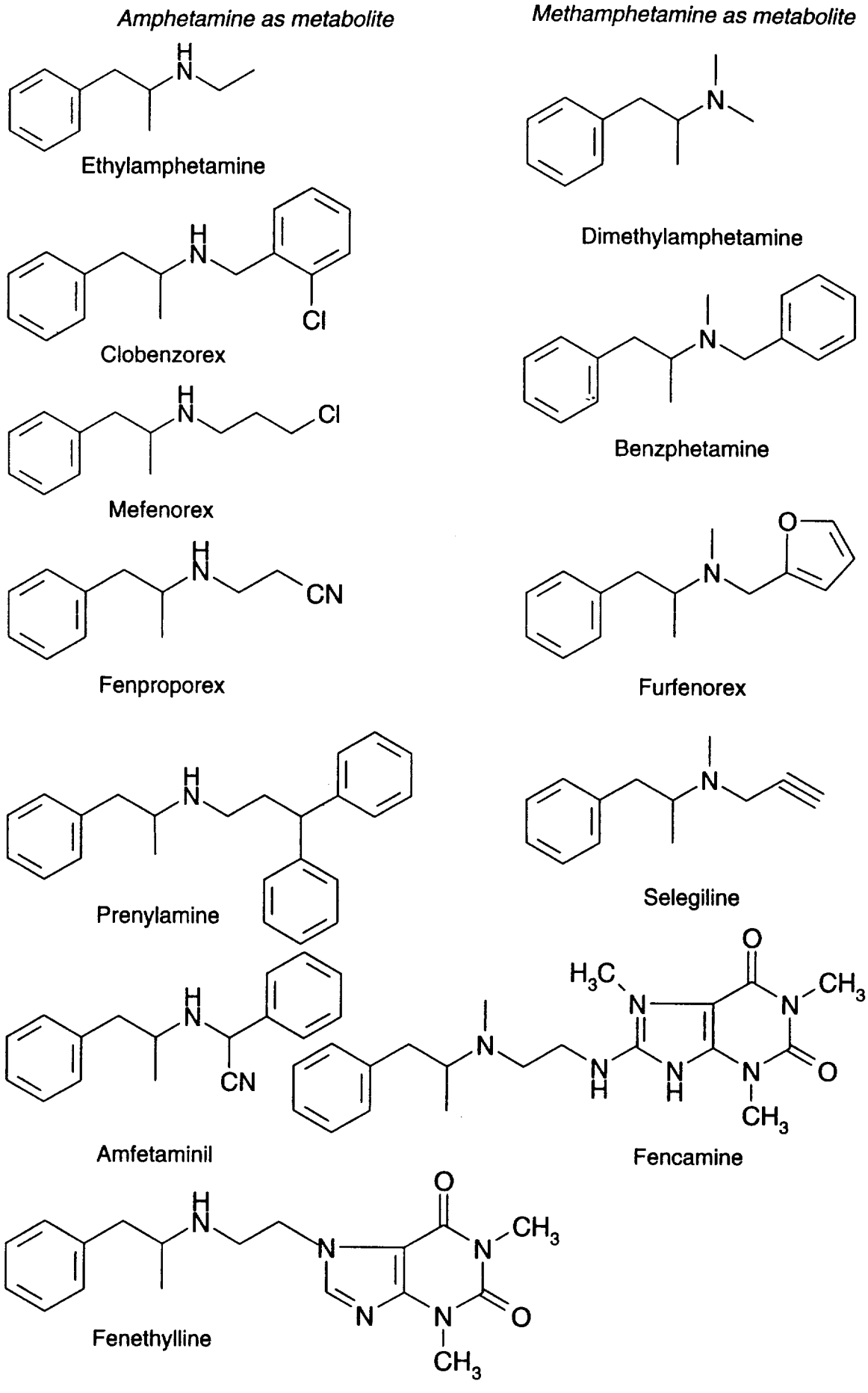
Amphetamine	0.57
Methamphetamine	0.70
Phentermine	1.00 (25.12 min)

E. Interpretation of results

Unchanged amphetamine has been detected in the urine up to 29 h after a single oral dose of 5 mg amphetamine. Unchanged methamphetamine has also been detected up to 23 h following a single oral dose. A positive amphetamine analysis generally indicates the use of amphetamine or methamphetamine within the previous 24-48 h.

Three additional points should be considered. Firstly, several non-proprietory drug preparations used as decongestants and anorectics contain ephedrine and phenylpropanolamine, which are capable of producing positive results with both EMIT and RIA tests if present in the urine in significant concentration. Secondly, several prescription drugs such as benzphetamine, fenfluramine, mephentermine, phenmetrazine and phentermine can also produce positive immunoassay results. Lastly, some drugs give amphetamine and methamphetamine in urine as their metabolites (figure V.4). It is therefore most important that, if any doubt exists concerning the provenance of amphetamine or methamphetamine detected in a urine sample, the urine should be re-examined for the presence of the parent drugs.

Figure V.4 Drugs metabolizing to amphetamine or methamphetamine



VI. Recommended methods for the detection and assay of ring-substituted amphetamine derivatives in biological specimens

A. Introduction

Illicit ring-substituted amphetamine derivatives are generally less frequently encountered than amphetamine and methamphetamine and differ from these also in their chemistry, pharmacology and methods of analysis. The following substances will be considered in this manual:

3,4-Methylenedioxyamphetamine	MDA
3,4-Methylenedioxymethamphetamine	MDMA, Ecstasy
3,4-Methylenedioxyethylamphetamine	MDE, MDEA
5-Methoxy-3,4-methylenedioxyamphetamine	MMDA
4-Methoxyamphetamine	PMA
4-Methoxymethamphetamine	PMMA
2,5-Dimethoxyamphetamine	DMA
2,5-Dimethoxy-4-methylamphetamine	DOM, STP
2,5-Dimethoxy-4-ethylamphetamine	DOET
3,4,5-Trimethoxyamphetamine	TMA
4-Bromo-2,5-dimethoxyamphetamine	DOB, Bromo-STP

Many other analogues of amphetamine and methamphetamine have been synthesized and some of these have been distributed illicitly on a limited scale in different localities, for example 3,4-methylenedioxyethylamphetamine and *N,N*-dimethylamphetamine. With the exception of PMMA they are also contained in the List of Substances in the Schedules to the Convention on Psychotropic Substances (1971). Background information concerning the history, physical properties and chemistry of these derivatives is given in the United Nations manual, *Recommended Methods For Testing Illicit Ring-Substituted Amphetamine Derivatives* [129] and will not be reproduced here. Additional information concerning the pharmacology and toxicology may be found in recent literature reviews [142-144].

1. Routes of administration

Virtually all of the illicit substances contain the drug in the form of the hydrochloride salt and appear as white or off-white powders, in tablets or capsules and, in the case of DOB, impregnated on paper (blotters) for oral administration. However, they can also be injected intravenously or can be inhaled by smoking, as for "ice" (see chapter V.A.2). The usual dose of MDA and MDMA ranges from 80 to 125 mg. DOM and DOB are generally taken orally in doses of from 15 to 25 mg.

2. *Metabolism and excretion*

Most amphetamine derivatives are rapidly absorbed from the gastrointestinal tract and readily cross the blood-brain barrier. The appearance of the psychotropic effects of the drugs is equally rapid.

The human metabolism of ring-substituted amphetamines has not been extensively studied. However, some generalisations can be made using the available human data and the results of metabolic studies in laboratory animals [144-153]. For all drugs, a significant fraction of the dose is excreted unchanged in urine. Therefore, the parent compounds are the target analytes for the detection of the use of these drugs by analysis of urine.

Known information concerning metabolism of these substances is summarized in table VI.1.

In a similar manner, little is known concerning the plasma half-lives of these derivatives and their excretion in urine. However, published studies involving DOM and DOET indicate that up to 20% of the former and 10-40% of the latter are excreted unchanged in the urine in the first 24 h. The peak urinary excretion occurs at 3-6 h for both drugs [150, 151].

In a controlled clinical study with 1.5 mg/kg body weight of MDMA, maximum plasma levels in the range of 0.33 μg/ml were observed after 2 h, with a plasma half-life of 8 h. Small amounts of MDA, the *N*-demethyl metabolite of MDMA, could be observed in plasma. Average urine levels of MDMA were about 1.4, 14 and 23 μg/ml after 1.5, 10 and 22 h, respectively. The main urinary metabolite were 4-hydroxy-3-methoxymethamphetamine and 3,4-dihydroxymethamphetamine, both excreted as glucuronides [153].

Table VI.1 Metabolism of ring-substituted amphetamine derivatives

Compound	*Species*	*Target compound*	*Other metabolites*	*References*
MDA	Rat	MDA	4-Hydroxy-3-methoxyamphetamine	145
MDMA	Human	MDMA	4-Hydroxy-3-methoxymethamphetamine	146, 153
			3,4-Dihydroxymethamphetamine (conjug.)	153
			3,4-Methylenedioxyamphetamine	153
PMA	Human	PMA	4-Hydroxyamphetamine	147
DOM	Human	DOM	4-Carboxy-2,5-dimethoxyamphetamine	148-150
DOET	Human	DOET		151
DOB	Human	DOB		152

B. Sampling and sample preparation procedures for the assay of ring-substituted amphetamine derivatives

The general procedures and precautions given in chapter I.C and G.5. are applicable to samples used for assay of ring-substituted amphetamine derivatives.

Precautions

Care must be taken when concentrating extracts containing ring-substituted amphetamine derivatives by evaporation as the free bases may be lost during evaporation of the solvent. These losses may be avoided by adding methanolic hydrochloric acid (methanol-concentrated hydrochloric acid (9:1, v/v; 50 μl)) to the extract to form the corresponding hydrochloride salts of the drugs before the solvent is evaporated.

1. *Sample preparation for immunoassay*

In general, very little sample preparation is required for initial immunoassay tests. It is unnecessary to hydrolyse the urine samples because immunoassays measure both the free and conjugated forms of the drug and/or metabolites. It may be necessary to adjust the pH or centrifuge the urine to remove turbidity. For optimum results, the manufacturer's instructions should be followed.

2. *Sample preparation for chromatography*

(a) Hydrolysis

For ring-substituted amphetamines there is generally no need for hydrolysis, except where low concentrations of mono- or dihydroxylated metabolites (e.g. of MDMA) have to be analysed [153].

(b) Extraction

The target analytes are the unchanged ring-substituted amphetamine derivatives which are extracted from urine using procedures similar to those described for amphetamine and methamphetamine (chapter V.B). Note that some of the metabolites of substances in this group may contain both acidic (carboxylic and phenolic) and basic (amine) groups. If these metabolites are to be analysed, a careful selection must be made of the extraction conditions, especially the pH.

Several solid-phase extraction procedures for MDMA, using mixed [154] or ion-exchange sorbents [138, 153], have been proposed. The following SPE method is recommended.

Urine (2 ml) plus internal standard (0.25 ml of an 8 μg/ml solution of the selected internal standard) are mixed with phosphate buffer (0.1 M, pH 6; 2 ml) [154]. The pH is adjusted to pH 5-7 with sodium hydroxide (0.1 M) or hydrochloric acid (0.1 M) if necessary. Solid-phase extraction cartridges (5 ml capacity, packed with modified silica containing mixed non-polar and cation-exchange groups, for example Bond Elut Certify®) are conditioned with methanol (2 ml) and phosphate buffer (0.1 M, pH 6; 2 ml). Urine samples are drawn through the cartridges slowly over a period of at least 2 min. The cartridges are washed with acetic acid (1 M; 1 ml) and then dried by drawing air through under full vacuum for 5 min. The cartridges are rinsed again with methanol (6 ml) and then dried again for 2 min. The analytes are eluted with ethyl acetate containing 2% conc. ammonia (freshly prepared; 2 ml). The eluate is evaporated carefully under a stream of nitrogen at a temperature of less than 40° C. Note that the addition of methanolic hydrochloric acid to these extracts to avoid evaporative loss of analyte (see chapter VI.B above) is inappropriate as it will result in a precipitate of ammonium chloride. This extract may be purified if necessary, depending on the subsequent method of analysis, by partitioning the residue between aqueous sodium hydroxide (1 M; 1 ml) and chlorobutane (2.5 ml). The upper organic layer is transferred to a clean glass tube and carefully evaporated to dryness. Methanolic hydrochloric acid may be added in this instance prior to evaporating the solvent.

(c) Internal standards

Selection of a suitable internal standard should observe the general criteria given in chapter I.G if possible. Suggested internal standards for GC or HPLC

analysis of ring-substituted amphetamines in urine are phentermine, propylamphetamine, methylenedioxypropylamphetamine and other amphetamine analogues. For GC/MS, the preferred internal standards are deuterated analogues of the target analytes, but if these are unavailable, one of the standards listed above for GC should be used.

(d) Calibration standards

Prepare a stock solution of each target analyte and of the internal standard in methanol at a concentration of 1 mg/ml. From these stock solutions prepare urine standards containing the target compound(s) in the range 0-5 μg/ml and internal standard at a concentration of 5 μg/ml. A set of calibration urine standards should be processed simultaneously with the test samples.

C. Screening methods

1. Immunoassay methods

General comments concerning the selection and use of immunoassays are given in chapter I.G.1. Most immunoassays for amphetamine-like drugs are designed to detect amphetamine and/or methamphetamine. However, some of the immunoassays show significant cross-reactivity with members of the family of ring-substituted amphetamine derivatives. Because immunoassays are not specific, positive results must always be confirmed by a second, more specific method.

Cut-off levels and detection limits have not been established for the ring-substituted amphetamines. However, some cross-reactivity data have been reported and these can be compared to cross-reactivities and cut-off levels for amphetamine and methamphetamine to estimate cut-off levels for the ring-substituted derivatives (see table VI.2).

Table VI.2 Cross-reactivities of commercial immunoassays for ring-substituted amphetamine derivatives

	Cross-reactivity (%)						
Assay	*MDA*	*MDMA*	*MDE*	*DMA*	*TMA*	*DOB*	*DOM*
EMIT-d.a.u. monoclonal	147 (1 000)[a,b]	74 (1 000)	--	--	--	--	--
FPIA-TDx amphetamine	15 (1 000)[c,d]	25 (1 000)	32 (1 000)	0.6 (5 000)	0.4 (5 000)	0.5 (5 000)	0.6 (5 000)
FPIA-TDx amphetamine/ Metham-phetamine II	148 (1 000)[c,d]	97 (1 000)	43 (1 000)	7 (5 000)	3 (5 000)	5 (5 000)	4 (5 000)
Abuscreen-Online	41 (1 000)[a,b]	0.2 (1 000)	66 (1 517)	0.5 (219 298)	--	--	--

[a]Cross-reactivity calculated by dividing the target concentration (1 000 ng/ml) by the concentration equivalent to 1 000 ng/ml d-amphetamine and multiplying by 100 (in parenthesis equivalents, ng/ml).

[b]Manufacturer's information.

[c]Cross-reactivity calculated by dividing the apparent (measured) concentration by the actual concentration and multiplying by 100 (in parenthesis concentration, at which cross-reactivity was determined, ng/ml).

[d]See J. T. Cody [128].

2. *Thin-layer chromatography*

Standard TLC technique

General comments concerning the application of thin-layer chromatography as a screening technique are given in chapter I.G.2. Details of standard TLC materials and procedures are outlined elsewhere [10] and these are applicable to the analysis of biological extracts.

TLC plates

Coating:	Activated silica gel G containing an additive which fluoresces when irradiated with UV light, wavelength 254 nm
Layer thickness:	0.25 mm
Size of plates:	Glass plates 20 × 20 cm, 20 × 10 cm or 10 × 5 cm; the optimum run is approximately 10 cm

Standard solutions

Make all standard solutions at a concentration of 1 mg/ml in methanol and apply 5 µl of each solution to the plate.

Procedure

Urine extracts are evaporated to dryness in a test tube, noting the precaution given in chapter VI.B, and redissolved in methanol (50 µl). The entire extract is spotted on the plate with a glass capillary.

Developing solvents [129]

System A:	Methanol	100
	Conc. ammonia	1.5
System B:	Ethyl acetate	85
	Methanol	10
	Conc. ammonia	5

Visualization

The plates must be dried prior to visualization. This can be done at room temperature, in an oven at 120° C for 10 min, or, more quickly, by use of a hot air blower. It is important for proper colour development, however, that all traces of ammonia be removed from the plate.

Fast Black K reagent [129-132]. (For preparation see chapter V.C.2)

Ninhydrin reagent. (For preparation see chapter V.C.2)

Fluorescamine reagent (Fluram). (For preparation see chapter V.C.2)

For the detection of low concentrations of primary amines the use of fluorescamine reagent (Fluram) is recommended (see chapter V.C.2).

Results

Table VI.3. $R_f \times 100$ values

Compound	Developing system	
	A	*B*
MDA	41	62
MDMA	31	62
PMA	41	62
DMA	37	65
DOM	35	63
DOET	36	61
DOB	37	62

D. Confirmatory chromatographic methods

1. Gas chromatography

(a) Sample derivatization

Heptafluorobutyric anhydride (HFBA) [134]

HFBA (50 µl) is added to the dry residue. The tube is capped, vortexed and incubated at 75° C for 20 min. The tube is uncapped and dried under air or nitrogen at 30° C. The contents are dissolved in 50 µl of ethyl acetate and 1-2 µl are injected onto the GC column.

Alternative procedure [135]

Potassium hydroxide (0.5 M; 50 µl) is added to the dry residue followed by 500 µl of toluene. After mixing and centrifugation, the organic layer is transferred to a clean test tube and 5 µl of HFBA is added. The solution is mixed thoroughly and sodium bicarbonate (10%, w/v; 500 µl) is added immediately with continuous mixing. The tube is centrifuged and 1 µl of the toluene (upper) layer is injected onto the GC column.

Trifluoroacetic anhydride (TFAA) [125, 136]

Ethyl acetate (100 µl) and TFAA (50 µl) are added to the dry residue. The tube is shaken and incubated at 60 °C for 20 min. The mixture is evaporated carefully to 50 µl final volume under a gentle stream of air or nitrogen at room temperature and 1-2 µl are injected onto the GC column.

(b) Method without derivatization: packed column technique

Operating conditions

Detector: FID, NPD

Columns: Glass packed columns (2 m × 3-4 mm I.D.) with dimethylsilicone (e.g. OV-1, SE-30) or methylphenyl silicone (e.g. DB-1, OV-17) liquid stationary phases

Carrier gas: Nitrogen at 30 ml/min.

Fused silica capillary columns with chemically bonded nonpolar stationary phases (e.g. SE-54) are a useful alternative to the packed columns described above. Helium carrier gas is used at a flow rate of 1 ml/min

Operating temperatures:
- Injector: 250-280° C
- Oven: 90-280° C (programmed, depending on the column used)
- Detector: 280-300° C

(c) Method with derivatization

Operating conditions

Detector: FID, NPD, ECD or MS with EI ionization, operated in the selected ion monitoring (SIM) mode.

Columns and temperatures: See method without derivatization above.

Results

Table VI.4 Retention data for ring-substituted amphetamines and their derivatives

Compound	Column		
	OV-1 or SE-30 underivitization[a]	*DB-1 underivitazation*[a]	*SE-54 HFBA derivitization*[b]
MDA	1 477	1 444	5.77
MDMA	1 585	1 501	6.87
PMA	1 412	1 346	4.77
DMA	1 558	1 527	6.24
DOM	1 618	1 593	6.65
DOET	1 654	1 654	7.23
DOB	1 809	1 786	8.70

[a]Retention indices.

[b]Retention times (min) on a SE-54 capillary column, 25 m × 0.3 mm I.D., film thickness 0.17 μm, temperature programmed from 120° C (1 min) to 280° C at 10°/min. The retention time of amphetamine on this system is 2.74 min.

2. *Gas chromatography-mass spectrometry*

When a mass spectrometer is used as a detector, the principal ions (m/z) in the EI^+ spectra are as given in table VI.5.

Table VI.5 Principal ions in the mass spectra of ring-substituted amphetamine derivatives

Compound	*Principal fragment ions (m/z)*	
	Underivatized	*HFBA derivative*
MDA	44, 135, 136	135, 162, 240
MDMA	58, 77, 135, 136	135, 162, 254

Table VI.5 *(continued)*

Compound	Principal fragment ions (m/z)	
	Underivatized	*HFBA derivative*
PMA	44, 78, 122	121, 148, 240
PMMA	58, 77, 78, 121	--
DMA	44, 72, 91	151, 178, 240
DOM	44, 151, 166	91, 135, 165, 193, 405
DOET	44, 165, 180	179, 206, 240
DOB	44, 215, 217, 230, 232	229, 231, 256, 258, 240

3. *High Performance liquid chromatography*

Two methods are given below for the assay of ring-substituted amphetamine derivatives in urine by HPLC [138-141].

(a) Method without derivatization [138]

Sample preparation

Extraction of samples is carried out as described earlier (chapter VI.B) using phentermine as internal standard. The extracts are evaporated to dryness under a stream of nitrogen, noting the precautions given in chapter VI.B.2, and then redissolved in 100 µl acetonitrile or mobile phase.

Standard solutions

Standard solutions are prepared by dissolving reference material in acetonitrile to give a concentration of 0.01 mg/ml.

Operating conditions

Column:	Octadecylsilica (Spherisorb ODS-1 or equivalent), 3 or 5 µm, 12.5 cm × 4.0 mm I.D. + precolumn
Mobile phase:	Acetonitrile-water (57:943, w/w) + phosphoric acid (8.5 g/l) + hexylamine (0.28 ml/l). Depending on the type of column used, k' values can be optimized by variation of the acetonitrile-water ratio
Flow rate:	0.8 ml/min
Detection:	UV at 190 nm
Injection volume:	10 µl
Quantitation:	By peak areas, using the internal standard method

(b) Method with derivatization [141]

Sample preparation

Extraction of samples is carried out as described earlier (chapter VI.B) using phentermine as internal standard. The extracts are evaporated to dryness in a glass

vial under a stream of nitrogen. They are then redissolved in aqueous sodium bicarbonate (2%, w/v; 200 µl) and aqueous sodium ß-naphthoquinone-4-sulphonate solution (0.5%, w/v; 200 µl) is added. The vials are capped and heated at 60° C for 30 min. After cooling, the aqueous solution is extracted with hexane-diethyl ether (2:1, v/v), using a vortex mixer for 1 min. The organic layer is transferred and evaporated to dryness under a stream of nitrogen. The residue is dissolved in acetonitrile (100 µl).

Standard solutions

Standard solutions are prepared by dissolving reference material in blank urine to give a concentration of 5 µg/ml.

Operating conditions

Column:	Octadecylsilica (µ-Bondapack C-18 or equivalent), 3 or 5 µm, 15 cm × 3.9 mm I.D.
Mobile phase:	Acetonitrile-methanol-0.01 M sulphuric acid (20:20:60, v/v/v)
Flow rate:	0.8 ml/min
Temperature:	40° C
Detector:	UV at 248 nm or electrochemical detector at 0.0 V
Injection volume:	5-10 µl
Quantitation:	By areas using the internal standard method

Results

Table VI.6 Retention times of ring-substituted amphetamine derivatives relative to phentermine (I.S.)

Compound	*Method without derivatization*	*Method with derivatization*
MDA	0.83	0.49
MDMA	1.19	--
PMA	0.90[a]	0.57
PMMA	--	0.76
Phentermine	1.00 (8.1 min; 3.4 min[a])	1.00 (25.12 min)
DMA	1.88	--
DOM	2.15[a]	1.44
DOET	4.06[a]	--
DOB	2.59[a]	1.72

[a]Determined with a mobile phase containing acetonitrile-water (182:816, w/w) + phosphoric acid (8.5 g/l) + hexylamine (0.28 ml/l).

E. Interpretation of results

Little has been published in the scientific literature concerning the range of concentrations which might be anticipated in casual or chronic users of ring-substituted amphetamines.

Following a single dose of MDA, plasma and urine concentrations of unchanged drug are reported to be less than 0.4 and 10 µg/ml, respectively. For PMA, the corresponding plasma and urinary drug concentrations are less than 0.2 and 5 µg/ml, respectively [84].

In abuse situations, plasma and urine levels of MDA have been found to be in the ranges 5-25 and 50-150 μg/ml, respectively. For PMA the corresponding ranges are 0.3-2 μg/ml plasma and 5-200 μg/ml urine [84].

After administration of 1.5 mg/kg body weight of MDMA, maximum plasma levels in the range of 0.33 μg/ml were observed, whereas urine concentrations of about 1.4, 14 and 23 μg/ml could be found after 1.5, 10 and 22 h, respectively [153].

Fatalities associated with these drugs have been reported. Blood and urine concentrations for MDA fatalities were in the ranges 2.3-26 μg/ml blood and 46-175 μg/ml urine, respectively [154-156]. Five fatalities associated with MDMA and MDEA were found to have blood levels ranging from 0.9 to 2 μg/ml blood [157]. Nine deaths involving PMA had blood and urine concentrations of PMA in the ranges 0.3-1.9 and 6.0-175 μg/ml, respectively [158].

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