UNITED NATIONS INTERNATIONAL DRUG CONTROL PROGRAMME
Vienna

Recommended Methods for the Detection and Assay of

Lysergide (LSD), Phencyclidine (PCP), Psilocybin and Methaqualone

in Biological Specimens

MANUAL FOR USE BY
NATIONAL LABORATORIES

Scientific Section

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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. illicit demand for drugs. Drugs seized are not only traditional drugs already under national and international control, but also include 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 the 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 pressure on nations to intensify regulatory effort, 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 analysis, it is now required to also produce reliable 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 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 achievement of these objectives.

An Expert Group in Kuala Lumpur in 1986[1], while working on recommended methods for testing seized cannabis and amphetamine/metamphetamine, recognized that an issue of increasing importance to all Member States was 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.

This proposal was endorsed by the Commission on Narcotic Drugs (CND) 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 substances in body fluids.

The International Conference on Drug Abuse and Illicit Trafficking (ICDAIT) 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[2].

In response to the suggestions of the CND and the ICDAIT, the former Division of Narcotic Drugs convened an Expert Group Meeting in 1987 on Guidelines for the Establishment of National Testing Programmes and Laboratories for Drugs of Abuse in Body Fluids. That Group recommended *(a)* the "publication of follow-up working manuals on the subject to serve as guidelines for laboratory and programme development" and *(b)* "the setting up of an expert review group that would periodically review methodology and drug-testing procedures"[3].

The CND, 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"[4].

In response to the Commission's request and at the invitation of the Government of Singapore, the former Division of Narcotic Drugs convened in 1989 an Expert Group Meeting on the Detection and Assay of Controlled Drugs in Biological Specimens and Recommended Methods for the Detection and Assay of Heroin/Morphine and Cannabinoids in Biological Specimens. A subsequent meeting was held in Madrid in 1990 on Methods for the Detection and Assay of Cocaine, Amphetamine, Metamphetamine, and Ring-Substituted Amphetamine Derivatives in Biological Specimens. At the invitation of the Government of Hong Kong, the UNDCP convened in 1995 an Expert Group Meeting on the Detection and Assay of Barbiturates and Benzodiazepines in Biological Specimens. This manual resulted from a Consultative Meeting in Barcelona in 1997 on Methods of Detection and Assay of LSD and other Hallucinogens and Methaqualone in Biological Specimens

B. Purpose of the manual

This manual, prepared by the Scientific Section, of the United Nations International Drug Control Programme, is part of a series related to the assay of drugs in biological specimens. It reflects the conclusions of the Consultative Meeting held in Barcelona in 1997 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 LSD and other hallucinogens and Methaqualone in biological specimens. 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. In this context, the reader is referred to the United Nations manual on Recommended Guidelines for Quality Assurance and Good Laboratory Practices (ST/NAR/25)[5].

In selecting methods, the Expert Group was aware that many laboratories in existence today utilize methods that meet or may exceed legislative requirements. However, it was noted that there was great diversity with regard to the structure of national programmes and laboratory equipment and methodologies 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 that may not have access to sophisticated equipment and methods. Each method has been recommended as suitable and reliable. In the process of identifying these methods the Expert Group was 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/her 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 analysis by following current literature on the subject. Useful adjuncts to this manual would be the United Nations manuals on Recommended Methods for Testing LSD, (ST/NAR/17)[6], Recommended Methods for Testing Psilocybin, (ST/NAR/19)[7], and Recommended Methods for Testing Methaqualone, (ST/NAR/15)[8]. The reader is also referred to the United Nations manuals:

- Recommended Methods for Detection and Assay of Heroin, Cannabinoids, Cocaine, Amphetamine, Metamphetamine and Ring-Substituted Amphetamine Derivatives in Biological Specimens (ST/NAR/27)[9].
- Recommended Methods for Detection and Assay of Barbiturates and Benzodiazepines in Biological Specimens (ST/NAR/28)[10].

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

United Nations International Drug Control Programme
Scientific Section
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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, i.e. the analysis of biological specimens 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, i.e. 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 specimen donor.

Because punitive action may be the consequence of positive analytical results, procedures and methods used must follow strict standards based on 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 radioimmunoassay (RIA), enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA) etc. This should 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 chromatographic techniques coupled to a suitable detector, preferably a mass spectrometer (MS).

B. Guidelines for the collection and 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[3], urine is the sample of choice for drugs-of-abuse testing. 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. In this booklet blood methods have also been included since the Expert Group considered these a useful addition to urine testing for LSD and other hallucinogens and methaqualone. The use of other biological materials like hair, sweat and saliva for the present purposes, i.e. establishing the illicit consumption of drugs, is the subject of another manual (ST/NAR/30)[11].

In order to maintain the validity of analytical results in the forensic context, particular care should be given to the **supervision** of specimen collection, transportation and storage.

Trained personnel having a clear understanding of the legal implications of the procedure must do supervision 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 is conducted at sites that 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.

Collection

☐ Staff at the **collection site** is responsible for the collection, labeling, packaging and transportation of samples, ensuring that the collection and storage procedures have the proper documentation and necessary security methods (chain of custody).

☐ All staff at the collection site must be provided with sufficient training to understand the collection process and its significance to the laboratory results.

☐ The collection site 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 that could be used to invalidate the sample and should be set up without soap dispensers or cleaning agents.

☐ The urine specimen (50 ml) should be collected in two suitable bottles (aliquots A and B). The bottles should not be totally filled.

☐ Immediately after collection, the temperature (32-38°C within 4 min) should be estimated and recorded. If adulteration is suspected the laboratory should be notified.

☐ The bottles should be securely stoppered, sealed and labeled. Steps should be taken to ensure that the integrity of the specimen is maintained, e.g. by use of a security seal consisting of sealing wax imprinted with a departmen-

Possible ways to invalidate urine specimens (adulteration)

- False results may be the consequence of specimen adulteration, for example by addition of pH-modifying (vinegar, ascorbic acid, lemon juice, lime solvent etc.), oxidizing (sodium hypochlorite, sodium nitrite), surface-active (detergent, soap etc.) and enzyme-deactivating (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 the urine specimen.
- In certain circumstances, adding illicit substances to the urine to produce positive results.
- Placing a pinhole in the bottom of the container that 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 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.
- Drinking large volumes of liquid to dilute the urine.

tal seal or some other measure to indicate that tampering with the specimen has occurred. It is important that the donor witnesses the sealing of the bottles and sign or initials the seal or label.

- Specimen labels (which are not easily removed) should be affixed to the urine container and not to the lids. This will prevent accidental or intentional switching of specimens and/or identifying labels.

The label should contain the following information, so that the correct identity of the donor and the specimen are assured:

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: ....

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*Some laboratories use a code number rather than the donor's name for reasons of confidentiality.

- Personnel details of each specimen donor are filled in a request form for analysis. The 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 labeling, 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.

Transport and storage

- After the request form has been completed, the specimen and request forms 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 specimens are protected from prolonged exposure to light and heat during the storage and shipment.
- The designated dispatch person is responsible for transporting the specimens to the laboratory and maintaining appropriate chain of custody records for ensuring that the specimens are not tampered with during transit.

Submission to the laboratory

- At the laboratory an authorized person should receive and carefully check the specimens and documents. One bottle of each urine specimen (or tube of blood) should be used for analysis and the other stored frozen for further analysis if necessary.
- After ensuring the specimens and request form are in order, a written acknowledgment of receipt should be made, signed and given to the dispatch person.
- Samples not complying with the correct collection, transportation and storage procedures should be rejected.
- The laboratory should maintain well-documented records and strict security to ensure integrity of specimens and confidentiality of results.
- If the analysis is delayed beyond one or two days, specimens should be stored frozen, in a locked refrigerator. Recommended Methods for Detection and Assay of Barbiturates and Benzodiazepines in Biological Specimens, (ST/NAR/28), United Nations 1995. When frozen, specimens will generally be stable for several months.

Request form for drug analysis

- A drug analysis request form that accompanies the specimens will allow the laboratory to check the individual specimens 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, specimen number, date and time of collection.

☐ Additional information that may be included on the form is a specification of the drugs for which the specimen is to be screened and a note of any suspicions concerning the validity of the specimen.

☐ After completion, the form should be signed by an authorized person and stamped with an official seal.

C. 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 analysis must be kept locked and secure.

☐ Reports should be accessible only to authorized persons.

D. 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.

E. Summary of security procedures

☐ In addition to the specimens, 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 specimen—labeling, packing and transportation to the laboratory.

☐ It is important that the donor witnesses the sealing of the container and signs or initials 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 (or blood) 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 should be kept strictly confidential and be accessible only to authorized persons.

F. Methodology

A screening test is initially performed. If it is positive, a confirmatory test must be performed on a second aliquot of the sample.

The 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 specificity and may result in cross-reactivity.

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

Immunoassay methods

Immunoassays are the methods of choice when large numbers of specimens must be screened within a limited time. Several immunoassay kits are available commercially for the screening of drugs of abuse. Laboratories should only use validated immunoassays. The most commonly used methods are radioimmunoassay (RIA), enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA), etc. RIA, FPIA and EIA require instrumentation that is relatively expensive.

The choice of technique would depend in most instances on the workload (number of specimens per day) handled by the laboratory, using either instrumental or non-instrumental methods.

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.

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.

Thin-layer chromatography (TLC)

TLC 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 is 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.

Gas chromatography (GC) and high performance liquid chromatography (HPLC)

GC and HPLC offer high sensitivity and specificity for confirmation of presumptive positive results in screening assays. The equipment is, however, relatively expensive and training and experience for these highly technical systems are critical.

Mass spectrometry in combination with either gas chromatography (GC-MS) or liquid chromatography (LC-MS)

GC-MS and LC-MS are the most sensitive methods available for confirmation of drug presence in a specimen. They require the greatest outlay in capital, training and maintenance costs. They are the methods least likely to be challenged in court and should be considered as a necessary and important asset in programmes where the control laboratory will be the final source of confirmation for questioned assays.

Sample preparation

In general, very little sample preparation is required for initial immunoassay tests. It is unnecessary to hydrolyze the urine specimens because immunoassays measure both the free and conjugated forms of the drug and/or metabolites. For optimum results, the manufacturer's instructions should be followed.

For chromatographic procedures, good sample preparation is extremely important. This is necessary because urine and blood are complex containing a mixture of large amounts of numerous organic and inorganic compounds in which the specific target analyte is found in minute amounts. Urine sample preparation usually involves hydrolysis and the extraction and purification of the analyte.

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 specimen 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 can provide greater sensitivity.
- The derivatised compounds may be more thermally stable.
- Chromatographic properties may be improved, i.e. peak shape, retention times and separations.
- The mass spectra may contain ions that are more suitable for GC-MS in the selected ion monitoring (SIM) than those of the underivatised forms.

Quantitative analysis

For the purpose of establishing the illicit use of drugs, it is not absolutely necessary to use quantitative analytical methods. However, when there is an established cut-off it is essential to accurately determine whether a drug's concentration is above or below the cut-off concentration. There are also 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 specimen.

Quantitative analysis by GC, HPLC, GC-MS or LC-MS requires an internal standard to be added to the specimen prior to extraction. An internal standard also permits the measurement of relative retention time. Internal standards should resemble the target analytes such that they can be extracted, derivatised and analyzed 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 the specimen such as other drugs or endogenous materials.

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

Usually, cut-off concentration values are established for the substances to be determined in biological matrices. Those values are chosen considering the expected concentrations after the use of the drug but simultaneously avoiding misinterpretation of results in those circumstances when inadvertent or passive exposure has occurred.

All analytical methods used for the detection of substances for which cut-off values are established must be considered as quantitative methods with limit of quantification (LOQ) equal to or lower than the required cut-off value.

In those cases, when the quantification is carried out using one calibration sample (or "standard"), it must be chosen with a concentration equal to the cut-off.

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

Concentration of analyte $X = C_{RS} \times A_X/A_{IS1} \times A_{IS2}/A_{RS}$

Where:

A_X = Peak area for analyte X obtained from the sample chromatogram
A_{IS1} = Peak area of internal standard obtained from the sample chromatogram
A_{RS} = Peak area for reference standard obtained from the standard chromatogram
A_{IS2} = Peak area of internal standard obtained from the standard chromatogram
C_{RS} = Concentration of analyte X in the reference standard solution

Once calculated, the result will only be used to know if the concentration is "greater than or lower than" the cut-off value.

A semi-quantification of a sample can also be performed in the same way but choosing the calibration sample with a concentration near the expected concentration of the sample.

G. Quality assurance

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

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 specimens and/or 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 over a period of time.

External quality assessment

Where possible, the laboratory should participate in an external proficiency testing programme. Such a programme should be conducted by an independent external agency. A programme under the aegis of the United Nations (UNDCP Inter-collaborative exercises) is available to laboratories interested in participating at the contact address:

United Nations International Drug Control Programme
Scientific Section
Vienna International Centre
P.O. Box 500
A-1400 Vienna
Austria
Telephone: 43 1 26060 4303
Fax: 43 1 26060 5866

H. Interpretation of results

The qualitative or quantitative analysis of a biological specimen 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 administered.

A positive result means that a drug or metabolite is present in the specimen at a concentration above or equal to the cut-off concentration (if established). Elimination from the body and drug concentrations in urine and blood depend on such factors as the route of administration, frequency and duration of use, rate of drug metabolism, subject's physical condition, collection time and fluid intake etc.. It is important to note, however, that the concentration of drug in urine can in no way be related to the level of impairment

II. LSD and other hallucinogens

A. Introduction

The hallucinogenic substances included in this manual which are controlled under the United Nations Convention on Psychotropic Substances (1971) are (+)-Lysergide (LSD), Psilocin and Psilocybin (Schedule I) and Phencyclidine (PCP, Schedule II). Other controlled drug substances producing hallucinogenic effects include THC (in high doses) and mescaline. Some of these are considered elsewhere (ST/NAR/27).

Hallucinogens are a diverse group of naturally occurring and synthetic drugs that fall into three main chemically related groups, based on tryptamine (for example, LSD and Psilocin), phenethylamine (for example, mescaline) and tropane alkaloids (for example, atropine, scopolamine and belladonna). PCP is synthetic, LSD is semi-synthetic and Psilocin and Psilocybin are naturally occurring. Not all of them have any accepted medical uses, although one exception to their general prohibition is the use of some hallucinogens in traditional religious or tribal practices.

Abuse and misuse of hallucinogens is internationally widespread which means that any forensic laboratory may encounter these compounds. All synthetic hallucinogens are produced in illicit laboratories while the naturally occurring substances are usually harvested locally in a seasonal pattern. There is no evidence that any significant international trade exists in these naturally occurring substances

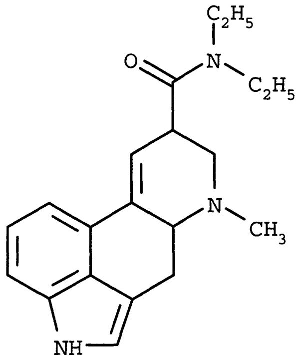
From INCB statistics, the significant hallucinogens in the last decade have been LSD, cannabinoids and some ring-substituted amphetamines.

Analysts should be aware of the particular hallucinogens commonly available in their area. For information on their characteristics and methodologies for their identification and analysis reference should be made to the United Nations manual on Recommended Methods for Testing Lysergide (LSD) (ST/NAR/17) and Recommended Methods for Testing Peyote Cactus (Mescal Buttons)/Mescaline and Psylocybe Mushrooms/Psilocybin (ST/NAR/19). The Multilingual Dictionary of Narcotic Drugs and Psychotropic Substances under International Control (ST/NAR/1/REV.1) published by UNDCP includes a listing of synonyms for hallucinogens.

Illicit synthesis of LSD

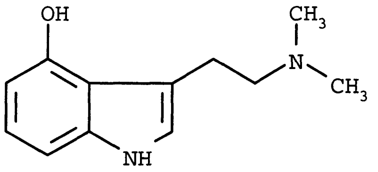
LSD is one of the most potent hallucinogenic substances known. Its properties were first discovered in the 1930s and for many years it was occasionally used experimentally in psychiatry. There has been no licit use for LSD in over 20 years and LSD products encountered today on the illicit market are produced only in clandestine laboratories.

Figure 1. LSD and other hallucinogens under international control

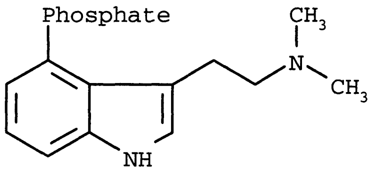


(+)-Lysergide (LSD),
d-Lysergic acid diethylamide
$C_{20}H_{25}N_{30}$
M.W. = 323.4
PKa : 7.8

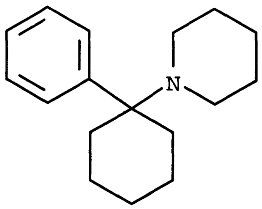
LSD tartrate
$(C_{20}H_{25}N_{3}O)_{2}C_{4}H_{6}O_{6}\bullet 2CH_{3}OH$
M.W. = 860.9



Psilocin
4-Hydroxy-N, N-dimethyl-tryptamine
$C_{12}H_{16}N_{2}O$
M.W. = 204.3
pK_a : 8.47



Psilocybin
4-Phosphoryl-N,N-dimethyl-tryptamine
$C_{12}H_{17}N_{2}O_{4}P$
M.W.= 284.3
pK_a : not available at time of publication



Phencyclidine
1-(1-phenylcyclohexyl) piperidine
$C_{17}H_{25}N$
M.W. = 243.4
pK_a :8.5

Phencyclidine hydrochloride
$C_{17}H_{25}N\bullet HCl$
M.W. = 279.9

LSD can be produced by several different methods, the majority of which use lysergic acid as the starting material. Lysergic acid itself is also produced in clandestine laboratories using, most commonly, ergometrine or ergotamine tartrate as starting material. Other ergot alkaloids may be substituted for these purposes although they are not used frequently. It is not known which synthetic method is most commonly employed by clandestine laboratory operations.

LSD synthesis from lysergic acid

There are three reported methods for producing LSD using lysergic acid as the precursor. The first involves treatment of lysergic acid with lithium hydroxide to form lithium lysergate that is then reacted with a sulphur-trioxide dimethylformamide complex and diethylamine to form the crude LSD product.

The second method employs the reaction of lysergic acid with N,N-carbonyldiimidazole followed by treatment with diethylamine. The last involves the reaction of lysergic acid with trifluoroacetic anhydride and treating the resulting mixed anhydrides with diethylamine.

LSD synthesis from ergot alkaloids

In this method an ergot alkaloid or a mixture of ergot alkaloids are used as the starting material. The alkaloid(s) is treated with hydrazine hydrate to form lysergic acid hydrazide. Using sodium nitrite, the hydrazide is converted to the azide that is then reacted with diethylamine to form the finished product.

All of the methods described for the synthesis of LSD produce a crude product that contains large amounts of iso-LSD and other by-products. Removal of these impurities is generally accomplished by chromatographying the crude product on an alumina column or by a series of partitioning between weak organic acids and weak bases with a suitable organic solvent. Tartaric acid, sodium bicarbonate and methylene chlorides are examples of the types of chemicals that have been used. Also, because of the instability of LSD base, the tartrate salt is generally produced.

This is done by precipitating the salt from a methanol solution of LSD base using a solution of tartaric acid in methanol as the precipitating reagent. Further details of the synthesis of LSD are contained in ST/NAR/10 (Clandestine Manufacture of Substances under International Control).

Lysergic acid synthesis

The most common method for producing lysergic acid in clandestine laboratories is the conversion of ergometrine or ergotamine tartrate to lysergic acid. This is accomplished by refluxing the ergot alkaloid with potassium hydroxide and hydrazine in an alcohol/water medium. Alternatively, lysergic acid can be produced by extracting lysergamide from Morning Glory or Hawaiian Baby Woodrose seeds and treating the purified extract of lysergamide in the same manner as described for ergotamine.

Lysergic acid can also be produced by fermentation of cultures of *Claviceps paspali* or *Aspergillus clavatus* or through a multi-step process beginning with methyl-6-methylnicotinoate.

Illicit synthesis of phencyclidine (PCP)

The preferred route in clandestine laboratories starts with condensation of 1-phenylcyclopentylamine with pentamethylene dibromide. The ethyl ether and other volatile solvents used in the process give off a distinctive odour that often gives away the location of the laboratory.

B. Physical and chemical characteristics of illicit products

Lysergide (LSD)

When LSD was first introduced into the illicit market in the 1960s, it was common to apply it to a variety of substrates by adding a drop of an LSD solution to an absorbent material. Among the substrates commonly used were sugar cubes, blotter or other absorbent paper and pharmacologically inert powders that were then used to fill empty gelatine capsules. Another common dosage form was termed "window panes" or "pyramids" in which the LSD was incorporated into a gelatine matrix and the solidified gelatine cut into small squares. The most common dosage forms, however, were tablets of various sizes, shapes and colours.

The content of the tablets was extremely variable, ranging from 20 to 500 micrograms of LSD due to the difficulty of obtaining a homogenous powder for tableting. Thus, even though LSD tablets continued to be the predominant dosage form in the 1970s, the number of types of tablets decreased, being limited to those laboratories which were able to produce a more uniform product. One type of tablet in particular, the "microdot", became prevalent and consisted of round tablets, approximately 1.6 mm in diameter, containing reasonably uniform dosage of about 100 micrograms of LSD per tablet.

In the 1980s, paper dosage forms became much more common. However, unlike earlier paper forms, where the LSD was dropped onto the paper and which are still frequently encountered in several countries, the new paper dosage forms are produced by soaking pre-printed paper in a solution of LSD, thereby ensuring a more uniform product. Typically, these sheets are perforated into squares of approximately 5 mm x 5 mm in size each containing a typical dose of 30-50 micrograms of LSD. A variety of designs have been encountered on these sheets, ranging from abstract art to cartoon figures.

At the present time, the great majority of the types of LSD dosage forms found in the illicit market are either paper dosage units, small tablets similar to the "microdots" and gelatine forms. The content of these forms is generally about 50 micrograms of LSD. Nevertheless, due to the ease with which LSD solutions can be applied to a variety of substrates, other forms should not be discounted.

Psilocybe mushrooms/psilocybin

Psylocybe mushrooms played a major role in the divinatory and magic rites of the ancient inhabitants of the Aztec Empire. Important religious cults based on the sacramental consumption of these "sacred" mushrooms called "teonanacatl" ("divine flesh"). Still today the hallucinogenic mushroom cult, e.g. by using *Psilocybe mexicana,* is deeply rooted in the native tradition of Mexican Indians.

The hallucinogenic mushrooms so far recognized all belong (except for *Amanita muscaria*) to a single group within the Basidiomycotina called the agarics (Agaricales). The term agaric is the general name applied to those fungi which basically possess an umbrella-shape, consisting of a cap (or pileus) on a centrally placed stem (or stipe), and radiating plate-like structures, called gills (or lamellae) on the underside of that cap. The gills bear on their surfaces the tissue that produces the basidia, those reproductive cells on which the spores develop. Note that some agarics, which may be mistaken for *Psilocybe* species, contain toxic material as for example gastroenteric irritants, cytolytic and/or haemolytic compounds.

The genus *Psilocybe* belonging to the family of Strophariaceae, is undoubtedly the most important, almost cosmopolitan genus of the hallucinogenic mushrooms. More than 140 different species are known, 80 of them contain psychotropic substances. They are found from the arctic to the tropics, although its main distribution is temperate. The species grow in the soil and on a variety of organic substrata such as humus, dung, rotting wood, peat, and also in clumps of mosses. Other Psilocybin containing mushrooms belong for example to the genus *Panaeolus* (Coprinaceae), *Conocybe* (Bolbitiaceae), *Inocybe* (Cortinariaceae), and *Pluteus* (Pluteaceae). The most important of these species, from the aspect of drug abuse, are *Psilocybe semilanceata* and *Psilocybe cubensis*. Since a few years, a marked increase can be observed in the abuse—by ingestion of fresh or dried fruit-bodies—of these potent narcotic and therefore in many countries illicit drugs.

Psilocybe semilanceata (FR.) QUEL., "Liberty Caps", is the most widespread psilocybin containing mushroom occurring for example in North and Middle Europe, North America, Russia and Australia. It grows very scattered to gregarious on rich soil, among grass in fields near farmyards, in well-manured pastures or meadows.

Psilocybe cubensis (EARLE) SINGER (synonym: *Stropharia cubensis* EARLE) is known from southern Mexico, Central America, West Indies, Florida, South America and south-eastern Asia. It grows singly or in small groups, usually on dung or rich pasture soil.

The main alkaloids present in these species are the phosphorylated indoleamines psilocybin (4-phosphoryloxy-N,N-dimethyltryptamine) and Baeocystin (4-phosphoryloxy-N-methyltryptamine, norpsilocybin), possibly the direct biochemical precursor of psilocybin. Psilocin (4-hydroxy-N,N-dimethyltryptamine), the de-phosphorylation product and psychotropic metabolite of psilocybin, is usually only present in traces. It is formed enzymatically or more often by inappropriate drying and storage. Until now it is not known whether Baeocystin produces psilocybin-like psychotropic effects. The content of Psilocybin and Baeocystin varies between 0.2-2% and 0.05-0.7%, respectively.

Phencyclidine

Phencyclidine comes in a wide variety of forms like tablets, capsules, powders and liquids. It can be found on papers, leaf mixtures (e.g. parsley, oregano, pepper, mint, tea or marihuana) or cigarettes (e.g. dipped in liquid phencyclidine). A number of phencyclidine analogues or precursors appeared in street drugs, e.g. 1-(1-(2-thienyl)cyclohexyl) piperidine (TCP), N-ethyl-phenylcyclo-hexylamine (PCE), 1-(1-phenylcyclohexyl)pyrrolidine (PHP or PCPy), 1-piperidinocyclo-hexanecarbonitrile (PCC), 1-phenylcyclohexylamine and phenylcyclopentyl-piperidine (PCPP)[12]. Leaf mixtures typically contain about 1 mg of phencyclidine per 150 mg of plant material (range 0.25%-8.0% PCP)[13].

III. Recommended methods for the detection and assay of LSD

A. Pharmacology

Current patterns of use

LSD continues to be widely abused, particularly by teenagers in various areas of the world, including Europe and the United States[14, 15]. Its popularity is partly due to wide availability and relatively low cost. A street dose generally contains between 20 and 80 g of LSD, and is ingested orally.

Development of tolerance and dependence

After administration, the effects of LSD typically last for 6 to 12 hours[16]. Tolerance develops after 3 or 4 days of continuous use, but disappears after a short period of abstinence. Discontinuing use of LSD does not result in withdrawal symptoms.

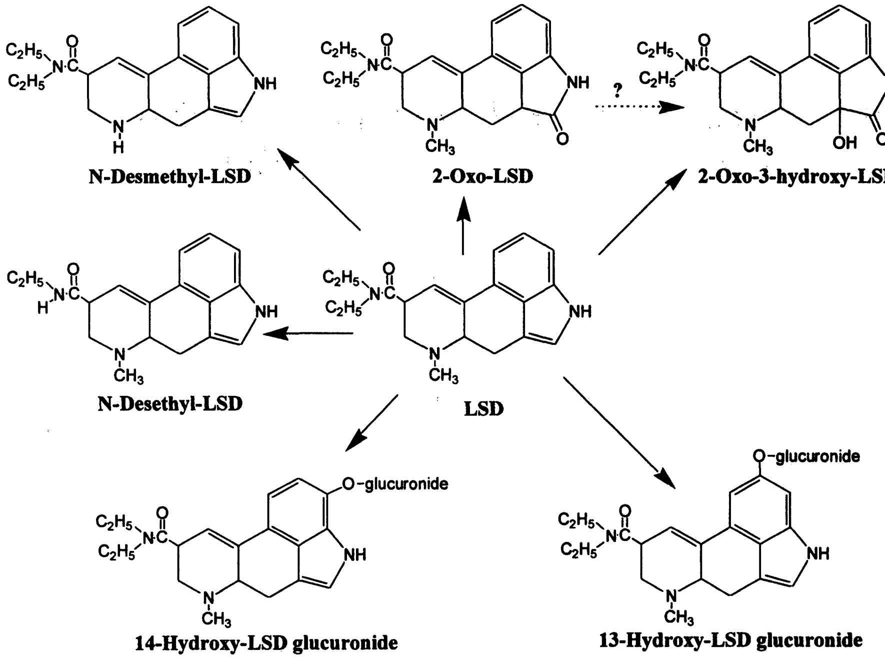
B. Disposition

Metabolism and disposition

LSD is rapidly metabolized. Only about 1 to 3% of an oral dose of LSD is excreted in the urine as unchanged LSD[17]. Investigation of the metabolism of LSD in humans has been severely limited by restrictions to clinical studies involving administration of the drug. However, N-desmethyl LSD ("nor-LSD") and 2-oxo-3-hydroxy-LSD have been conclusively identified in urine from LSD users[17, 18]. In addition, glucuronide conjugates of 13- and 14-hydroxy-LSD have been tentatively identified in LSD-positive urine samples[17], and lysergic acid ethylamide has been identified in human liver microsomes in vitro[19]. The known and tentatively identified human metabolites of LSD are summarized in figure 2.

Contrary to statements in the literature that 2-oxo-LSD is the major metabolite of LSD in humans, recent studies indicates that it is only a minor metabolite. Very little quantitative data have been reported for metabolites of LSD. However, unpublished LC/MS/MS data show that 2-oxo-3-hydroxy-LSD and the glucuronide conjugate of 13-hydroxy-LSD can be detected in urine for a longer period than LSD. The relative proportions of an LSD dose excreted in urine and faeces have not been determined, although radiolabelled LSD has been administered to monkeys[20] and to baboons [unpublished].

Figure 2. Known and suspected human metabolites of LSD



Iso-LSD, an inactive diastereoisomer of LSD, is often present in urine samples from LSD users, sometimes at concentrations higher than LSD itself. However, iso-LSD is not formed metabolically from LSD, and therefore its presence in the urine samples must be due to its presence as a contaminant in the ingested LSD.

Blood concentrations

Blood concentrations following LSD ingestion are typically less than 10 ng/ml. The peak concentration following a 1-μg/kg (70 μg) dose was 1.9 ng/ml at 3 h[21]. In two separate studies, a dose of 2 μg/kg of LSD gave a peak concentration of 5 ng/ml at 1 hour[22] [9] and 9 ng/ml within 5 h[23]. After administration of a 4-μg/kg dose to each of two volunteer subjects, the peak plasma concentrations were 9.7 and 7.4 ng/ml[18].

Reported elimination half lives have ranged from 2.4 to 5.0 h[18,21]. The volume of distribution is reported to be; 0.3 l/kg, and protein binding is 90%.

Urine concentrations

Urine concentrations of LSD as high as 26 ng/ml have been reported[24]. However, peak urine concentrations following oral ingestion of a typical street dose (20 to 80 μg) are normally less than 10 ng/ml and drop below 1 ng/ml within 12 to 24 hours. Thus, extremely sensitive analytical methods are required to detect LSD use for more than 1 day after ingestion of the drug.

C. Toxicology

In pharmacological terms, LSD is not highly toxic. Nevertheless, LSD is considered dangerous because it can produce panic, delirium and bizarre behaviour, sometimes resulting in irrational and injurious acts. These acts can occur with usual doses, even to an experienced LSD user[14].

One of the well-known side effects of LSD is the occurrence of flashbacks. These are repetitions of hallucinatory and other perceptual experiences that occur after the initial LSD experience has subsided. They may last for minutes to hours and may occur up to several years after the last LSD use. The mechanism is unknown, but it is thought to result from some permanent neuronal damage that shows up later as flashbacks upon a particular stimulation of the serotoninergic system. Flashbacks occur in approximately half of the users, whether they have used LSD a few times or hundreds of times.

D. Methods of analysis

Stability of LSD

LSD is sensitive to ultraviolet light, elevated temperatures, and extremes of pH. However, instability is not a major analytical problem if reasonable precautions are taken in the handling and storage of standards and specimens containing the drug.

In terms of photosensitivity, only light containing wavelengths in the ultraviolet (e.g. sunlight) rapidly degrades LSD. For example, after exposure of solutions of LSD in clear glass vials to sunlight for 13 hours, the LSD concentrations decreased by 90%[25]. However, LSD concentrations in urine stored for one month in polyethylene bottles at room temperature and exposed to normal room light changed by less than 10%[26].

LSD partially epimerizes to iso-LSD under alkaline conditions. At a pH of 7.0 or higher, a LSD/iso-LSD ratio of 9:1 is achieved after one week at 45°C, or two weeks at 37°C[27]. LSD is also unstable at pH < 4[28].

Availability of standards. Standards are available; contact the UNDCP for details.

Screening methods

Immunoassay

A number of different immunoassay methods are available for screening for the presence of LSD in urine. Most screening methods use a cut-off of 500 pg/ml, although 100 and 250 pg/ml have also been used, mainly with RIA methods. The commercially available immunoassays are listed in table 1, together with some of their characteristics. They can be subdivided in homogeneous (i.e. requiring no separation between bound and free fraction) and heterogeneous assays.

Table 1. Available immunoassays for LSD screening in urine

Assay	*Homogeneous or heterogeneous*	*Principle*	*Sample volume (µl, including dead volume)*	*Typical duration of analysis*	*Remarks*
RIA DPC	Heterogeneous	RIA	100	2 h	
EIA Cozart	Heterogeneous	ELISA	25	2.5-3 h	
EMIT	Homogeneous	Enzyme multiplied immunoassay technique	< 100	12 min*	
CEDIA	Homogeneous	Enzyme linked (cloned enzyme donor)	< 100	12 min*	Uses 3 reagents
Online/KIMS	Homogeneous	Particle agglutination	< 100	< 20 min	Online uses 2 reagents

*On a Hitachi (917 or 911) analyser.

Note. The cross reactivity for LSD metabolites and analogues is given in table 2.

Table 2. Cross reactivity (CR,%) of LSD metabolites and analogues in different immunoassays at the mentioned concentrations (in ng/ml): Analytical technique

	RIA DPC		*EIA*		*EMIT*		*CEDIA*		*KIMS*	
Compound tested	*Conc*	*CR*	*Conc*	*CR*	*Conc*	*CR*	*Conc*	*CR*	*Conc*	*CR*
nor-LSD		1	1	25	20	1.7	100	0.57	1.8	28
iso-LSD	ND	ND	ND	ND	280	0.18	2500	0.037	11	4.5
LAMPA	100	5.6	ND	ND	ND	ND	1	44	16	3.1
2-oxo-3-OH LSD	ND	ND	ND	ND	29.36	1.7	30	1.82	4.4	11
Lysergic acid	100000	0	10000	<0.05	100000	0	100000	0.01	86000	0.0006
2-oxo-LSD		11	ND	ND	ND	ND	ND	ND	ND	ND

Note. ND: not done.

Interferences. A high rate of false positive results has been demonstrated for the EMIT assay[29]. Reported causes include the presence of sertraline, metoclopramide, pipamperone, or haloperidol[30]. False positive results have also been encountered with the CEDIA assay[31].

Analysis of urine samples from controlled studies showed that DPC RIA had the best agreement with GC-MS/MS, followed by Roche RIA, EMIT and CEDIA, Roche Online and STC. These discrepancies can best be explained by the presence of LSD metabolites that cross-react with the antibodies used in the immunoassay[32].

Thin layer chromatography (TLC)[24]

Methods based on thin layer chromatography are not sensitive enough to detect LSD at concentrations lower than 1 ng/ml. An instrumental high performance thin layer chromatography (HPTLC) method has a detection limit of 0.4 ng/ml.

Sample preparation

☐ To 8 ml of urine in a screw-cap tube (16 x 125 mm), add 100 µl of a 1µg/l methysergide (internal standard), 100 µl 6 N sodium hydroxide and 100 µl of saturated ammonium carbonate.

☐ Vortex mix for 15 s and add 5ml of extraction solvent (petroleum ether-dichloromethane-isoamyl alcohol (70:30:0.5)).

☐ Cap the tubes and rotomix for 15-20 minutes at approximately 15 rpm.

☐ Centrifuge the tubes for 10-15 min. at 4000 rpm.

☐ Transfer the upper organic layer into correspondingly labelled 16 x 125 mm screw-cap tubes and to each tube, add 5 ml of 0.1 M ammonium hydroxide solution.

☐ Cap the tubes, rotomix for 15-20 min. at approximately 15 rpm and then centrifuge for 5 min. at 4000 rpm.

☐ Transfer the upper organic layers to correspondingly labelled 13 x 100 mm tubes, being careful not to transfer any of the aqueous lower layer.

☐ Add a spatula tip (approximately 0.2 g) of sodium sulphate to each tube, vortex for approximately 15 s.

☐ After standing at room temperature for 2-3 minutes, decant the solvent into correspondingly labelled 12 x 75 mm tubes and evaporate to dryness under nitrogen at approximately 50°C.

☐ Reconstitute each residue with 25 µl of dichloromethane-isopropyl alcohol (19:1).

HPTLC analysis

☐ Apply the reconstituted residue to the pre-absorptive region of the HPTLC plate (Whatman 10 x 10 cm LHP-K linear-K high-performance silica gel with a layer thickness of 200 m and a pre-absorptive region) in a 1 cm vertical streak using a 25 µl Hamilton syringe. On each plate, include a LSD neat spot and at least one extracted spiked standard and/or control.

☐ Place the spotted plates in a paper-lined developing tank and allow to equilibrate with the pre-developing solvent, ethyl acetate, for 10-15 min.

☐ After the clean-up development in the ethyl acetate to a height of approximately 95 % of the plate to remove interfering substances, remove the plates from the tank and permit to air dry at room temperature protected from the light.

☐ Place the plates in a second paper-lined developing thank and equilibrate with chloroform-methyl alcohol (90:10) for 10-15 min. followed by development to 60 % of the plate height.

☐ Scan the plates with a fluorimetric scanner (excitation wavelength from mercury lamp = 313 nm, emission wavelength filter 320-400 nm).

☐ Spray the plates with DMAB spray reagent (2.5 g of p-dimethylaminobenzaldehyde dissolved in a mixture of 225 ml ethyl alcohol and 25 ml concentrated hydrochloric acid) to visualise the spots chromogenically. LSD and methysergide are blue-coloured spots against a yellow background at R*f* 0.53 and 0.51, respectively.

High performance liquid chromatography (HPLC) with fluorescence detection (HPLC-FL)

The native fluorescence of LSD is used for detection. Methods using different columns and mobile phases have been published:[25, 33, 34, 35, 36, 37]

Lysergol, methysergide and LAMPA have been used as internal standards. The detection limit of HPLC-FL methods is approximately 0.5 ng/ml.

Sample preparation [36]

- Blood, serum or urine (1-3 ml) are diluted to 20 ml with borate buffer pH 9.5 (5 g $Na_2B_4O_7$.10 H_2O in 1 L). The determinations are carried out with external standardisation.
- Apply the diluted specimen to an Extrelut® (or equivalent) column.
- Elute the column with dichloromethane-isopropanol (85:15).
- Evaporate to dryness.
- Reconstitute the residue with 200 L methanol.
- Inject 10 µl in the HPLC system.

Instrumental conditions

The analysis is carried out with a HPLC system with a fluorimetric detector, with the excitation wavelength set at 325 nm and the emission wavelength filter at 430 nm.

The specificity of the method is increased, either by performing the analysis with two mobile phases (A and B) on a reversed-phase column (column 1), or by using a column switching system, where the columns are connected one after the other.

Column 1: Merck Hibar steel column EC 250 x 4 mm with C_8 reversed-phase material LiChrosorb (7 m).

Column 2: Merck Hibar steel column EC 125 x 4 mm with Kieselgel Merck LiChrosorb Si 60 (5 m).

Flow: 1.5 ml/min.

Oven temperature: 60°C

Mobile phase A: methanol-water with 3 g KH_2PO_4/L (adjusted to pH 3 with H_3PO_4 (50:50))

Mobile phase B: methanol-water with 1% $(NH_4)_2CO_3$ (60:40)

Single column mode A: use column 1 and mobile phase A

Single column mode B: use column 1 and mobile phase B

Column-switching mode: first column 1 with mobile phase A; after 3.2 minutes, switching to column 2. There is no interference from iso-LSD, lysergic acid and ergotamine.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS is the technique most often employed for quantification and confirmation of LSD in biological specimens because of the high degree of sensitivity and specificity that is required in view of the very low concentrations found in blood and urine specimens from LSD users. All published GC-MS methods for the determination of LSD and/or its metabolites in physiological specimens include one or more extraction steps, derivatisation to improve the chromatographic behaviour of the analytes, and analysis by gas chromatography in combination with either single-stage or tandem mass spectrometric detection. Methods of ionisation have included electron ionisation (EI), negative ion chemical ionisation (NCI), and positive ion chemical ionisation (PCI).

Sample preparation

At a pH > 8.0 LSD can be efficiently extracted from urine or serum samples with a relatively non-polar solvent such as n-butyl chloride[15, 26]. Additional cleanup can be achieved by back-extraction of the basic LSD into a phosphate buffer (pH 4.5), and then re-extraction into n-butyl chloride after addition of ammonium hydroxide to the aqueous layer[28].

Solid-phase extraction methods have also been used for isolation of LSD from biological matrices[25, 38, 39, 40]. Most of the solid-phase extractions employ an adsorbent possessing both hydrophobic and cationic characteristics. After the biological specimen is adjusted to a pH of 5 to 6, it is added to the extraction column, and after washing the column with a dilute acid and methanol, the LSD is eluted with an organic solvent such as ethyl acetate containing 2 to 4% ammonium hydroxide.

Immunoaffinity resins can achieve very selective extraction of LSD and some of its metabolites from biological samples[25, 41, 42].

Derivatisation

Trimethylsilylation of the indole nitrogen is the derivatisation most often used for GC-MS analysis of LSD. Underivatised LSD can be gas chromatographed, but sensitivity is generally severely limited due to adsorptive losses during the chromatographic process. The specific trimethylsilylating reagents used include bis (trimethylsilyl)trifluoroacetamide (BSTFA)[26, 28] and N-methyl(trimethylsilyl)- trifluoroacetamide (MSTFA) in pyridine (1:1 v/v)[15].

Treatment of extracts containing LSD with trifluoroacetylimidazole in the presence of 10% 1,4-dimethylpiperazine in toluene converts LSD and the N-desmethyl metabolite of LSD to their respective trifluoroacetyl derivatives, which are efficiently ionized by negative ion chemical ionization[17].

Gas chromatography

Both dimethylsilicone and methylphenylsilicone capillary GC columns have been used for LSD analysis. However, in order to achieve optimum sensitivity, it is critically important that the capillary column be well deactivated. Even derivatised LSD has a strong tendency to undergo adsorptive losses at active sites in the chromatographic column.

Mass spectrometry

Most published GC-MS assays for LSD are based on electron ionisation (EI-MS) of the trimethylsilyl (TMS) derivative and selected ion monitoring of the molecular ion at m/z 395 and two abundant fragment ions. The selection of which fragment ions to monitor is influenced by the choice of internal standard. If N-CD_3 LSD is the internal standard, the major fragment ion at m/z 253 is not suitable for monitoring because it is common to the EI mass spectra of the TMS derivatives of LSD and N-CD_3 LSD. For this reason the structural analogue, lysergic acid methylpropylamide (LAMPA), is often chosen as the internal standard. The TMS derivatives of LSD and LAMPA are easily separated chromatographically and give very similar mass spectra. Therefore, the molecular ion (m/z 395) and the two most abundant fragment ions (m/z 253 and 293) can be monitored for both LSD and LAMPA.

Recommended methods

Recommended GC-MS method for determination of LSD in urine[26]

The following procedure has been used successfully to measure LSD concentrations in urine over the range 0.5 to 10 ng/ml. It employs a relatively simple liquid/liquid extraction, derivatisation with BSTFA, and analysis using capillary column gas chromatography and electron impact mass spectrometry with selected ion monitoring.

The sensitivity of this method is limited primarily by interferences from background peaks. A lower limit of quantification can be achieved with a more selective extraction procedure that incorporates a back-extraction cleanup step, and/or additional purification by solid-phase extraction[28]

Sample preparation

☐ To 5 ml of urine in a silanized, screw-cap tube (16 x 100 mm), add 25 ng of the internal standard, lysergic acid methylpropylamide (LAMPA), in methanol.

☐ The urine is made basic (pH > 8) by addition of approximately 0.5 g of $NaHCO_3$ and 0.25 ml of concentrated NH_4OH.

☐ After addition of 5 ml of n-butyl chloride, the sample is mixed for 10 min and then centrifuged for 5 min at 2000 rpm.

☐ The upper organic layer is transferred to a 5-ml conical, screw-cap vial and evaporated to dryness under a stream of air at 60°C.

☐ The extract is derivatised by adding 20 µl of BSTFA and heating the capped vial at 70°C for 10 min.

☐ Approximately 2 µl of the derivatised extract is injected into the GC-MS.

Instrumental conditions

Injector and transfer line temperatures: 250°C

Injection mode: splitless

Carrier gas: helium, 1 ml/min flow rate

GC column: a well deactivated methylsilicone or 5% phenylmethylsilicone fused silica capillary column, 12 m x 0.20 mm i.d., 0.33 m film thickness

Oven temperatures:

Initial temperature: 190°C
Temperature programme: 20°C/min
Final temperature: 300°C
Final time: 6 min

Mass spectrometer:

Electron ionization (EI)
Electron energy: 70 ev
Electron multiplier voltage set for optimum signal-to-noise
Selected ion monitoring of the following m/z values:
    m/z 395 (molecular ion of LSD-TMS and LAMPA-TMS)
    m/z 253 (fragment ion for both LSD-TMS and LAMPA-TMS)
    m/z 293 (fragment ion for both LSD-TMS and LAMPA-TMS)
Dwell time at each m/z: 100 ms
Selected ion monitoring window: ± 0.3 amu
Typical retention times:
    LSD-TMS, 7.0 min
    LAMPA-TMS, 7.3 min

Recommended GC-MS method for determination of LSD in serum[15]

The following procedure is similar to the recommended GC-MS method for determination of LSD in urine; the main difference being addition of a back-extraction cleanup step. The LSD extraction efficiency is reported to be 76% and the lower limit of quantification is 0.1 ng/ml. Also, this procedure calls for derivatisation of LSD with MSTFA in pyridine (1:1 v/v).

Sample preparation

☐ To 1 ml of serum add 10 µl of a methanolic solution containing 2 ng of CD$_3$ LSD as the internal standard.

☐ Add 0.1 ml of a saturated aqueous solution of Na$_2$CO$_3$ and 0.2 ml of NH$_4$OH.

☐ Add 5 ml of n-butyl chloride, mix for 5 min and centrifuge for 5 min at 2000 rpm.

☐ Transfer the n-butyl chloride layer to a silanized glass vial and evaporate to dryness under a stream of air at 60°C.

☐ Dissolve the residue in 5 ml of phosphate buffer (pH 4.5) and add 5 ml of a mixture of n-butyl chloride and cyclohexane (1:1 v/v). Mix for 10 min and centrifuge for 5 min at 2000 rpm.

☐ Discard the upper organic layer. Add 0.1 ml of the saturated solution of Na$_2$CO$_3$ and 0.5 ml of NH$_4$OH. Add 5 ml of n-butyl chloride. Mix for 5 min and centrifuge for 5 min at 2000 rpm.

☐ Transfer the organic layer to a silanized glass vial and evaporate to dryness under a stream of air at 60°C.

☐ Add 20 µl of MSTFA in pyridine (1:1 v/v) and heat the capped vial at 80°C for 15 min.

☐ Add 50 µl of hexane to the derivatized extract. Vortex and inject approximately 2 µl into the GC-MS.

Instrumental conditions

The same instrument conditions can be used as described for the recommended method for GC-MS determination of LSD in urine.

Recommended GC-MS/MS method for determination of LSD in blood, plasma or urine samples[39]

Because extremely low concentrations of LSD are often present in blood and urine from LSD users, even more sensitive analytical methods than those described above are sometimes needed. Of the methods published to date, the combination of gas chromatography and tandem mass spectrometry (GC-MS/MS) provides the best sensitivity, with a reported lower limit of quantification of 20 pg/ml and a limit of detection of; 5 pg/ml.

The recommended GC-MS/MS method consists of a solid-phase extraction, derivatisation with BSTFA, analysis by capillary chromatography and positive ion chemical ionization, and detection by selective reaction monitoring tandem mass spectrometry. Only the initial sample preparation procedures differ for urine and blood samples.

Preparation of urine samples

☐ To 4 ml of urine in a 16 x 100 mm screw cap tube, add 75 µl of a methanolic solution containing 16 pg/µl of LAMPA to give a concentration of 300 pg/ml of the internal standard. Vortex.
☐ Add 2 ml of 0.1 M phosphate buffer (pH 6). Vortex.
☐ Check the pH to insure that it is 6.0 ± 0.5. If not, adjust pH accordingly.

Preparation of blood and plasma samples

☐ To 2 ml of whole blood or plasma in a 16 x 100 mm screw cap tube, add 50 µl of a methanolic solution containing 16 pg/µl of LAMPA to give a concentration of 400 pg/ml of the internal standard. Vortex.
☐ Add 2 ml of 0.1 M phosphate buffer (pH 6) to each tube. Vortex.
☐ Add 5 ml of blank urine to each sample. Vortex.
☐ Centrifuge for 15 min at 3000 rpm.
☐ Pour supernatant into a clean 16 x 100 mm screw cap tube.
☐ Check pH of each sample to insure that it is 6.0 ± 0.5. If not, adjust pH accordingly

Solid-phase extraction

☐ Place a 24 x 100 mm culture tube in the centrifuge for each sample.
☐ For each sample, fit a Varian BondElut Certify I extraction column (or equivalent) with a plastic sleeve and place in 24 x 100 mm culture tubes.
☐ Condition the extraction columns for each sample as follows:
☐ Add 2 ml of methanol and centrifuge for 1 min. at 400 RPM
☐ Add 2 ml of 0.1 M phosphate buffer (pH 6) and centrifuge for 1 min. at 400 RPM.
☐ Add each diluted urine sample to an extraction column and centrifuge for 5 min. at 400 RPM.

☐ Add 1 ml of 1.0 M acetic acid to each extraction column and centrifuge for 2 min at 400 RPM (Prepare 1.0 M acetic acid fresh daily).

☐ Add 5 ml of methanol to each extraction column and centrifuge for 5 min. at 1800 RPM.

☐ Transfer each column to a clean 13 x 100 mm tube. Discard waste in 24 x 100 mm culture tubes.

☐ Elute analyte and internal standard with 2 ml of ethyl acetate containing 4% ammonium hydroxide (96:4, $EtOAc:NH_4OH$). Centrifuge for 5 min. at 200 RPM.

☐ Evaporate to approximately 400 ml in a Savant Speed Vac.

☐ Transfer extract to 0.7 ml ALS vial and evaporate to dryness in Speed Vac.

☐ Add 10 µl of BSTFA, cap, and incubate for 30 min. at 85 °C.

Instrumental conditions

Injector and transfer line temperatures: 298°C
Injection mode: splitless
Carrier gas: hydrogen
GC column: a well deactivated 5% phenylmethylsilicone fused silica capillary column, 15 m x 0.25 mm i.d., 0.25 m film thickness

Oven temperatures:

Initial temperature: 175°C; hold for 0.8 min
Temperature programme: 20°C/min
Final temperature: 300°C; hold for 0.6 min
Total time: 7.6 min

Tandem Mass spectrometer:

Ionisation: positive ion chemical ionisation
Reagent gas: ammonia, approximately 3000 mTorr source pressure
Ion source temperature: 200°C
Collision gas: argon, approximately 2.0 mTorr pressure
Collision energy: 35 eV
Ions monitored by selected reaction monitoring (SRM):
m/z 396 (MH+) 353
m/z 396 295
m/z 396 280

IV. Recommended methods for the detection and assay of psilocybin

A. Pharmacology

Current patterns of use

The most common way of using psilocybin is by ingestion of fresh or dried *Psilocybe* species occurring in nature or cultivated in vitro[43]. The psilocybin content of *Psilocybe* mushrooms varies between 0.2-2% (maximum can be up to 3%, but this is unusual). Tolerance develops very rapidly which limits chronic use.

Pharmacodynamics

The hallucinogenic effects and somatic side effects of psilocybin and psilocybin-containing mushrooms are mainly due to Psilocin, the dominant Psilocybin metabolite. Psilocin interacts with serotonergic (5-HT_{1A}, 5-HT_{2C}, etc.) and noradrenergic receptors. An oral dose of 12-25 mg (equivalent to 0.6-12.5 g of dried mushrooms) of psilocybin is necessary to produce psychotropic effects such as derealisation and depersonalisation phenomena including virtual hallucinations, thought disorders and changes in affect and mood[44].

B. Disposition

Metabolism

Alkaline phosphatase and non-specific esterases of the intestinal mucosa rapidly and extensively cleave the phosphoric acid group of psilocybin before entering the systemic circulation. This indicates that psilocybin acts as a pro-drug and that its 4-hydroxy metabolite psilocin represents the true pharmacologically active agent. Psilocin undergoes demethylation and consecutive deamination and oxidation to 4-hydroxy-indole-acetic acid, presumably by the influence of liver enzymes such as monoaminooxidases and aldehyde dehydrogenase. Other metabolites found are 4-hydroxy-indole-acetaldehyde and 4-hydroxy-tryptamine. Presumably psilocin is excreted as a glucuronide[44, 45].

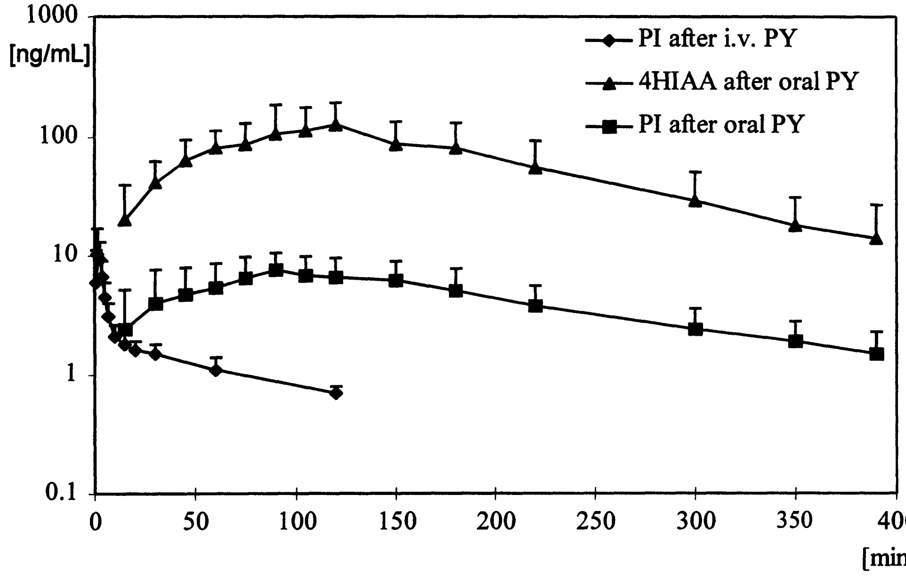
Pharmacokinetics, urinary excretion

The data in table 3 is from a controlled clinical study with 5 volunteers receiving an oral dose of 16.8 mg per 75 kg[44, 45].

Table 3. Pharmacokinetics of psilocin (PI) and 4-hydroxy-indole-acetic acid (4HIAA) following an oral dose of psilocin (mean ± SD, n = 5)

PI				*4HIAA*		
C_{max} [ng/ml]	t_{max} [min]	$t_{1/2\beta}$ [min]	F_{abs} [%]	C_{max} [ng/ml]	t_{max} [min]	$t_{1/2\beta}$ [min]
8.2 ± 2.8	105 ± 37	163 ± 64	53 ± 20	150 ± 61	113 ± 41	145 ± 97

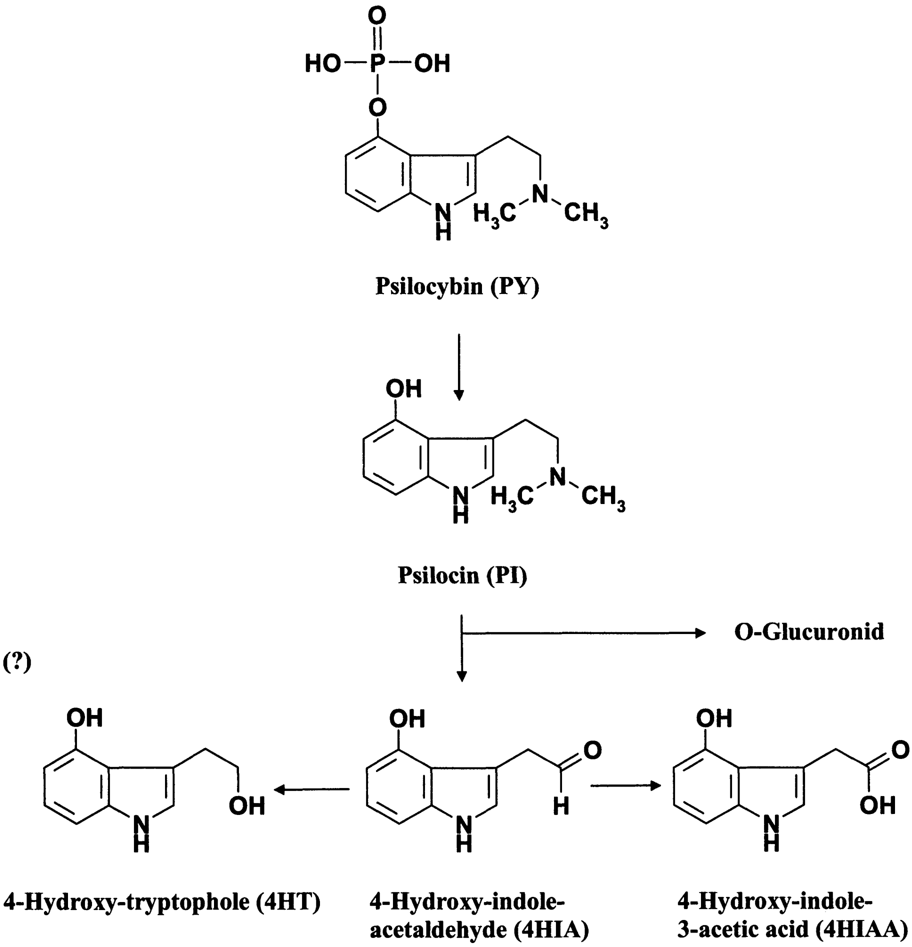
Figure 3. Plasma concentration-time profiles (mean + SD) of psilocin and 4-hydroxy-indole-acetic acid after oral administration of 16.8 mg psilocybin (PY) per 75 kg b.wt. and plasma concentrations (mean + SD) after intravenous administration of 1 mg psilocybin



Detection time in urine

Following an oral dose of 16.8 mg per 75 kg³, the urine concentrations were as shown in table 4. The urine concentration in the first collection interval (0-2 hours after dosage) ranged from 0 to 964 ng/ml. In the second interval (2-4 hours after dosage) 86-871 ng/ml PI were found. In the third interval (4-6 hours), the minimum was 179 ng/ml and the maximum 1163 ng/ml. In the next interval (6-12 hours), the minimum was 173 ng/ml to 939 ng/ml. In the last interval (12-24 hours), the range was between 100 and 266 ng/ml. After enzymatic hydrolysis the concentration of the total PI could be increased by a factor of about two.

Figure 4. Metabolism of psilocybin[44, 45]



Note. No pharmacokinetic data are available for the ingestion of psilocybin-containing mushrooms, but it is assumed that following absorption, the pharmacokinetics are similar to those for oral psilocybin.

Table 4. Urinary concentrations following an oral dose of psilocybin (mean ± SD, n = 5)[45]

Subject	*Urine concentration of unconjugated psilocin (ng/ml) at different time intervals after oral dosage [h]*				
	0-2	*2-4*	*4-6*	*6-12*	*12-24*
1	964	746	438	173	236
2	17	251	255	469	266
3	919	741	475	316	187
4	21	254	179	311	100
5	0	871	1163	286	162
6	0	416	432	939	199
7	32	224	402	358	113
8	0	86	370	624	117

C. Toxicology

Uncontrollable hallucinations and severe somatic side-effects (nausea, cardiovascular symptoms etc.) result from oral doses of 40-50 mg psilocybin. The acute toxicity of psilocybin is very low. From animal studies it is estimated that gram dosages are required for toxicity.

No information is available concerning blood and urine concentrations after high doses of psilocybin-containing mushrooms.

Interpretation of results

The detection of psilocin in blood and urine would indicate the use of psilocybin or psilocybin-containing mushrooms, although the administration of psilocin cannot be excluded.

D. Method of analysis

(Psilocin is the target analyte.)

Sample preparation

The selection of a solvent system for extraction procedures should take into account the health and safety of laboratory personnel by avoiding, if possible, hazards of toxicity and flammability. These issues are discussed in the United Nations manual on Recommended Guidelines for Quality Assurance and Good Laboratory Practices (ST/NAR/25)[5].

Psilocin decomposes very quickly as a result of the oxidation of the phenolic hydroxy group at position C-4. Therefore stabilisation is recommended by adding ascorbic acid to urine and plasma as an anti-oxidant.

Urine

6ml urine plus 350 l of ascorbic acid solution (0.5M) plus 1 ml of absolute ethanol is frozen (for example in dry ice) and lyophilised, and the residue is redissolved in 1 ml methanol. After sonication for 5 minutes, the solution is filtered through a membrane filter (5 micron) and 10 microlitres are injected into the HPLC system.

An enzymatic hydrolysis can be performed to increase the concentration of psilocin.

Plasma

Blood is collected without adding an anticoagulant (which interferes with the method) and plasma is separated by centrifugation (15 min at 3000 rpm) immediately after sampling. 3.0 ml of the clear supernatant are transferred to polypropylene test tubes (in order to avoid absorption to glassware). 150 µl of a concentrated solution of ascorbic acid (900 mg/10ml water) were added to give a final concentration of 25 mM of ascorbic acid, a sufficiently high concentration to stabilise the analyte. After vortexing for 30 seconds, the sample is frozen (dry ice) and lyophilised. After reconstitution of the residue with 700 microlitres of water, the analytes were separated from plasma proteins by *in vitro* microdialysis using two polycarbonate-membrane probes per sample (membrane diameter 0.5 mm, length 16 mm, molecular weight cut-off 20,000 Da). Bi-distilled water was used as perfusion liquid at a flow rate of 2 µl per minute. Total perfusion time of 2.5 hours. The total volume of perfusate (600 µl) was collected in light-protected HPLC vials and concentrated by freeze-drying. The residue was redissolved in 60 µl of the mobile phase prior to HPLC analysis.

Note that the recovery of analytes, including psilocin, using microdialysis is typically about 15%. Instead of microdialysis, an alternative extraction procedure can be used avoiding multiple steps which might result in decomposition of the analytes of interest.

Screening methods

There are no commercially available immunoassay methods for psilocin. Also, TLC is insufficiently sensitive and does not have sufficient separating power for use in the analysis of psilocin in biological specimens.

Confirmatory methods

Gas chromatography and GC-MS

For the determination of psilocin in plasma or urine, derivatisation is necessary using BSTFA containing 1% TMCS. Note that even following derivatisation to the trimethylsilyl derivatives there are likely to be losses of psilocin by absorption and/or decomposition. Alternatively, acetylation can be used[46]. The use of a nitrogen detector will increase both sensitivity and specificity.

High performance liquid chromatography

Determination of psilocin in urine:

The HPLC-ECD method uses a column switching system with two columns:

Column I: Spherisorb RP-8, 3 micron, 50 x 4.6 mm i.d.
Column II: Spherisorb RP-8, 3 micron, 150 x 4.6 mm i.d.

Column switching was initiated after 2.2 minutes. The mobile phase contained 0.3 M aqueous ammonium acetate and 0.3 M methanolic ammonium acetate 46:54 v/v, adjusted to pH 8.3 with 10% ammonium hydroxide. The flow rate is 0.5 ml/minute. Electrochemical detection (ECD) in oxidation mode is at +150 mV, range 5 microamperes, and the cell is thermostated at 34 °C.

Determination of psilocin in plasma:

The HPLC-ECD method uses a column switching system with two columns:

Column I: Spherisorb RP-8, 3 micron, 50 x 4.6 mm i.d.
Column II: Spherisorb RP-8, 3 micron, 150 x 4.6 mm i.d.

Column switching was initiated after 2.2 minutes. The mobile phase consisted of 47% (v/v) water containing 0.3 M aqueous ammonium acetate, adjusted to pH 8.3 with 25% ammonium hydroxide and 53% methanol. The flow rate is 0.45 ml/minute. Electrochemical detection (ECD) in oxidation mode is at +150 mV, range 1 microampere, and the cell is thermostated at 34 °C.

Quantification uses an external standard method for calibration based on peak area.

V. Recommended methods for the detection and assay of phencyclidine

A. Pharmacology

Phencyclidine was initially synthesised in 1956 as an anaesthetic. Due to its behavioural side effects all clinical trials were stopped in 1965 and phencyclidine was never marketed for human use. Later it was reintroduced in the market under the trade name of Sernylan as a veterinary anaesthetic.

Phencyclidine is a dissociative anaesthetic with sympathomimetic and hallucinogenic properties. It is believed to stimulate alpha-adrenergic receptors, potentiating the effects of norepinephrine (noradrenaline), epinephrine (adrenaline) and serotonin[12, 13, 47, 48, 49].

Current patterns of use of phencyclidine

Phencyclidine is self-administered by inhalation (smoking), intranasal application ("snorting"), intravenously ("mainlining") and by oral, rectal or vaginal routes or even taken in eye drops or directly absorbed through the skin. Typical street doses contain 3-5 mg PCP, usually as the hydrochloride salt.

Effects of phencyclidine

Phencyclidine can induce psychosis clinically indistinguishable from schizophrenia. Adverse effects reported include bizarre and violent behaviour, hallucinations, euphoria, agitation, catatonic rigidity, disorientation, incoordination, nystagmus, hypersalivation, vomiting, convulsions, numbness, hypertension, tachycardia, rhabdomyolisis leading to renal failure, acidosis and some times malignant hyperpyrexia. Duration of the "high" is 2-4 hours. Psychosis may last weeks.

Development of tolerance and dependence

Anecdotally, physiological dependence and tolerance (four-fold) on phencyclidine have been reported in abusers. Cross-tolerance with other drugs has not been reported nor withdrawal syndrome. In contrast, psychological dependence appears relatively common in those who use the drug.

Potential for abuse

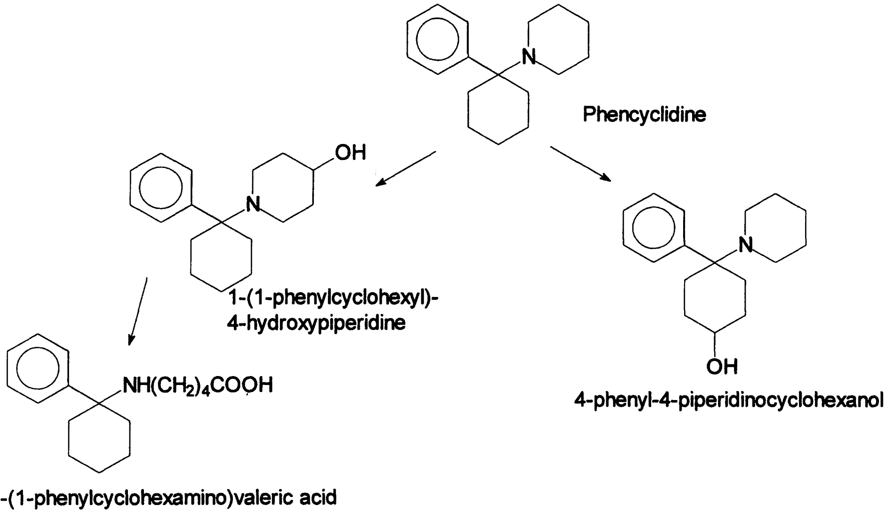
Although there is no evidence of physiological dependence, due to its psychological dependence properties a clear potential for abuse must be considered.

B. Disposition

Routes of metabolism

PCP undergoes extensive oxidation hydroxylation in the liver to, at least, 2 inactive metabolites, 4-phenyl-4-piperidino-cyclohexanol (PPC) and 1-(phenylcyclohexyl)-4-hydroxypyperidine (PCHP). Both are excreted conjugated as glucuronides. The major urinary excretion product in pregnant women is the 5-(N-(1’-phenylcyclohexylamino) pentanoic acid(PCA)[12, 13, 47, 48, 50].

Figure 5. Metabolism of PCP



Urinary excretion and plasma elimination half-life

Excretion patterns show 4-19% of PCP is eliminated unchanged in urine and 25-30% of conjugated metabolites. Concentrations of PCP unchanged in urine have ranged from 0.04 to 3.4 mg/l after recreational doses.

Urinary excretion increases (100 fold) when urine is acidified to pH 5.5 or lower.
Renal clearance: 33 +/- 8 ml/min.
Total clearance: 380+/- 80 ml/min
Vd: 5.3-7.5 l/kg

Plasma elimination half-life is dose dependent ranging from 1h when small doses are administered to 18 h (range 7 to > 50h) in overdose cases.

C. Toxicology

Blood concentrations

After recreational doses (1-6 mg PCP•HCl) associated blood concentrations found in subjects arrested for driving under the influence of drugs or being intoxicated in public were 7-250 ng/ml (with an average of 75 ng/ml).[12,48,51]

After a threshold toxic dose (10-20 mg), associated blood concentrations of 1 μg/ml have been measured.

After a fatal dose (120 mg) blood concentrations of 0.3 to 25 mg/l have been found.

Fraction bound to proteins in plasma ranges from 65 to 80%.

Detection time limits in urine

Phencyclidine can be detected in urine for 7-8 days after drug administration. In chronic users drug can be found for up to 2-4 weeks.[52]

Interpretation of results

Results must be interpreted in accordance with the expected blood concentrations specified under "toxicology" in this manual.

The level of impairment caused by this drug can not be correlated with the plasma concentrations as no controlled human pharmacokinetic studies have been performed using psychoactive doses. Concentrations found in 124 individuals (car drivers checked at traffic stops) ranged between 12 to 118 ng/ml. Nevertheless, at concentrations as low as 12 ng/ml, behavioural effects could be detected by DRE (Drug Recognition Expert) Evaluation[53].

D. Methods of analysis

Screening methods

A number of immunological tests are commercially available. Some of the instrumental ones are based on FPIA, EIA, RIA, etc. Others are non-instrumental on-site kits. Some of these are listed together with their cut-off levels in table 5.

Table 5. Immunological test

Assay	*Principle of immunoassay*	*Cut-off ng/ml*	*References*
EMIT II d.a.u.	Enzyme-linked	25	54, 55, 56
Adx/TDx	Fluorescence polarisation		57, 58
Coat-A-Count RIA	Radioimmunoassay	10	59
Abuscreen RIA	Radioimmunoassay		60
CEDIA	Enzyme-linked		61
Triage	Competitive binding		62

Adulterants added to urine can affect FPIA and RIA assays.[57] Diphenydramine has shown cross-reactivity with the FPIA test for PCP, which was not observed with PCP II.[63]

Where immunological methods are not available, chromatographic methods described for confirmation can be used for screening purposes as well. A recent review on chromatographic methods of analysis of phencyclidine can be found in the literature.[64]

Confirmation methods[65, 66]

Gas-chromatography method A

Based on Drummer et al, 1994[65]

This procedure has only been checked for blood although potentially it can be used for urine and vitreous humour as well.

Sample preparation

☐ Add 1 ml blood (or control or standard) to silanised glass extraction tubes.
☐ Add 1 μg cyclizine (100 μl of a 10 μg/ml methanolic solution) as internal standard to each tube, mix.
☐ Add 1 ml of 1M Tris buffer to each tube, mix.
☐ Add 8 ml butyl chloride and extract for 30 min on a horizontal shaker.
☐ Centrifuge briefly at 2000 rpm, 5 min.
☐ Transfer solvent to a fresh glass extraction tube.
☐ Evaporate to dryness, either in a vacuum centrifugal concentrator at room temperature, or in heating block (at 30°C under nitrogen).
☐ When dry, reconstitute residue with 100 μl methanol.
☐ Transfer to GC injection vials (screw-on with teflon seal) and inject 1-2 μl into GC.

Instrumental conditions

Injector: capillary split/splitless
  Temperature: 250°C
  Injection volume: 1-2 μl
  Mode: splitless

Column: 5% phenyl-methylsilicone 12 m x 0.53 mm I.D. 10 μm film thickness capillary column
Carrier gas: Helium
Flow: 2-3 ml/min

Oven:
  Initial temperature: 100°C
  Initial time: 2 min
  Temperature programme: 7.5°C/min
  Final temperature: 280°C
  Final time: 10 min

Detector:

NPD: Hydrogen flow: 3.7 ml/min

Air flow: 90 ml/min?

Make-up gas flow: 30 ml/min

Bead current: 5 µA

MS: Characteristic ions (rel. abundance): 200 (100%), 91 (45%), 243 (22%), 242 (25%), 186 (20%).

High peformance liquid chromatography method B

Based on Drummer et al, 1993 [66]

This procedure is the HPLC method used for blood, plasma, urine and vitreous humour for PCP and many other drugs.

Sample preparation

☐ Add 1.0 ml blood (or plasma etc), standard or control to 10-ml silanised glass extraction tubes or to polypropylene extraction tubes.

☐ Add 1 µg pentazocine (100 µl of a 10 µg/ml methanolic solution) as internal standard to each tube, mix.

☐ Add 0.5 ml 2% sodium tetraborate solution, vortex briefly.

☐ Extract with 8 ml hexane/n-butanol (95:5), for ~15-30 min on a wheel or shaker.

☐ Centrifuge for ~5 minutes, 3000 rpm.

☐ Place tubes into an alcohol bath and when aqueous layer is frozen (~2 min) decant solvent into a fresh extraction tube, or aspirate off lower aqueous quantitatively.

☐ Extract with 200 µl 0.2% phosphoric acid for a minimum 15-30 min on a wheel or shaker.

☐ Centrifuge for ~5 minutes, 3000 rpm.

☐ Place tubes into an alcohol bath and when aqueous layer is frozen (~2 min) decant off solvent waste, or aspirate off solvent quantitatively. If there is residual solvent left "air" in a fume cupboard.

☐ Thaw acid (if necessary) and transfer in a vial.

☐ Inject 30 µl into the HPLC.

Instrumental conditions

Column: Novapak Phenyl, 3.9 x 150 mm, 5 m particle size.

Mobile phase: acetonitrile/10 mm phosphate, pH 3.0 (55:45)

Flow: 1.5 ml/min

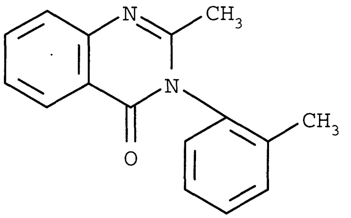
Detection: UV at 214 nm.

VI. Recommended methods for the detection and assay of methaqualone

A. Introduction

Methaqualone [CAS 72-44-6, MW 250.3] is a quinazoline derivative first synthesised in 1951 and introduced as a barbiturate substitute with similar effects in 1965. It soon gained in popularity as a drug of abuse and in 1984 was removed from the US market due to its extensive misuse. In other countries it is still used clinically as a sedative and hypnotic but is also used illicitly, for example, mixed with heroin or benzodiazepines[8]. It is also available as the hydrochloride salt [CAS 340-56-7]. Common names include Dormigoa, Mandrax (with diphenhydramine), Mequin, Normi-Nox, Noxybel, Paxidorm, Quaalude, Revonal, Sopor, Toquilone, Toraflon.

Figure 6. Structure of methaqualone



M.W. = 250.3
Hydrochloride M.W. = 286.8

pK_a = 2.4

The two sources of illegally distributed methaqualone are diversion from legitimate pharmaceutical trade and illegitimate manufacture in clandestine laboratories. Methaqualone was first prepared in 1951 and introduced pharmaceutically in 1965 for use as a non-addictive, non-barbiturate "sleeping pill". Mecloqualone was synthesised in 1960 and is available as a legitimately dispensed hypnotic in some European countries.

Although found to be useful at first as legitimate pharmaceuticals, the abuse of these substances has become so widespread that several Member States have banned them in their country under Article 13 of the Convention on Psychotropic Substances 1971.

In North America, the legitimate manufacture of methaqualone ceased in 1983. In Canada, only the combination product containing methaqualone and diphenhydramine is commercially available and is a controlled drug preparation. On the other hand, there are indications that these substances are still produced in clandestine laboratories.

Illicit synthesis of methaqualone

The synthetic routes for these drug substances are not complicated and are easily performed in clandestine laboratories. Two basic methods have been encountered. The first is a two-step reaction involving the preparation of N-acetyl anthranilic acid (from anthranilic acid and acetic anhydride) followed by condensation with either o-toluidine to produce methaqualone or o-chloroaniline to produce Mecloqualone. Phosphorus trichloride is used to remove water produced in the reaction. The second method is a one-step reaction carried out by refluxing anthranilic acid, o-toluidine and acetic acid. Polyphosphoric acid is usually added to remove water. Purification, if carried out, involves dissolution of the solid residue in methanol and precipitation of the hydrochloride salt from a methanol-diethyl ether solution.

B. Physical and chemical characteristics of illicit products

Clandestinely produced methaqualone appears on the illicit market as a brown, grey or black tacky powder with 30-70% purity. The colour depends upon the amount of impurities present. It is also available as tablets and capsules from illicit manufacture. Recently it has been used as a cutting agent for heroin and in those cases is usually present at about 30% concentration. Several years ago, many counterfeit methaqualone tablets, flooding the market, actually contained diazepam. Both the free base and the hydrochloride salts of methaqualone from licit and illicit production may be encountered in capsule, tablet or powder form.

C. Pharmacology

Current patterns of use of methaqualone/mecloqualone

Methaqualone is usually taken orally. It is used for patients who do not respond adequately to other hypnotics. Usual daily doses are 75-300 mg as the free base, however it is subject to misuse being self administered in doses as high as 3 g daily. Driving under the influence of methaqualone has been reviewed[67, 68].

Effects of methaqualone

In high doses it acts as a CNS depressant causing slurred speech, ataxia, drowsiness and nystagmus. It is just as potent as pentobarbital and Phenobarbital and is effective as a sleeping tablet. The side effects include headache, dizziness, drowsiness, anorexia, nausea, vomiting, dryness of the mouth, diarrhoea, tachycardia, and skin rashes. None of the hydroxylated metabolites have any significant biological activity.

Development of tolerance and dependence

Originally advertised as non-addictive, both physical and psychological dependence were reported as early as 1966 in Great Britain. Tolerance, physical and psychological dependence develop from the chronic use of Methaqualone, and the use of 7.5

grams per day.[68] According to clinical studies, 2 grams of Methaqualone used for one month are sufficient to produce withdrawal seizures.[68] Death has occurred from methaqualone in combination with alcohol.

Potential for abuse

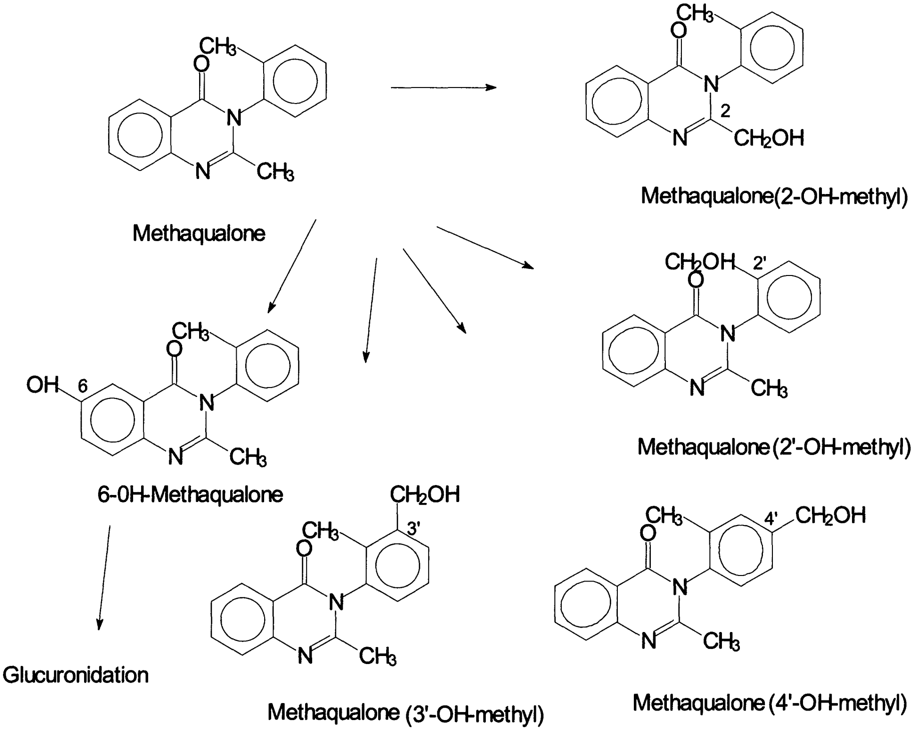
The risk potential of Methaqualone is roughly equivalent to that of the short-acting barbiturates.[69] Acute and chronic toxicity from Methaqualone use does occur. After ingestion of large doses, convulsions and eventually coma result.

D. Disposition

Routes of metabolism

Methaqualone is rapidly absorbed after oral administration and is extensively metabolized in the liver. Less than 1% of the parent drug is excreted unchanged. It is oxidized to the N-oxide and a number of biologically inactive hydroxylated derivatives and these appear in the urine as the glucuronide conjugates.[69]

Figure 7. Metabolism of methaqualone



Urinary excretion and half-life

In a controlled clinical study with six volunteers receiving an oral dose of 200 mg Methaqualone a high inter-individual variation was observed in excretion profiles, as shown in the table below. Over the 72 hours after dosing, 19.8-29.0% of the dose was excreted in the urine. The main metabolites of Methaqualone were 2'-OH-methaqualone and, 3'-OH-methaqualone, both excreted as glucuronides. Only 1-5% of the dose is excreted unconjugated.

Table 6. Urine excretion profiles (8-72) hours after oral administration of 200 mg Methaqualone (GC-MS, ng/ml, before and after hydrolysis, n=6).[69]

Subject	*Methaqualone*	*2-OH-M*	*2'-OH-M*	*3'-OH-M*	*4'-OH-M*	*6-OH-M*
A	42-161	0	65-158	12-102	3-53	5-69
	21-278	80-3510	830-8850	432-5660	74-2900	99-2320
B	57-203	0	22-104	29-182	13-351	43-316
	46-519	107-7980	898-25500	263-49100	48-20000	47-11350
C	0-303	0-29	0-233	0-375	0-193	0-813
	58-360	0-3660	0-12700	0-8030	0-3630	0-2520
D	77-206	0	4-83	0-168	0-76	8-489
	37-245	58-3990	31-6550	111-19600	286-6780	10-5960
E	80-211	0	93-190	19-148	0-84	0-111
	54-226	127-2680	1600-9810	495-11600	600-4200	77-1680
F	66-151	0	34-104	30-204	10-87	22-1050
	35-160	131-1310	1910-6780	2200-10500	671-2840	574-3400

E. Toxicology

Blood concentration

Data on blood (and plasma) concentrations can be found in Stead and Moffat,[70] Baselt, and Cravey[48], Clarke[51] and TIAFT Bulletins.[71]

Blood concentrations range up to 2.2 mg/L following a dose of 250 mg methaqualone (range 1-4 mg/L) by 5 h. Proportionally higher levels are achieved with higher doses. Therapeutic blood concentrations usually range from 0.4 to 5 g/ml. The terminal elimination half-life ranges from 20 to 60 h with a mean of ~35-40 h. Volume of distribution is ~ 6 L/kg. Protein binding is 75-95%.

Toxic effects occur at blood concentrations greater than 2 mg/L of blood. Death has been reported at concentrations greater than 5mg/ml, average approximately 20 mg/L of blood. Corresponding liver levels are about 5-10 fold higher. A review of 246 fatalities has been published.[72] Acute intoxications have been reviewed.[73]

F. Methods of analysis

Sample preparation

Liquid-liquid extraction

The selection of a solvent system for extraction procedures should take into account the health and safety of laboratory personnel by avoiding, if possible, hazards of toxicity and flammability. These issues are discussed in the United Nations manual on Recommended Guidelines for Quality Assurance and Good Laboratory Practices (ST/NAR/25).[5]

Urine for TLC

20 ml of alkalinised urine are extracted with dichloromethane, dried over sodium sulphate, and evaporated to a small volume.

Plasma and Blood

Methaqualone/Mecloqualone are normally detected in plasma using radioimmunoassay (RIA), TLC, GC or HPLC.

A method for the analysis of Methaqualone in blood has been published[74] which uses 200 l of blood, basified with 100 l of ammonium hydroxide and extracted with 5 ml diethyl ether by vortexing. After centrifuging, the ether layer is transferred to another tube and the solvent careful evaporated at 40 °C under nitrogen. The residue is reconstituted in 50 l of methanol for analysis. The recovery is reported to be quantitative.

Solid-phase extraction

Urine

A solid-phase extraction method has been published for methaqualone confirmation from human urine.[75] This method uses a Bond Elut Certify column. Eluant was hexane/ethylacetate (3:1). Diatomaceous earth (Celite) has also been used to extract methaqualone and other weak acid and neutral drugs from whole blood.[76] A solid-phase method using XAD-5 resin is also described.[77]

Screening methods

Immunoassay methods

Both the EMIT II and On-Line immunoassay kits easily detect methaqualone use in urine for 72 h after a 200 mg oral dose using a 0.6 g/ml cut-off.[78,79]

Table 7. ONLINE vs EMIT II cross-reactivity to major methaqualone hydroxy-metabolites

Metabolite	*OnLine (ng/ml)*	*Cross-reactivity (percentage)*	*Emit II*	*Cross-reactivity (percentage)*
2'-OH-Methaqualone	469	64	2000	15
3'-OH -Methaqualone	375	80	550	55
4'-OH- Methaqualone	259	116	300	100

The cross-reactivity data above may vary depending on the batch of antibody used in any individual immunoassay kit. Reference should be made to manufacturers' information sheets that normally accompany kits for data pertaining to the materials being used.

It is important that immunoassay kits are used according to the manufacturers' instructions concerning dilution of specimens and reagents, volumes of reagents and storage/shelf-life of reagents. If changes are made to the manufacturers' recommended procedures, the reliability of the procedure will be affected and the modified method will have to be reassessed to establish its suitability for the intended purpose.

Interferences are known to occur with immunoassays. These depend on the type of immunoassay, the type and quality of specimen and of course, the presence of substances other than the class sought to be measured in the specimen that may cross-react with the antibody reaction. Therefore, the analyst should always consider the possibility of interfering substances in an analysis (see chapter I.F. of this manual for more information).

Thin-layer chromatography

Developing a Merck F_{254} silica plate in ethyl acetate/methanol/ammonia (170:20:10) shows under UV_{254} a methaqualone metabolite as a dark spot at Rf 0.35; spraying with 1 mg/ml Fast Blue B in 75% methanol/water shows a blue-mauve spot.[80]

Confirmatory methods

Gas chromatography

Packed column[81] and capillary column technique[65]

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 15h. Conditioning is carried out with the normal carrier gas flow and with the column disconnected from the detector.

Packed column method A

Based on Peat et al.[81]

☐ 1 ml plasma and 50 µl of internal standard solution (equivalent to 500 ng mecloqualone) are vortexed
☐ 1 ml saturated sodium borate is added and the sample gently vortexed.
☐ 5 ml n-butyl chloride are added and the extraction tube is mechanically shaken for 10 minutes.
☐ After centrifugation for 5 min the solvent is transferred to a conical centrifuge tube and evaporated to dryness at 60°C under a stream of nitrogen.
☐ The residue is reconstituted with 30 µl of hexane:ethanol (1:1 v/v)
☐ 2 µl are used for GC-NPD analysis of methaqualone.

Silanisation of the glassware is recommended.

The chromatographic system uses a short packed column (approx. 1.5 metres) x 3 mm I.D., packed with 1% SP-1000 on Gas Chrom Q (100-120 mesh). Carrier gas is nitrogen at a flow rate of 35 ml/min, and a nitrogen detector is used. Injector and detector temperatures are 250 and 300°C, respectively. The temperature programme is: initial temperature 220°C (for 4 min), programmed at 8°C per min to 240°C (held for 4 min).

Capillary column method A
(FID/NPD)

Based on Drummer et al., 1995[65]

This procedure is the single step method used for methaqualone estimation in blood, plasma, urine and vitreous humour. Use a 10 m or 25 m x 0.3 mm I.D. capillary column, BP-5, or equivalent, temperature programme from 100°C to 280°C at 7.5°C/min. Detector can be an FID, NPD or MS.

☐ Add 1 ml blood (or control or standard) to silanised glass extraction tubes.

☐ Add 100 µg internal standard cyclizine to each tube, mix.

☐ Add 1 ml Trizma buffer to each tube, mix.

☐ Add 8 ml butyl chloride and extract for 30 min on a horizontal shaker.

☐ Centrifuge briefly at 2000 rpm, 5 min.

☐ Transfer solvent to a fresh glass extraction tube.

☐ Evaporate to dryness, either in Savant Concentrator at room temperature, or in heating block (at 30°C under nitrogen).

☐ When dry, reconstitute residue with 100 µl methanol.

☐ Transfer to GC injection vials (screw-on with teflon seal) and inject 1-2 µl into GC.

Detectors

Detectors suitable for the analysis of Methaqualone/Mecloqualone by GC include the Flame Ionization Detector (FID) and the Nitrogen Phosphorous Detector (NPD) which is more selective and yields a smaller solvent front than a FID.

Gas chromatography-Mass Spectrometry
Capillary column method B
(GC/MS)

Based on Brenner et al., 1996[78]

Hydrolysis

☐ 1 ml of a 1.1 M solution of sodium acetate buffer (pH 5.2) plus 300 µl of internal standard solution (d4-methaqualone, at a concentration of 300 ng/ml) is added to 1 ml of urine (sample, calibrator, or control).

☐ Adjust pH of the mixture to 5-6, if necessary, by the addition of 0.1 M HCl.

☐ Add 100 µl (130,000 units' glucuronidase/ml) of β-glucuronidase (Glusulase) are added and the tubes are placed in an oven at 30 °C for 4 hours.

☐ Removed tubes and brought to room temperature prior to extraction.

Extraction

☐ Add 1 ml of a 1.5 M sodium carbonate buffer (pH 9.0) and 8 ml of chloroform:isopropanol 9:1 v/v to each tube.

☐ Tubes are capped mixed for two minutes and centrifuged if necessary.

☐ Aspirate the top aqueous layer and transfer the organic layer into test tubes

☐ Evaporate organic phase under a stream of nitrogen at 50 °C to a volume of approximately 1 ml.

☐ Concentrate is transferred into GC vials and evaporated to dryness.

Derivatisation

☐ 100 µl of pentafluoropropionic anhydride are added to each GC vial.

☐ Cap each vial and heat at 70 °C for 30 minutes.

☐ After cooling to room temperature, and removing the excess of reagent under a stream of nitrogen, 300 µl of ethyl acetate are added to the residue and the resulting solution used for GC or GC-MS.

GC-MS analysis

The GC column is a dimethylsilicone column (DB-1 or equivalent), 10 m x 0.18 mm I.D., film thickness 0.4 microns. The oven temperature was maintained at 150 °C for 3 minutes then slowly heated to 285 °C at a rate of 20 °C/minute with a final temperature hold of 3 minutes. Analysis is carried out in the SIM mode, monitoring ions as shown in the table.
The limit of detection of this analytical method is 25 ng/ml and the limit of quantification is 50 ng/ml.

It is recommended that a relatively new column be used in order to obtain the required separating power. As the column ages, slight tailing is observed.

Compound	*Retention time (min)*	*Ions for identification (m/z)*	*Ions for quantification (m/z)*
Methaqualone	7.12	250	235
2-OH-methaqualone	7.36	412	235
2'-OH-methaqualone	7.42	412	265
3'-OH-methaqualone	7.52	412	397
4'-OH-methaqualone	7.63	412	397
6-OH-methaqualone	7.68	412	397
d4-methaqualone (internal standard)	7.10	254	239

High performance liquid chromatography method A

This procedure is the single step method used for blood, plasma, urine and vitreous humour. Use a Spherisorb S5-ODS, or equivalent, 3.9 x 150 mm, 5 µm particle size column with methanol/acetonitrile/15 mm phosphate, pH 4.0 (40:10:50) and a UV detector 214 nm.

☐ Add 0.25 ml blood or plasma unknown or standard to 500 µl microcentrifuge extraction tubes.

☐ Add 0.25 ml extraction solvent A (acetonitrile containing p-methylphenylphenylhydantoin (MPPH) (IS) at 20 µg/ml). Prepare Solvent A by dissolving 200 µl of a 1mg/ml MPPH stock in 9.8 ml of acetonitrile; use a glass vial to all tubes, vortex mix immediately after addition.

☐ Allow to stand for 10 min and vortex briefly again at end.

☐ Centrifuge for ~10 minutes at full speed on a microfuge.

☐ Transfer supernatent into a vial.

☐ Inject 20 µl into the HPLC.

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