UNITED NATIONS
INTERNATIONAL DRUG CONTROL PROGRAMME
Vienna

Recommended Methods for the Detection and Assay of Barbiturates and Benzodiazepines in Biological Specimens

MANUAL FOR USE
BY
NATIONAL LABORATORIES

UNITED NATIONS
New York, 1997

This publication has been based on the deliberations of the Expert Group Meeting on the Detection and Assay of Barbiturates and Benzodiazepines in Biological Specimens, held at Hong Kong from 13 to 17 November 1995. The views do not necessarily reflect those of the United Nations. This publication has not been formally edited.

ST/NAR/28

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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 amfetamine/metamfetamine, 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 32nd 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 Organization (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 i) the "publication of follow-up working manuals on the subject to serve as guidelines for laboratory and programme development" and ii) "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 Commision'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, Methamphetamine, 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.

B. Purpose of the Manual

This manual, prepared by the Laboratory Section, Technical Services Division 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 Expert Group Meeting held in Hong Kong in 1995 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 barbiturates and benzodiazepines 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 which 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 which 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 Barbiturate Derivates under International Control (ST/NAR/18) [6] and Benzodiazepine Derivatives under International Control (ST/NAR/16) [7]. The reader is also referred to the United Nations manual on Recommended Methods for Detection and Assay of Heroin, Cannabinoids, Cocaine, Amphetamine, Methamphetamine and Ring-Substituted Amphetamine Derivatives in Biological Specimens (ST/NAR/27) [8].

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:

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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 specimens 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 radio immunoassay (RIA), enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA) and latex agglutination inhibition (LAI). 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 a different chemical or physical principle like thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC) or mass spectrometry (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 barbiturates and benzodiazepines. The use of other biological materials like hair and saliva for the present purposes, i.e. establishing the illicit consumption of drugs, has not yet been generally accepted.

In order to maintain the validity of analytical results in the forensic context, particular

care should be given to the **supervision** of specimen collection, transportation and storage.

Supervision must be done by trained personnel having a clear understanding of the legal implications of the procedure and this ought to be carried out by direct visual observation. Proper surveillance must be maintained at all times but every effort must be made to maintain the privacy and dignity of the individual. The supervisor should also ensure that no attempt is made to add contaminating or reactive substances to the urine.

When it is necessary to transport samples to an analytical laboratory, security and a clearly established chain of custody must be maintained.

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

1. Collection

(i) Staff at the **collection site** are responsible for the collection, labelling, packaging and transportation of samples, ensuring that the collection and storage procedures have the proper documentation and necessary security methods.

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

(iii) The collection site must be supervised and witnessed by trained authorized personnel.

(iv) Suitable toilet facilities for the purpose of urine collection must be available before collection of urine is considered.

(v) The collection room must be surveyed for any substance which could be used to invalidate the sample and should be set up without soap dispensers or cleaning agents.

(vi) The urine specimen should be collected in duplicate in two 50 ml bottles. Each bottle should be filled at least 2/3 full. Plastic containers and rubber stoppers should be avoided whenever possible as non-polar drugs and their metabolites are very prone to absorb to some plastic and most rubber surfaces. If, for practical reasons, disposable plastic containers are used, individual laboratories should conduct tests to ensure that the plastic containers do not alter the composition or concentration of the drug(s) or metabolite(s) in the urine.

POSSIBLE WAYS TO INVALIDATE URINE SPECIMENS

1) Introducing various chemical substances to the specimen. Table salt, detergents or some commonly used household items such as hypochlorite (bleach) can destroy the drugs or affect the assay to generate false negative results.
2) In certain circumstances, adding illicit substances to the urine to produce positive results.
3) Placing a pinhole in the bottom of the container which results in leaks.
4) 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.
5) Obtaining urine from friends not using drugs.
6) Scooping water from the WC into the collection container to dilute the urine.

(vii) Immediately after collection, the temperature (32°-38°C within 4 min) and the pH of the fresh urine specimen should be measured and recorded. If adulteration is suspected the laboratory should be notified.

(viii) The bottles should be securely stoppered, sealed and labelled. 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 departmental seal or some other measure to indicate that tampering with the specimen has occurred. It is important that the donor witness the sealing of the bottles and sign or initial the seal or label.

(ix) Specimen labels 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 at least the following information

Name: . . . . . .

I.D. number: . . . . . .

Date and time of collection: . . . . . .

Place of collection: . . . . . .

Name of person supervising the collection: . . . . . .

Drug(s) to be tested for: . . . . . .

Sample Number: . . . . . .

(x) Personnel details of each specimen donor are filled in a request form for analysis. The form will accompany the specimen to the laboratory.

(xi) The specimen donor should not be allowed to have any involvement in the post collection handling of the sample-labelling, packing and transportation to the laboratory.

(xii) Strict security should also be observed in the storage and dispensing of empty cups, request forms, labels and packing materials.

2. Transport and Storage

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

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

(ii) 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.

3. Submission to the Laboratory

(i) 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.

(ii) After ensuring the specimens and request form are in order, a written acknowledgement of receipt should be made, signed and given to the dispatch person.

(iii) The laboratory should maintain well-documented records and strict security to ensure integrity of specimens and confidentiality of results.

(iv) If the analysis is delayed beyond one or two days, specimens should be stored frozen, in a locked refrigerator. When frozen, specimens will generally be stable for several months.

4. Request Form for Drug Analysis

(i) A drug analysis request form which 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.

(ii) 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, and the temperature and pH of the specimen at the time of collection.

(iii) Additional information which 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.

(iv) 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.

(i) Any information related to the donor and to the results of the analysis must be kept locked and secure.

(ii) 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

(i) 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.

(ii) The specimen donor should not be allowed to have any involvement in the post collection handling of the specimen - labelling, packing and transportation to the laboratory.

(iii) It is important that the donor witnesses the sealing of the container and signs or initials the seal or label.

(iv) Accurate and complete records of all individuals involved in the urine collection, storage and transport should be maintained.

(v) 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.

(vi) Information on sample donors and results should be kept strictly confidential and be accessible only to authorized persons.

F. Methodology

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

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

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

1. Immunoassay Methods

Immunoassays are the methods of choice when large numbers of specimens must be assayed within a limited time. Several immunoassay kits are available commercially for the screening of drugs of abuse. The most commonly used methods are radio immunoassay (RIA), enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA) and latex agglutination inhibition (LAI). RIA, FPIA and EIA require instrumentation that is relatively expensive. Any of the above assays may be used by laboratories which have access to them.

The choice of technique would depend in most instances on the workload (number of specimens per day) handled by the laboratory. The enzyme immunoassay and radio immunoassay techniques for instance are available in either the single- or multi-test versions. For laboratories with small numbers of specimens, the single-test versions, or latex agglutination inhibition may be utilized but are expensive when considered in terms of the cost of analysis per specimen. For large workloads, the multi-test enzyme immunoassay or the fluorescence polarization immunoassay are more appropriate.

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

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

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

Table I.1 General characteristics of immunoassays

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

[a] Single test (st) [b] Drug abuse urine assay (dau)

2. 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 are available.

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

3. Gas Chromatography (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 in comparison to TLC or immunoassay and training and experience for these highly technical systems are critical.

4. Gas Chromatography - Mass Spectrometry (GC-MS)

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

5. Sample Preparation

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

For chromatographic procedures, good sample preparation is extremely important. This is necessary because urine (and blood) is a complex containing a mixture of large amounts of numerous organic and inorganic compounds in which the specific target analyte is found in minute amounts. Sample preparation usually involves hydrolysis of urine 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 derivatization is frequently recommended for the following reasons:

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

6. Quantitative Analysis

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

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

Quantitative analysis by GC, HPLC or GC-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, derivatized and analysed under the same conditions as the target analytes, but be readily distinguished from them during the chromatographic procedure. Care must be taken, however, to avoid using substances which might occur in the specimen such as other drugs or endogenous materials.

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

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

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

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

Where:

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

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

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

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

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

G. Quality Assurance

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

1. Internal Quality Control

A good and well-documented quality assurance programme must be an integral part of the drug laboratory set-up and it should at least incorporate some means of assessing the accuracy and precision of all analyses done. The precision of methods should be assessed either by multiple analyses of individual 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.

2. External Quality Assessment

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

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

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

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 absorbed into the body.

A positive result on initial screening means that a drug or metabolite is present in the specimen at a concentration above or equal to the cut-off concentration. 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. Recommended Methods for the Detection and Assay of Barbiturates in Biological Specimens

A. Introduction

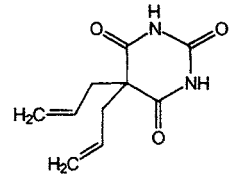
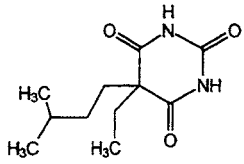
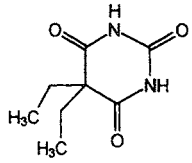
Barbiturates are a relatively homogeneous group of synthetic drugs. They are therapeutically used as sedatives, hypnotics, anesthetics and anticonvulsants in relatively high doses. For many of their uses they have been replaced by the benzodiazepines in many of the developed countries.

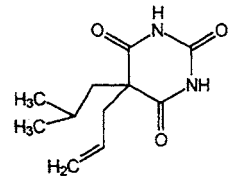
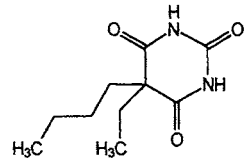
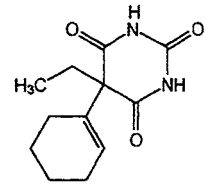
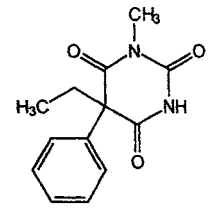
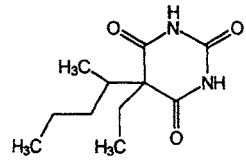
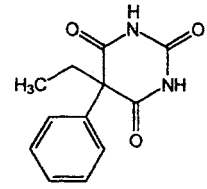
Reportably, over 2500 barbiturates have been synthesized. More than 50 are presently marketed for clinical use throughout the world. Barbiturates often occur as mixtures with other barbiturates (amobarbital/secobarbital) as well as with other substances (caffeine, acetyl salicylate, ephedrine, theophylline, codeine).

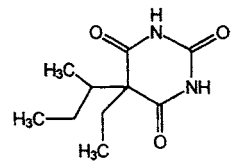
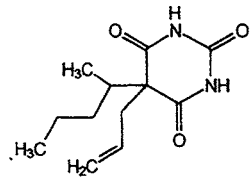
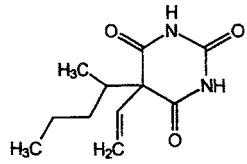
Abuse/misuse of barbiturates is internationally widespread which means that any forensic laboratory may encounter a range of these compounds. Virtually all of the barbiturates in the illicit market result from diversion from legitimate sources and there is no evidence of clandestine manufacture.

Out of the presently marketed barbiturates twelve are subject to international control under the 1971 Convention on Psychotropic Substances.

Table II.1 Barbiturates under international control

Allobarbital 	$C_{10}H_{12}N_2O_3$ M.W. 208.2 pKa 7.8 Schedule IV	
Amobarbital 	$C_{11}H_{18}N_2O_3$ M.W. 226.3 pKa 7.9 log P (octanol/pH 7.4) 1.6 Schedule III	
Barbital 	$C_8H_{12}N_2O_3$ M.W. 184.2 pKa 8.0 Schedule IV	

Butalbital		$C_{11}H_{16}N_2O_3$ M.W. 224.3 pKa 7.6 Schedule III
Butobarbital		$C_{10}H_{16}N_2O_3$ M.W. 212.2 pKa 8.0 log P (octanol/pH 7.4) 1.7 Schedule IV
Cyclobarbital		$C_{12}H_{16}N_2O_3$ M.W. 236.3 pKa 7.6 log P (octanol/pH 7.4) 1.8 Schedule III
Methylphenobarbital		$C_{13}H_{14}N_2O_3$ M.W. 246.3 pKa 7.8 Schedule IV
Pentobarbital		$C_{11}H_{18}N_2O_3$ M.W. 226.3 pKa 8.0 log P (octanol/pH 7.4) 1.9 Schedule III
Phenobarbital		$C_{12}H_{12}N_2O_3$ M.W. 232.2 pKa 7.4 log P (octanol/pH 7.4) 1.4 Schedule IV

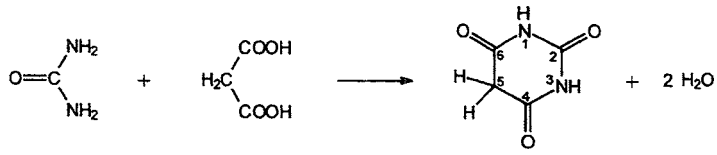
Secbutabarbital 	$C_{10}H_{16}N_2O_3$ M.W. 212.2 pKa 8.0 log P (octanol/pH 7.4) 1.3 Schedule IV	
Secobarbital 	$C_{12}H_{18}N_2O_3$ M.W. 238.3 pKa 7.9 Schedule II	
Vinylbital 	$C_{11}H_{16}N_2O_3$ M.W. 224.3 Schedule IV	

From INCB statistics [9], the significant barbiturates in the last decade have been amobarbital, butalbital, cyclobarbital, pentobarbital and phenobarbital.

Analysts should be aware of the particular barbiturates 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 Barbiturate Derivates under International Control (ST/NAR/18) [6] as well as to national pharmacopoeias. The Multilingual Dictionary of Narcotic Drugs and Psychotropic Substances under International Control (ST/NAR/1/REV.1) [10] published by UNDCP includes a listing of many brand names and other synonyms for barbiturates under international control.

B. Physical and Chemical Characteristics

The parent compound of the barbiturate group of drugs is barbituric acid, which is formed by the condensation of urea and malonic acid:



The two hydrogen atoms at position 5 can be replaced by different organic radicals to form a large number of barbiturate compounds, i.e. the 5,5'-disubstituted barbiturates. In some cases also the hydrogen atom at position 1 is replaced by an alkyl group (e.g. methylphenobarbital). The oxygen atom linked to the carbon in position 2 can be replaced by sulfur, i.e. the thiobarbiturates (e.g. thiopental).

In the free acid form, barbiturates are soluble in most organic solvents such as ethyl ether, ethyl acetate, chloroform and methanol, but are insoluble in water. Amobarbital, pentobarbital, phenobarbital, secbutabarbital and secobarbital are available as sodium salts, cyclobarbital as a calcium salt. These salts are generally insoluble in ethyl ether, ethyl acetate and chloroform, but are soluble in methanol and water.

C. Pharmacology

The therapeutic use of barbiturates has declined in recent years because they do not have specificity with respect to their central nervous system (CNS) effects. They have a lower therapeutic safety margin than alternatives such as the benzodiazepines and tolerance occurs more often than with benzodiazepines. They have a greater abuse liability and exhibit a significant number of drug interactions [11,12].

1. Current Uses of Barbiturates

Current uses include:

- as hypnotics and sedatives - for example phenobarbital is used in a variety of drug mixtures for gastrointestinal disorders, urethral inflammation, hypertension, asthma and coronary artery disease;
- as antagonists for unwanted side-effects of central stimulants such as to ephedrine;
- phenobarbital is used as an anticonvulsant in the treatment of epilepsy;
- as intravenous anesthetics (most often thiopental (or also known as Pentothal), which is not controlled under the 1971 Convention).

2. Effects of Barbiturates

The barbiturates can cause all degrees of depression of the CNS from mild sedation to general anaesthesia. Some have selective anticonvulsant activity. They show a lower anxiolytic effect than benzodiazepines compared to the sedative effect. They can exert a euphoriant effect similar to that of morphine, but do not have any significant effect on the perception of pain. They cannot be relied on to produce sleep or sedation in the presence of even moderate pain. In some users, the presence of pain can cause a paradoxical excitement. When injected by drug abusers, they can cause violent behaviour [12].

3. Development of Tolerance and Dependence

Tolerance[1] to the effects of barbiturates occurs during the chronic administration of barbiturates, over a period of weeks to months. Drug metabolism is also induced[2], but within a few days to a week. It is important to note that tolerance to the effects of barbiturates does not significantly elevate the blood concentration that can cause toxicity.

Physical dependence that accompanies the development of tolerance and sudden withdrawal from the drugs in a dependent individual can be dangerous, resulting in delirium, convulsions and occasionally death. Phenobarbital readily crosses the blood/placental barrier and is associated with a higher than normal incidence of birth abnormalities in infants born to mothers using barbiturates. Withdrawal symptoms may be present in infants born to mothers dependant on barbiturates.

4. Potential for Abuse

As a result of their sedative and hypnotic properties, barbiturates are potentially habit-forming, with tolerance, and psychological and physiological dependence occurring with continued use. Patients who develop such a dependence will frequently increase the dosage or decrease the dosage interval without consulting a physician. Deliberate abuse of barbiturates is relatively uncommon, at least in comparison with other illicit drugs. Barbiturates (and other drugs) may be added to folk or patent medicines for their anxiolytic properties and may be consumed unwittingly [13]. The use of barbiturates, often in conjunction with the use of a plastic bag placed over the head promoting suicide, has also been advocated in literature.

D. Disposition

1. Routes of Metabolism

Most barbiturates are biotransformed in the body to inactive metabolites by one of the four possible routes shown in Figures II.1 and II.2 below.

These routes are:

(i) oxidation of the C^5 substituents with formation of alcohols, phenols, ketones and carboxylic acids which may appear in the urine both as free compounds and as glucuronic acid conjugates;

(ii) desalkylation of the *N*-alkyl groups;

(iii) desulfuration of the S^2 group of thiobarbiturates;

(iv) destruction of the barbituric acid ring between N^1 and C^6.

Of particular relevance in the forensic context is the partial metabolism of thiopental to pentobarbital (Figure II.2). Consequently, if pentobarbital is detected in urine, the use of thiopental should be excluded.

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[1] Tolerance refers to a term used to describe an adaptation to the effects of a drug, usually following repeated use, requiring larger doses to exhibit a standard response.

[2] This refers to an accelerated metabolism produced by an effect of barbiturates on enzyme activity.

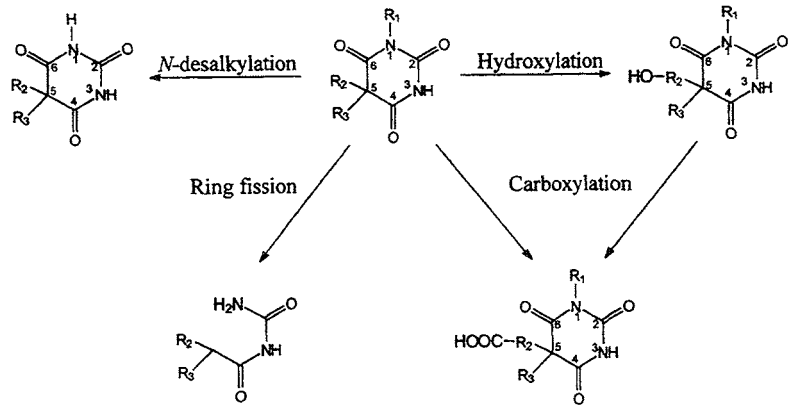
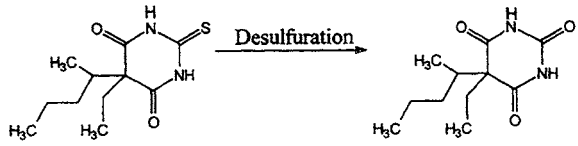


Figure II.1 General routes of metabolism of barbiturates



Thiopental Pentobarbital

Figure II.2 Metabolism of thiopental to pentobarbital.

2. Urinary Excretion and Half-Life

A summary of the main metabolites and some relevant pharmacokinetic data are given in Table II.2.

Table II.2 Metabolism, half-lives and excretion data for barbiturates under international control

Barbiturate	**Parent compound excreted**	**Known metabolites**	**Plasma $t_{½}$ (h)**
Allobarbital	10-35% slowly excreted over 1 week	Not known	40-48
Amobarbital	1-3%	- 3'-hydroxyamobarbital (30-50%) - *N*-glucopyranosylamobarbital (up to 30%) - 5-(3'-carboxybutyl)-5-ethylbarbituric acid (5%)	8-40 mean 24

Barbiturate	Parent compound excreted	Known metabolites	Plasma $t_{½}$ (h)
Barbital	almost all, 95% (2% in 8h, 16% in 32h)	(also produced as a metabolite of metharbital)	about 48
Butalbital	5%	- 5-(2,3-dihydroxypropyl) metabolite (20-60%) - 5-(3-hydroxy-2-methyl-1-propyl) metabolite (10%)	30-88
Butobarbital	5-9%	- 3'-hydroxybutobarbital (22-28%) - 3'-oxobutobarbital (14-18%) - 3'-carboxypropyl metabolite (4-8%)	about 40
Cyclobarbital	less than 10%	- 5-(3-oxocylcohex-1-enyl)-5-ethylbarbituric acid	8-17 mean 12
Methylphenobarbital	less than 2%	- phenobarbital (up to 10%) - *p*-hydroxymethylphenobarbital (30-35%, as free and conjugated forms) - *p*-hydroxyphenobarbital (small amounts) - 5-ethyl-5-(4-hydroxy-3-methoxyphenyl)-barbituric acid (small amounts) - 5-ethyl-5-(4-hydroxy-3-methoxyphenyl)-1-methylbarbituric acid (small amounts)	50
Pentobarbital [14-16]	less than 1%	- 3'-hydroxypentobarbital (7% *d*-, 30% *l*-) - 3'-oxypentobarbital (7-14%) - 3'-carboxy metabolite (10-15%) - *N*-glucoside conjugate (13%)	up to 48 mean 27
Phenobarbital [17-20]	25-67%	- *p*-hydroxyphenobarbital (17%, half of which is conjugated) - *N*-glucopyranosylphenobarbital (up to 30%) - two dihydrodiol metabolites - hydroxymethylphenobarbital	50-150 mean 100
Secbutabarbital	5-9%	- 5-(2-carboxy-1-methylethyl)-5-ethyl-barbituric acid (30%) - 2'-hydroxysecbutabarbital (3%) - 2'-oxosecbutabarbital (1%)	34-42
Secobarbital [16]	less than 5%	- 3'-hydroxysecobarbital (two diastereo-isomeric forms) - 5-allyl-5-(3-carboxy-1-methylpropyl)-barbituric acid (4%) - 5-(2,3-dihydroxypropyl)secobarbital (4%) - 3'-oxosecobarbital (3%)	19-34 mean 25
Vinylbital [21]	5%	- 3-hydroxy metabolite	18-33

Notes

1. Data derives from references cited as well as references 21 and 23.
2. The % parent drug excreted may be higher in overdose cases.
3. Only limited information is available on the metabolism and excretion of vinylbital.

E. Toxicology

1. Blood Concentration

Data on therapeutic and toxic blood (and plasma) concentrations can be found in Stead and Moffat [22], Baselt and Cravey [23], Clarke's [21] and TIAFT Bulletins [24]. Table II.3 summarizes these data.

Table II.3 Therapeutic and toxic blood concentrations of barbiturates

Barbiturate	Maximum therapeutic level (mg/l)	Minimum level for toxicity (mg/l)
Allobarbital	40	50
Amobarbital	12	10
Barbital	30	20
Butalbital	10	10
Butobarbital	15	14
Cyclobarbital	10	8
Methylphenobarbital	15	40
Pentobarbital	10	5
Phenobarbital	40	3
Secbutabarbital	14	20
Secobarbital	10	10
Vinylbital	10	5

Precautionary Note. The concentrations listed in Table II.3 are a guide only. Blood (or plasma) concentrations should be interpreted with caution. The toxic effects of barbiturates are dependent on the degree of tolerance developed by the person, the presence of any natural disease, particularly respiratory or cardiovascular, and the presence of any other psychoactive drugs, e.g. alcohol and other CNS depressant drugs (narcotics, benzodiazepines etc). Such factors usually reduce the concentration required to elicit a toxic response. An example frequently encountered is the high tolerance developed by epileptics to phenobarbital. The range of blood concentrations encountered in patients treated regularly with phenobarbital may overlap with the toxic range. Urine concentrations should not be interpreted with respect to dose and likely pharmacological effects.

Intravenous use of these drugs may lead to a lower blood concentration of drugs which causes a fatality than would generally be found following oral administration. This is especially true for phenobarbital, but this may also be true for other barbiturates.

2. Detection Time Limits in Urine

The time interval over which a barbiturate can be detected in urine is highly variable. Several factors contribute to the time interval of detection, including:

(i) dose of the drug,
(ii) chronic versus acute administration,
(iii) the co-ingestion of other drugs which may impair or enhance metabolism of the barbiturate,
(iv) the analytical methodology used,
(v) differences in the metabolism and excretion of drugs classified as barbiturates,
(vi) the pH of the urine.

In general, barbiturates can generally be detected in urine for approximately 24 hours after use for short-acting barbiturates such as pentobarbital and secobarbital, but much longer for the long-acting barbiturates such as phenobarbital (up to 14 days or more [25]).

3. Interpretation of Results

(a) Urine

Urine concentrations cannot usually be related to a particular drug dose or time since last dose, since urinary excretion depends on the volume of water excreted, creatinine clearance (kidney function), and time since last dose.

Individuals also vary greatly in their ability to metabolise drugs, hence there are variable amounts of parent drug excreted into urine. Most of the barbiturates are extensively metabolised (see Table II.2).

Some barbiturates such as barbital, pentobarbital and phenobarbital are metabolites of other barbiturates and other drugs such as primidone, although they can be used as drugs in their own right. This possibility should be considered in any interpretation of the presence of barbiturates detected in urine.

It is not possible to infer a likely degree of intoxication from urine concentrations.

(b) Blood, Serum and Plasma

Blood (or serum or plasma) can be used to obtain an estimate of the degree of drug use. Table II.3 illustrates the usual concentrations associated with therapeutic use and those possibly associated with the development of toxic symptoms.

There is, however, some overlap between these two ranges. Toxic symptoms are more likely to develop at the upper end of the therapeutic range in persons not normally exposed to barbiturates or in those persons using other drugs acting on the CNS (alcohol, benzodiazepines, narcotics etc.) and in those persons with significant respiratory and/or cardiovascular disease.

On the other hand, persons who have developed some degree of tolerance may not develop toxic symptoms unless rather higher concentrations are attained.

Toxic symptoms include slurred speech, unsteadiness and poor coordination. Serious toxicity usually manifests as convulsions and disturbance of breathing, leading to coma and death.

Barbiturates have a narrow therapeutic index (i.e. difference between therapeutic and toxic concentrations) and death can occur with excessive use of barbiturates at concentrations often not much higher than the upper limit of the therapeutic range.

(c) Interferences

1. Barbiturate-like ions may be observed in the GC-MS analysis of specimens from decomposed bodies, although barbiturates are not present.
2. Other drugs may be metabolised to phenobarbital, including methylphenobarbital and primidone, and pentobarbital is produced, in part, from thiopental.

F. Methods of Analysis

1. Introduction

The following chapters concern different aspects of methods for the analysis of barbiturates in biological specimens. The specimens covered are urine, the recommended specimen for detecting drug use and abuse [3], and blood, serum and plasma, which are used for therapeutic drug monitoring and detection of drug use in the clinical setting, the assessment of drug-induced impairment in, for example, the traffic safety context, and for assessment of the cause of death in drug-related fatalities in forensic medicine.

The procedures described in this manual are intended to guide the laboratory in the appropriate selection of suitable assay procedures. Many of the barbiturates available, but not under international control, may also be assayed by the procedures described.

To improve the readability of the text, plasma and serum are treated as synonymous although they are hematologically distinct.

When a complete method for the analysis of one or more barbiturates is required for a particular biological specimen such as urine or blood, appropriate selections should be made, as required, from the information provided in subsequent chapters. Alternatives have been provided to assist the analyst, who may not have access to all of the materials and equipment necessary for any complete method published in the literature. Each of these individual components of a method are recommended as being suitable for their specific purpose and can be depended upon to work reliably.

However, as stated in Chapter I., the ultimate choice of methodology depends inter alia on the type of specimen to be analysed, the context and purpose of the analysis (clinical or forensic), the facilities available in the laboratory and the experience of the laboratory staff in this field of analytical chemistry. The analyst has the final decision on the choice of which is the most appropriate method, for only the analyst is in possession of all the relevant facts concerning the specimen which is to be analysed.

Above all, it is imperative that any method, constructed from components listed below, is evaluated by the analyst testing it with standards made up in the same sample matrix as the specimens to be analysed and establishing if the method is suitable for the intended purpose.

A method may contain some or all of the components listed below. The decision on whether or not they should be included in a method depends on the type of specimen (urine or blood), the type of analysis (qualitative or quantitative), the purpose of the analysis (screening or confirmatory) and the type of equipment which will be used in the analysis

(immunoassay, TLC, GC, GC-MS or HPLC). The latter (analytical equipment) determines the sensitivity and specificity of the analysis and, as a corollary, the amount of analyte which must be available at the end step. This consequently determines the volume of specimen required for the analysis.

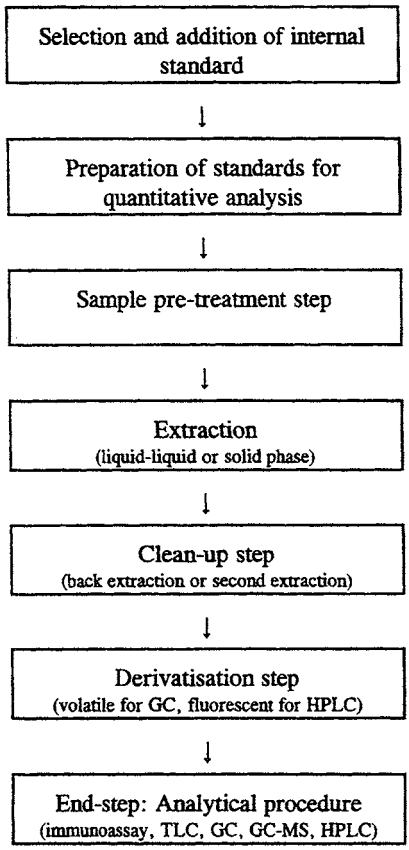


Figure II.3 Flow diagramme for the analysis of barbiturates in biological specimens

2. Choice of Internal Standard

The extraction procedure normally includes the addition of an internal standard. Suitable internal standards must take into account the method of extraction and the end-step procedure for detection, confirmation and quantitation. Suitable internal standards in all cases are barbiturate derivatives which are unlikely to be found in specimens because they are not used as drugs or are no longer prescribed in that country.

Solid-Phase Extraction (SPE) methods must use an internal standard with similar chemical properties (i.e. acidic) but this may not be true for solvent extraction unless a back

extraction step is included. Internal standards which have been reported include tetraphenylethylene [21], heptabarbital [21], tolylbarbital [26,27], aprobarbital [28], prazepam [29] and hexobarbital [30]. For GC-MS, deutero-labelled standards are suitable for quantitation by selected ion monitoring.

3. Extraction Procedures

(a) 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].

A study of extraction efficiencies of barbiturates from water (assume urine) and plasma has been carried out using 5 different solvents (hexane, ethyl ether, toluene, *n*-butyl chloride and chloroform) [31]. The results indicated that extraction of a 2 ml sample of water at acidic pH with 10 ml solvent gave the following recoveries shown in Table II.4. The corresponding efficiencies for plasma are also shown in the Table.

Table II.4 Extraction efficiencies of barbiturates from water and plasma*

Solvent	Recovery (%) water/urine	Recovery (%) plasma
n-butyl chloride	34-51	13-38
Chloroform	45-78	41-68
Ethyl ether	81-97	78-93
Hexane	0-14	0-8
Toluene	15-67	5-49

* after [31]; data only for barbiturates under international control.

Hexane and toluene gave relatively poor recoveries, whilst *n*-butyl chloride, chloroform and ethyl ether gave adequate recoveries.

Other solvent systems reported in the literature for the extraction of barbiturates in urine include dichloromethane [28], hexane - ethyl acetate (6:4, v/v) (75-84% recovery) [27] and toluene - ethyl acetate (4:1, v/v) [32].

(i) Urine

For the analysis of barbiturates in urine a hydrolysis step is not required.

The volume of specimen used depends on the sensitivity of the end-step procedure. For urine, the volume recommended varies between 0.5 ml [27,28] and 10 ml [21].

The pH is adjusted to acidic conditions by the addition of 1-2 ml of 1 M sulfuric acid,

phosphoric acid or tartaric acid [21] or phosphate buffer [27,28].

Solvent volume relative to the volume of urine can range from 1:1 (e.g. 10 ml for 10 ml of urine) to 6:1 [28].

Back extraction for clean-up of the initial extract uses 0.5 M sodium hydroxide (0.5 times the volume of solvent used for the initial extract, e.g. 5 ml for 10 ml of ethyl ether [21]) followed by acidification to acidic pH and re-extraction with solvent.

(ii) Plasma and Blood

Barbiturates are normally detected in plasma using radio immunoassay (RIA), GC or HPLC. TLC can also be used [33] if no other method is available. The volume of plasma used is typically 0.1 - 0.2 ml [21] or larger, up to 2 ml [34]. For TLC, the volume of plasma extracted is relatively high, being approximately 5 ml.

The pH is adjusted with phosphate buffer, e.g. 0.1 times the volume of phosphate buffer, pH 6.5 [21] or 0.25 volume phosphate buffer (50% saturated dihydrogen salt, pH 5) [33].

Solvents used include chloroform (0.25-10 times the volume of plasma), but the other solvents listed earlier for urine (notably ethyl ether and *n*-butyl chloride) may also be used. This extract is usually not back-extracted to clean it up, but is concentrated by forced evaporation and injected directly.

The volume of blood extracted has varied from 1 ml [26] to 7 ml [35]. Extraction solvents have included chloroform (10 times the volume) [35] and ethyl acetate (5 times the volume) [26]. The initial extract can be purified after evaporation to dryness by partition between hexane and acetonitrile and discarding the hexane (upper) layer [36].

b) Solid-Phase Extraction

(i) Urine

Methods which can be recommended are produced by most solid-phase column manufacturers, e.g. Varian (Bond-Elut Certify II), Waters (Sep-Pak) and International Sorbent Technology (isolut Confirm HCX) [37,38]. Diatomaceous earth, e.g. Extrelut (Merck), has also been used [36,39,40].

The following methods are recommended.

SOLID PHASE EXTRACTION METHOD A FOR URINE USING CERTIFY COLUMNS

after Pocci et al., 1992 [38]

Materials and Reagents

1. Bond Elut Certify II columns, 10 ml capacity (Varian Sample Preparation Products).
2. Vac Elute vacuum manifold (Varian AI 6000 or equivalent).
3. 100 mM sodium acetate buffer, pH 7.0.

Method

1. Treat 5 ml of urine with 2 ml of 100 mM sodium acetate buffer, pH 7.0 (adjust pH to between 5.0 and 7.0 if necessary).
2. Precondition columns with 2 ml of methanol followed by 2 ml of 100 mM sodium acetate buffer, pH 7.0 at a slow flow rate (control the flow rate by adjusting the vacuum of manifold).
3. Transfer a urine specimen to a column and elute at a slow flow rate until completely through. Do same for other urine specimens.
4. Wash the columns with 1 ml 100 mM sodium acetate buffer, pH 7.0, at a slow flow rate.
5. Dry the columns by leaving on vacuum for approx. 5 min further.
6. Wash columns with 2 ml hexane - ethyl acetate (95:5) and wipe tips of delivery needles dry.
7. Apply 2 ml hexane - ethyl acetate (75:25) to each column and allow to elute slowly into collection vials.
8. Add internal standard to eluate and evaporate at room temperature to dryness under a stream of nitrogen.
9. Reconstitute residue with 100 μl of ethyl acetate.

Notes

1. Internal standard can be added at the start of sample preparation, examples that can be used include other barbiturates such as hexobarbital.
2. Recoveries for pentobarbital and amobarbital are 82% and 90%, respectively.

SOLID PHASE EXTRACTION METHOD B FOR URINE
USING DIATOMACEOUS EARTH

after Ferrara et al., 1992 [40]

Materials and Reagents

1. Extrelut 3 columns (Merck).
2. 0.5 M phosphate buffer, pH 5.5.
3. 5% isopropanol in dichloromethane.

Method

1. Add 2 ml of 0.5 M phosphate buffer, pH 5.5, to 2 ml of urine.
2. Pour mixture into an Extrelut 3 column and wait approx. 10 min. Discard waste.
3. Elute with 15 ml of 5% isopropanol in dichloromethane and collect in suitable containers.
4. Evaporate the eluate to dryness under a stream of nitrogen at 40°C.
5. Reconstitute the extract with a solvent.

(ii) Blood

A solid-phase method for whole blood has been published (see below). Blood is sonicated, diluted with buffer and centrifuged. The supernatant is extracted using Bond-Elut Certify or Clean Screen DAU columns.

The following method is recommended.

SOLID PHASE EXTRACTION METHOD FOR BLOOD

after Chen et al., 1992 [29]

Materials and Reagents

1. Bond Elut Certify columns, 10 ml capacity (Varian Sample Preparation Products).
2. Clean Screen DAU columns, 12 ml capacity (Worldwide Monitoring Corporation).
3. SPE Vacuum manifold system (Baker-10 system or equivalent).
4. 0.1 M phosphate buffer, pH 6.0.
5. 0.01 M acetic acid, pH 3.3.

Method

1. Sonicate 1 ml of blood for 15 min in an ultrasonic bath at room temperature (alternate treatment procedures can be used if sonication is not possible, see original reference).
2. Vortex the blood specimens for 30 s after the addition of 6 ml of 0.1 M phosphate buffer, pH 6.0.
3. Centrifuge the blood specimen at 2000 rpm for 15 min and discard the pellet.
4. Precondition columns with 2 ml of methanol followed by 2 ml of phosphate buffer, pH 6.0 at a flow rate of approx. 2 ml/min (control the flow rate by adjusting the vacuum of manifold).
5. Transfer the pretreated blood specimen to a column at approx. 1.5 ml/min and elute until completely through. Do same for other columns.
6. Wash the columns with 1 ml purified water at approx. 1.5 ml/min.
7. Apply 0.5 ml of 0.01 M acetic acid, pH 3.3, to each column.
8. Dry the columns by leaving on vacuum for approx. 4 min further, then add 50 μl of methanol and dry for a further 1 min under vacuum.
9. Apply 4 ml of acetone - chloroform (1:1) to each column and allow to elute at approx. 0.8 ml/min into collection vials.
10. Add internal standard to eluate and evaporate at 40°C under a stream of nitrogen until approx. 100 μl of solvent is left.

Notes

1. Either Certify or Clean Screen columns can be used.

2. Internal standard can be added at the start of sample preparation, examples that can be used include other barbiturates or prazepam.
3. Recoveries for pentobarbital and hexobarbital are 92% and 94%, respectively.
4. Basic drugs can also be eluted using 2 ml ammoniated ethyl acetate after the acetone/chloroform solvent.

4. Screening Methods

(a) Immunoassay Methods

A number of different immunoassays are available for barbiturates. Some of these are listed together with their cut-off levels in Table II.5 [40].

Table II.5 Summary of immunoassays available for barbiturates

Assay	Principle of immunoassay	Calibration compound	Cut-off ng/ml
EMIT-1 or -2	enzyme-linked	Secobarbital	300
ADx/TDx	fluorescent polarisation	Secobarbital	500
Coat-A-Count RIA	radio labelled	Secobarbital	100
ONTRAK	latex agglutination	Secobarbital	200
EZ-SCREEN	enzyme-linked	Phenobarbital	300
Triage	competitive binding	Secobarbital	300
OnLine	kinetic interaction	Secobarbital	200
CEDIA	enzyme-linked	Secobarbital	200

Comparisons of immunoassays in terms of their reliability have been published [40,41].

Table II.6 Cross-reactivities of selected immunoassays

Barbiturate	Conc. equiv.	C.-r. (%)	Conc. equiv.	C.-r. (%)	Conc. (ng/ml)	C.-r. (%)	Conc. (ng/ml)	C.-r. (%)
KIT	ONLINE		ONTRAK		EMIT		TDx/AxSYM	
Allobarbital	358	76	200	100	10000	100	400	100
Amobarbital	913	22	200	100	2000	100	700	100
Barbital	1324	15	100	200	24000	100	2000	100
Butalbital	442	52	250	80	3000	100	200	100
Butobarbital					1000	100	200	100
Pentobarbital	550	45	500	40	1000	100	200	100
Phenobarbital	690	29	700	29	3000	100	200	100
Secbutabarbital					-	-	-	-
Secobarbital	200	100	300	100	300	100	300	100

Conc.equiv. = concentration equivalence
C.-r. = cross-reactivity
Conc. = concentration

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 which normally accompany kits for data pertaining to the materials being used.

It is important that immunoassay kits are used according to the manufacturer's instructions concerning dilution of specimens and reagents, volumes of reagents and storage/shelf-life of reagents. If changes are made to the manufacturer's 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 which 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.1. of this manual for more information.

(b) Thin-Layer Chromatography

Prepare urine specimens by extraction as per previous Chapter II.F.3..

The following methods are recommended.

TLC METHODS

Plates

Activated silica gel G on glass plates; the coating (0.25 mm thickness) contains an additive which fluoresces at 254 nm.

Developing Solvents

SYSTEM A:	Ethyl acetate	85
	Methanol	10
	25% ammonia	5
SYSTEM B:	Chloroform	80
	Acetone	20
SYSTEM C:	Isopropanol	90
	Chloroform	90
	25% ammonia	20

Preparation of solutions to be applied to the TLC plate

Sample
Extract the material using the method outlined in Chapter II.F.3. and prepare a solution in methanol containing the equivalent of approx. 5 mg/ml.

Standard solutions
All are made at a concentration of 5 mg/ml in methanol.

Visualization

The plates must be dried prior to visualization. This can be done at 120°C for 5 min in an air oven or, more quickly, by using a hot air blower.

1. UV light at 254 nm, both before and after exposure to ammonia vapour.
2. Mercuric chloride - diphenylcarbazone reagent.

   Spray reagent
   (a) Dissolve 0.1 g of diphenylcarbazone in 50 ml of ethanol.
   (b) Dissolve 1 g of mercuric chloride in 50 ml of ethanol. Prepare the solution daily.
   Mix (a) and (b) together just before spraying.

Method (*after Clarke's* [21])

Apply 1 to 2 μl of the sample and standard solutions to the plate. Develop with appropriate solvent system. Dry. First observe the plate under short wavelength UV light (254 nm). Expose the plate to concentrated ammonia vapours and observe again under UV light at the same wavelength. If necessary, spray with mercuric chloride - diphenylcarbazone reagent. Barbiturates give blue-violet spots on a pink background.

Detection limits are about 1-5 μg.

Note

Mercuric chloride - diphenylcarbazone is the most sensitive spray reagent amongst the many tested for the detection of barbiturates. However, the use of mercury salts cannot be recommended because of environmental concerns. Detection by visualization Method 1. is usually sufficient. Should the use of this reagent still be required, the spraying procedure must be performed with special care to guard against harmful mercury vapours.

Visualization can also use 1% silver acetate followed by diphenylcarbazone spray [33] or exposure to chlorine vapours followed by 2,7-dichlorofluorescein and Dragendorff's reagent [42]. The latter can detect down to 0.5 μg of barbiturate on the plate.

Table II.7 TLC hRf values for three solvent systems [43]

Barbiturate	Developing System* ($R_f \times 100$ values)		
	A	B	C
Allobarbital	31	50	53
Amobarbital	36	52	74
Barbital	31	41	51
Butalbital	38	54	67
Butobarbital	38	50	68
Cyclobarbital	35	50	59
Methylphenobarbital	43	70	72
Pentobarbital	45	55	76
Phenobarbital	28	47	38
Secbutabarbital	41	50	69
Secobarbital	44	55	78
Vinylbital	40	38	-

* See previous TLC methods for identity of solvent system A, B and C.

5. Confirmatory Methods

(a) Spectrophotometry

Barbiturates can also be detected by spectrophotometry because of their relatively high concentrations in biological specimens. This technique is not generally applicable to other groups of controlled drugs.

Barbiturates can be extracted from serum with chloroform and estimated by colorimetry at 550 nm after formation of a coloured derivative with mercuric chloride -diphenylcarbazone reagent. Alternatively, UV spectrophotometry can be used after back-extraction from chloroform into sodium hydroxide [21].

The following method is recommended.

EXTRACTION METHOD USING DIRECT DIFFERENTIAL SPECTROPHOTOMETRY [44]

This method is designed for blood. Barbiturates are extensively metabolised and therefore require alternative methods. To improve recovery, blood may also be treated with 10% sodium tungstate in 10% sulfuric acid to precipitate proteins prior to an ether extraction.

1. To 2 ml of blood in a glass stoppered tube add 2 ml of phosphate buffer, pH 7.
2. Add 20 ml of chloroform and shake vigorously for 5 min.
3. Following a brief centrifugation remove the upper layer with a pasteur pipette.
4. Filter the chloroform layer through filter paper into a dry conical tube and evaporate to dryness under a stream of air.
5. Dissolve the residue in 3 ml 0.45 M sodium hydroxide and place in a spectrophotometer cell.
6. Scan the extract from 220 to 320 nm. Use a reference cell containing only sodium hydroxide solution.
7. Add 0.5 ml of 16% ammonium chloride solution to the sample (to the 3 ml) and also to the reference cell.
8. Scan the extract from 220 to 320 nm.
9. Add a few drops of 50% sulfuric acid to both sample and reference cells.
10. Scan again from 220 to 320 nm.

Interpretation

Barbiturates show little UV absorption at acidic pH (last step) and a UV shift from pH 13 (first step) to pH 9-10 (second step). See Table II.8 for UV maxima [21]. Detection limits are poor. The method is generally only useful for situations involving large amounts of barbiturates, e.g. overdoses.

Table II.8 UV maxima of barbiturates at pH 9 and 13

Barbiturates	pH 9.2[a]	pH 13.0[b]
Allobarbital	241	256
Amobarbital	240	255
Barbital	239	254
Butalbital	240	255
Butobarbital	239	254
Cyclobarbital	239	256
Methylphenobarbital	244	243
Pentobarbital	239	255
Phenobarbital	239	254
Secbutabarbital	239	254
Secobarbital	239	254
Vinylbital	-	247

[a] = 0.05 M borax buffer, pH 9.2
[b] = 1.0 M sodium hydroxide solution, pH 13.0

(b) Gas Chromatography

(i) Packed Column Technique

The selection of stationary phases available for the gas chromatography of barbiturates on packed columns is large. While certain phases are recommended in this manual, this does not necessarily mean that other phases are not suitable. This comment also applies to column dimensions. The length and internal diameter, while affecting the retention characteristics of substances, can still be varied from those recommended, providing the chromatography conditions are well established by the analyst and the final procedure validated with respect to specificity and reproducibility and other critical performance indicators for the method at hand.

A recommended method is described below using dimethyl silicone (SE-30, OV-1). Other packed columns used include 2% SE-30 on Chromosorb G, 2% OV-17 on Chromosorb G-HP and 3% Poly A103 on Chromosorb W-HP [21,22]. One comparison of 12 difference

stationary phases included OV-25, OV-225 [45].

Alternate derivatizing methods have been described including formation of butyl derivatives (to distinguish derivatives from methylated barbiturate parent compounds), trimethylsilyl ethers, extractive alkylation and pentafluorobenzyl derivatives [46,47] as well as propyl derivatives [48]. One publication concerning propyl derivatives cautions that the final evaporation step must be carried out gently to avoid losses by volatilization [49].

It should be noted that derivatization is less likely to be needed for capillary columns using a chemically-modified fused silica column than for a packed column. Quantification of barbiturates by packed column GC will almost inevitably require derivatization.

The following method is recommended.

PACKED COLUMN METHOD

after Gill et al., 1981 [50]

Operating Conditions

Column:	2 m × 2-4 mm ID glass column, packed with 3% SE-30 on Chromosorb G-HP, 80-100 mesh
Carrier gas:	Nitrogen at 45-50 ml/min
Column temperature:	190-200°C
Injector temperature:	220°C
Detector temperature:	220°C

Derivatizing Conditions

Dissolve extract in a solution of trimethyl anilinium hydroxide (0.2 M) in methanol (Methelute) and inject directly into the gas chromatograph. On column methylation occurs.

Standards

Barbiturate standards are prepared as a 1 mg/ml stock solution in methanol. Dilutions can be made, as necessary, in methanol.

Internal Standards and Retention Time Markers

n-alkanes for use as retention time markers to calculate retention indices are prepared as 1 mg/ml solutions in ethyl acetate. These do not need to be derivatized.

Internal standards should be barbiturates which are not targeted in the analysis. These can be made up as solutions in methanol (1 mg/ml stock).

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 substentially 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.

(ii) Capillary Column Technique

Similar to packed column gas chromatography, the selection of capillary columns with respect to stationary phase, film thickness, column dimensions and type is large. The chromatographic characteristics of each column must be established as being adequate for the analytical procedure.

Recommended methods use a 25 m × 0.35 mm ID chemically-bonded fused silica column with a 0.52 μm coating of dimethyl silicone (SE-30 or equivalent, e.g. CP Sil-5, HP-1) or 50% phenyl 50% methyl silicone (OV-17). The carrier gas can be nitrogen at approx. 1 ml/min, although helium is often preferred [6].

Wide-bore capillary columns have also been used, e.g. 30 m × 0.53 mm ID, dimethyl silicone stationary phase, film thickness 0.88 μm or 30 m × 0.75 mm ID, dimethyl silicone stationary phase, film thickness 1.0 μm [29]. Shorter columns can be used [51].

The following methods are recommended.

CAPILLARY COLUMN METHOD A

after Jupp et al., 1987 [52]

Operating Conditions

Column:	25 m × 0.35 mm ID chemically bonded fused silica capillary column with 0.52 μm coating of dimethyl silicone (e.g. CP Sil-5, HP-1)
Carrier gas:	Nitrogen or helium at 1 ml/min
Split ratio:	20:1
Column temperature:	start at 200°C, then programmed at 4°C/min to 260°C
Injector temperature:	275°C
Detector temperature:	275°C

Derivatizing Conditions

Derivatizing is usually not necessary.

Standards

See packed column technique.

Internal Standards and Retention Time Markers

See packed column technique.

CAPILLARY COLUMN METHOD B

after Jupp et al., 1987 [52]

Operating Conditions

Column:	30 m × 0.53 mm ID chemically bonded fused silica capillary column with 0.88 μm coating of dimethyl silicone (e.g. CP Sil-5, HP-1)
Carrier gas:	Nitrogen or helium at 10 ml/min
Split ratio:	splitless mode
Column temperature:	start at 80°C for 2 min, then programmed at 20°C/min to 215°C and at 5°C/min to 285°C, hold at 285°C for 2 min
Injector temperature:	275°C
Detector temperature:	310°C

Derivatizing Conditions

Derivatizing is usually not necessary.

Standards

See packed column technique.

Internal Standards and Retention Time Markers

See packed column technique.

Table II.9 Retention indices on selected GC column types [52]

Barbiturate	Packed column technique	Capillary column technique	
	SE-30 (dimethyl silicone)	narrow bore: 0.22 mm ID, 0.25 μm film thickness dimethyl silicone	wide bore: 0.53 mm ID, 1.0 μm film thickness dimethyl silicone
Allobarbital	1606	-	-
Amobarbital	1718	1695	1705
Barbital	1497	1461	1478
Butalbital	1668	-	-
Butobarbital	1665	1642	1649
Cyclobarbital	1963	1947	1955
Methylphenobarbital	1891	-	-
Pentobarbital	1740	1720	1732
Phenobarbital	1957	1932	1937
Secbutabarbital	1662	-	-
Secobarbital	1791	1772	1777
Vinylbital	1720	-	-

(iii) Detectors

Detectors suitable for the analysis of barbiturates by GC include the Flame Ionization Detector (FID) and the Nitrogen Phosphorous Detector (NPD) (often termed a nitrogen-selective detector). The NPD is a more selective detector and will provide less interfering background and a smaller solvent front than a FID, although a FID is adequate for many applications.

Mass selective detectors such as mass spectrometers or ion traps are often used in combination with capillary GC and is the technique of choice for sensitivity and discriminating power. See Chapter II.F.6.c on Gas Chromatography - Mass Spectrometry.

(c) Gas Chromatography - Mass Spectrometry

Because of the similarity of many barbiturates, electron impact (EI) mass spectrometry of underivatized barbiturates does not discriminate between them easily and only weak molecular ions are obtained. Therefore, when identifying barbiturates by GC-MS, spectra must

be taken in the full scan mode. Identification of an analyte is obtained by comparing its retention time and three qualifier ions with those of reference standards. To ensure specificity, quantification should be carried out using reconstructed ion chromatogrammes, i.e. using ion chromatogrammes generated from full scan data, comparing the base peak ion with the representative area of the internal standard ion and the calibration curve. Ions below m/z 50 are unlikely to be of diagnostic value.

As some barbiturates have the same spectra, even after methylation (e.g. amobarbital and pentobarbital) using the EI mode, the current approach is to use positive ion chemical ionization (CI) with methane as reagent gas.

Mulé et al. have published a GC-MS method based on the methyl derivatives of the barbiturates [27], analysed by positive ion electron impace (EI^+) GC-MS using a 12.5 m × 0.2 mm ID fused silica capillary column coated with cross-linked dimethyl silicone. Selected ion chromatogrammes for each barbiturate are listed. The limit of quantitation was 20 ng/ml urine, plasma or blood. An alternative procedure was published by Pocci [38].

Table II.10 Prominent ions and abundances for barbiturates under international control

Barbiturates	**m/z and abundance***
Allobarbital	167(100), 124(97), 80(68), 193(23), 208(2)
Amobarbital	156(100), 141(73), 197(9), 198(6), 211(2)
Barbital	156(100), 141(97), 98(22), 112(20), 83(12)
Butalbital	168(100), 167(88), 181(30), 141(24), 209(3)
Butobarbital	141(100), 156(96), 98(19), 184(10), 197(2)
Cyclobarbital	207(100), 141(33), 236(3), 81
Methylphenobarbital	218(100), 117(39), 146(23), 246(10)
Pentobarbital	156(100), 141(84), 69(12), 98(10), 197(4)
Phenobarbital	204(100), 117(29), 232(23), 161(20)
Secbutabarbital	141(100), 156(87), 57(27), 98(13), 157(12)
Secobarbital	168(100), 167(80), 195(25), 141(11), 209(4)
Vinylbital	157(100), 83(29), 71(15), 209(1), 195(1)

* only ions of m/z > 50 included.

(d) High Performance Liquid Chromatography

Normal-phase as well as reversed-phase HPLC systems have been described for the analysis of barbiturates in biological specimens. Recommended methods use octadecyl and octyl silica columns as well as normal-phase silica columns [6,40,50,53]. To protect the analytical column the use of a guard column with the same packing is recommended.

Alternate methods have been described by Baselt [21], Chan and Chan [26] and White

[45]. Baselt uses a similar system as recommended in Method D giving a sensitivity of 0.25-1 mg/l plasma. Chan and Chan describe the use of a gradient system with a similar mobile phase (acetonitrile - phosphate buffer). White uses an octadecyl silica column with a mobile phase of methanol - 0.1% aqueous ammonium carbonate (40:60) to separate 27 out of the 29 barbiturates studied.

The following methods are recommended.

HPLC METHOD A	
	after ST/NAR/18 [6]
Column:	Octadecyl silica (ODS Hypersil or equivalent), 150 mm × 4.6 mm ID
Mobile phase:	0.1 M phosphate buffer, pH 3.5 - methanol (60:40, v/v)
Flow rate:	2 ml/min
Detector:	UV at 216 nm
Injection vol.:	20 µl

HPLC METHOD B	
	after ST/NAR/18 [6]
Column:	Octadecyl silica (ODS Hypersil or equivalent), 150 mm × 4.6 mm ID
Mobile phase:	0.1 M phosphate buffer, pH 8.5 - methanol (60:40, v/v)
Flow rate:	2 ml/min
Detector:	UV at 216 nm
Injection vol.:	20 µl

HPLC METHOD C	
	after Gill et al., 1981 [50]
Column:	Silica (Hypersil or equivalent), 250 mm × 4.6 mm ID
Mobile phase:	Isooctane - acetic acid - isopropanol (200:3:2, v/v/v)
Flow rate:	2 ml/min
Detector:	UV at 250 nm
Injection vol.:	20 µl

HPLC METHOD D

after Ferrara et al., 1992 [40]

Column:	Octyl silica (LiChrospher 100 RP8 or equivalent), 5 μm particle size, 250 mm × 4 mm ID, with a precolumn
Mobile phase:	0.01 M phosphate buffer, pH 3.5 - acetonitrile (60:40 to 70:30, v/v depending on separation characteristics required); solvent degassed prior to use.
Flow rate:	1 ml/min
Detector:	UV at 212 nm

HPLC METHODS A - D

Preparation of Standards

Dissolve accurately a weighed amount of barbiturate in methanol (or ethanol) at a concentration of 1 mg/ml. Prepare a 1:100 or 1:10 dilution in methanol (or ethanol) and inject to establish adequate chromatography of barbiturates.

Preparation of Extracts

Prepare extracts as per recommended procedures described earlier. Reconstitute extract either in HPLC mobile phase or methanol and chromatograph using one of the recommended procedures.

Internal Standards

Use an internal standard as a marker of recovery efficiency and retention times.

Results

Capacity factors (k') for the three suggested chromatographic conditions are shown below (Table II.11). The actual capacity factors may vary depending on the column characteristics, such as phase loading and degree of end-capping, consequently use these values as guides only.

Table II.11 HPLC retention data (k') for barbiturates [6,50]

Barbiturate	System A*	System B*	System C*
Allobarbital	2.46	1.33	9.03
Amobarbital	10.91	7.05	7.17
Barbital	1.11	0.63	11.95
Butalbital	6.17	3.48	6.68
Butobarbital	5.43	3.42	7.60
Cyclobarbital	5.25	2.61	10.04
Methylphenobarbital	7.27	3.84	2.91
Pentobarbital	10.96	8.07	6.78
Phenobarbital	3.09	1.23	12.57
Secbutabarbital	4.89	3.32	7.59
Secobarbital	16.28	11.47	5.63
Vinylbital	10.40	7.05	n.r.

* refer to HPLC methods A, B and C, respectively; n.r. = no data

III. Recommended Methods for the Detection and Assay of Benzodiazepines in Biological Specimens

A. Introduction

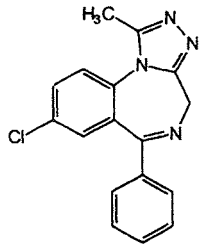
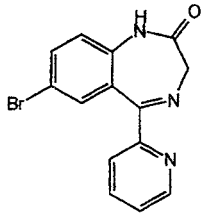
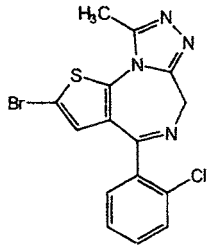
Benzodiazepines, therapeutically used as tranquilizers, hypnotics, anticonvulsants and centrally-acting muscle relaxants, rank among the most frequently prescribed drugs. They are given in a wide range of dosages from less than 0.1 mg to 100 mg or more each day.

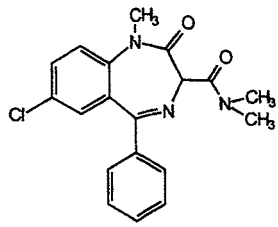
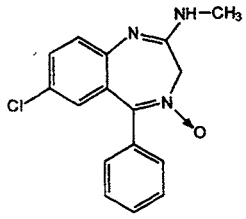
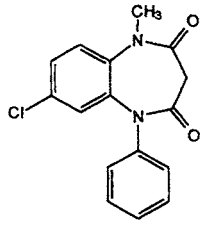
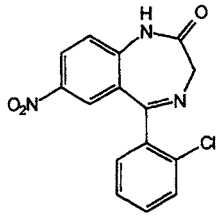
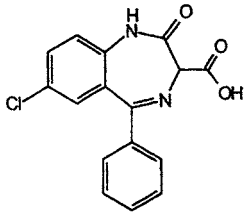
Numerous benzodiazepines have been synthesized. More than fifty of these are presently marketed for clinical use throughout the world. They appear mainly as capsules and tablets, however some are marketed in other pharmaceutical forms such as injectable solutions.

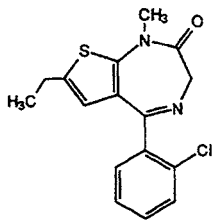
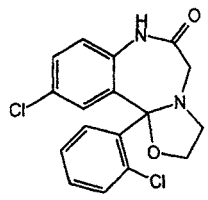
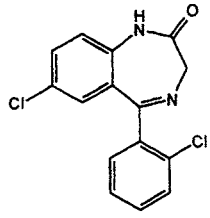
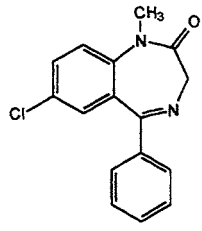
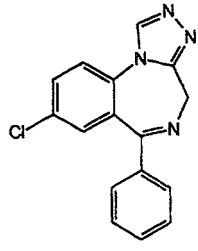
Abuse/misuse of benzodiazepines is internationally widespread which means that any forensic laboratory may encounter a range of these compounds. Virtually all of the benzodiazepines in the illicit market result from diversion from legitimate sources and there is no evidence of clandestine manufacture.

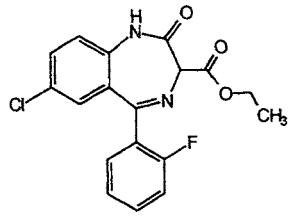
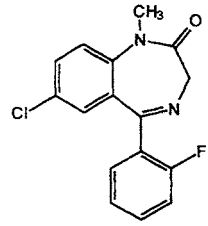
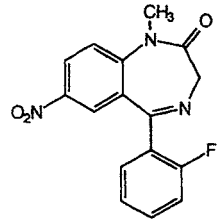
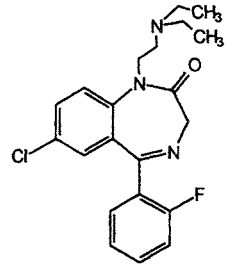
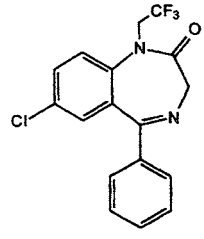
Out of the presently marketed benzodiazepines thirty-five are subject to international control under the 1971 Convention on Psychotropic Substances.

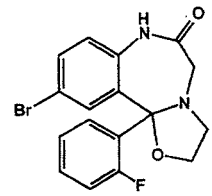
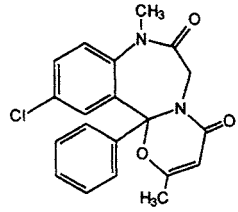
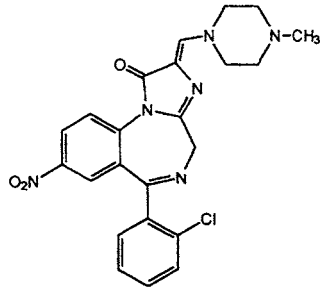
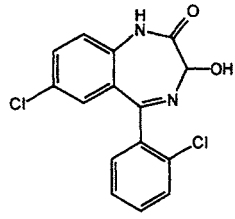
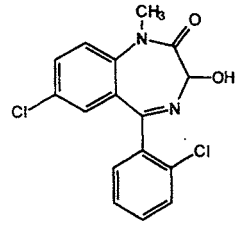
Table III.1 Benzodiazepines under international control

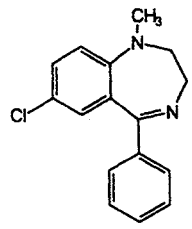
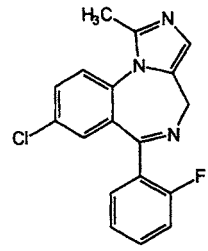
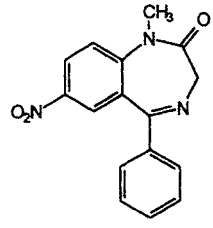
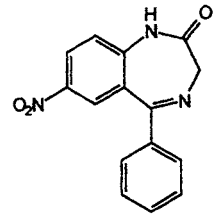
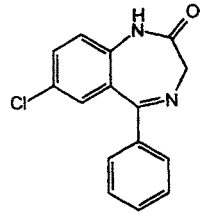
Alprazolam		$C_{17}H_{13}ClN_4$ pKa 2.4 Schedule IV	M.W. 308.8
Bromazepam		$C_{14}H_{10}BrN_3O$ pKa 2.9, 11.0 log P (octanol/pH 7.4) 1.6 Schedule IV	M.W. 316.2
Brotizolam		$C_{15}H_{10}BrClN_4S$ Schedule IV	M.W. 393.7

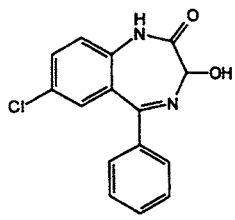
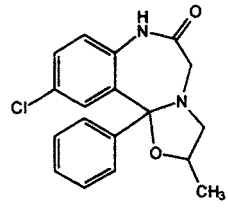
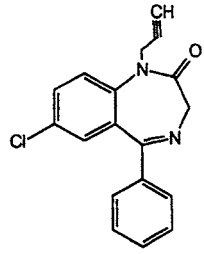
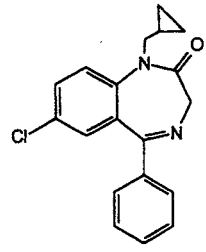
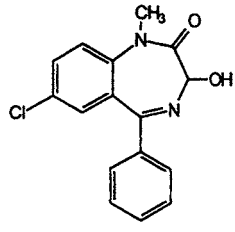
Camazepam 	$C_{19}H_{18}ClN_3O_3$ M.W. 371.8 Schedule IV	
Chlordiazepoxide 	$C_{16}H_{14}ClN_3O$ M.W. 299.8 pKa 4.6 log P (octanol/pH 7.4) 2.5 Schedule IV	
Clobazam 	$C_{16}H_{13}ClN_2O_2$ M.W. 300.7 Schedule IV	
Clonazepam 	$C_{15}H_{10}ClN_3O_3$ M.W. 315.7 pKa 1.5, 10.5 log P (octanol/pH 7.4) 2.4 Schedule IV	
Clorazepate 	$C_{16}H_{11}ClN_2O_3$ M.W. 314.7 pKa 3.5, 12.5 Schedule IV	

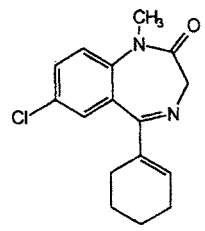
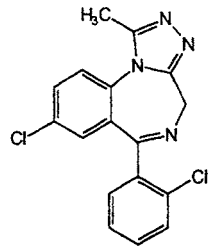
Clotiazepam 	$C_{16}H_{15}ClN_2OS$ M.W. 318.8 Schedule IV	
Cloxazolam 	$C_{17}H_{14}Cl_2N_2O_2$ M.W. 349.2 Schedule IV	
Delorazepam 	$C_{15}H_{10}Cl_2N_2O$ M.W. 305.2 Schedule IV	
Diazepam 	$C_{16}H_{13}ClN_2O$ M.W. 284.7 pKa 3.3 log P (octanol/pH 7.4) 2.7 Schedule IV	
Estazolam 	$C_{16}H_{11}ClN_4$ M.W. 294.7 pKa 9.6 Schedule IV	

Ethyl loflazepate 	$C_{18}H_{14}ClFN_2O_3$ Schedule IV	M.W. 360.8
Fludiazepam 	$C_{16}H_{12}ClFN_2O$ Schedule IV	M.W. 302.7
Flunitrazepam 	$C_{16}H_{12}FN_3O_3$ pKa 1.8 Schedule III	M.W. 313.3
Flurazepam 	$C_{21}H_{23}ClFN_3O$ pKa 1.9, 8.2 log P (octanol/pH 7.4) 2.3 Schedule IV	M.W. 387.9
Halazepam 	$C_{17}H_{12}ClF_3N_2O$ Schedule IV	M.W. 352.7

Haloxazolam 	$C_{17}H_{14}BrFN_2O_2$ M.W. 377.2 Schedule IV	
Ketazolam 	$C_{20}H_{17}ClN_2O_3$ M.W. 368.8 Schedule IV	
Loprazolam 	$C_{23}H_{21}ClN_6O_3$ M.W. 464.9 pKa 6.0 Schedule IV	
Lorazepam 	$C_{15}H_{10}Cl_2N_2O_2$ M.W. 321.2 pKa 1.3, 11.5 log P (octanol/pH 7.4) 2.4 Schedule IV	
Lormetazepam 	$C_{16}H_{12}Cl_2N_2O_2$ M.W. 335.2 Schedule IV	

Medazepam		$C_{16}H_{15}ClN_2$ M.W. 270.8 pKa 6.2 log P (octanol/pH 7.4) 4.0 Schedule IV
Midazolam		$C_{18}H_{13}ClFN_3$ M.W. 325.8 pKa 6.2 Schedule IV
Nimetazepam		$C_{16}H_{13}N_3O_3$ M.W. 295.3 Schedule IV
Nitrazepam		$C_{15}H_{11}N_3O_3$ M.W. 281.3 pKa 3.2, 10.8 log P (octanol/pH 7.4) 2.1 Schedule IV
Nordazepam		$C_{15}H_{11}ClN_2O$ M.W. 270.7 pKa 3.5, 12.0 Schedule IV

Oxazepam		$C_{15}H_{11}ClN_2O_2$ M.W. 286.7 pKa 1.7, 11.6 log P (octanol/pH 7.4) 2.2 Schedule IV
Oxazolam		$C_{18}H_{17}ClN_2O_2$ M.W. 328.8 Schedule IV
Pinazepam		$C_{18}H_{13}ClN_2O$ M.W. 308.8 Schedule IV
Prazepam		$C_{19}H_{17}ClN_2O$ M.W. 324.8 pKa 2.7 log P (octanol/pH 7.4) 3.7 Schedule IV
Temazepam		$C_{16}H_{13}ClN_2O_2$ M.W. 300.7 pKa 1.6 Schedule IV

Tetrazepam		$C_{16}H_{17}ClN_2O$ M.W. 288.8 Schedule IV
Triazolam		$C_{17}H_{12}Cl_2N_4$ M.W. 343.2 Schedule IV

From INCB statistics [9], the significant benzodiazepines in the last decade have been diazepam, lorazepam, alprazolam, temazepam, chlordiazepoxide, nitrazepam, triazolam, flunitrazepam and lormetazepam.

Analysts should be aware of the particular benzodiazepines 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 Benzodiazepine Derivatives under International Control (ST/NAR/16) [7] as well as to national pharmacopoeias and drug tablet and capsule identification guides for preliminary screening identification. The Multilingual Dictionary of Narcotic Drugs and Psychotropic Substances under International Control (ST/NAR/1/REV.1) [10] published by UNDCP includes a listing of many brand names and other synonyms for benzodiazepines under international control.

B. Physical and Chemical Characteristics

The classical benzodiazepines are based on a 5-aryl-1,4-benzodiazepine structure in which the benzene ring is fused to the 6-7 bond of the 1,4-diazepine. The aryl substituent at position 5 is usually phenyl (e.g. oxazepam or medazepam) or 2-halophenyl (e.g. 2-chlorophenyl for lorazepam or 2-fluorophenyl for flurazepam) [54,55].

The more recently introduced benzodiazepines include variations such as an imidazole (1,3-diazole) ring fused to the 1-2 bond of the 1,4-diazepine, i.e. the imidazo-benzodiazepines, e.g. midazolam or loprazolam. Similar but distinctly different are the triazolobenzodiazepines which have a 1,2,4-triazole ring instead of the imidazole. Examples of these compounds are alprazolam, estazolam and triazolam. Other structural modifications include annelation of heterocyclic rings at the 4-5 bond (e.g. haloxazolam, ketazolam and

oxazolam) or replacement of the benzene ring by a thienyl ring (clotiazepam).

Many benzodiazepines will hydrolyse in acid solutions to form the corresponding benzophenone which can be capitalized on for analytical purposes.

In the free base/acid form, benzodiazepines are generally soluble in most organic solvents such as ethyl ether, ethyl acetate, chloroform and methanol, but most are insoluble in water.

C. Pharmacology

The continuing popularity of the benzodiazepines is due mainly to their wide therapeutic index, minimal serious adverse reactions and the absence of undesirable autonomic nervous side effects, especially when compared with psychotropic agents used before, for example, meprobamate or barbiturates [56].

1. Current Uses of Benzodiazepines

Current uses include:

- as hypnotics and sedatives - e.g. triazolam, flunitrazepam, oxazepam;
- as anxiolytics and minor tranquilizers - e.g. diazepam, temazepam, alprazolam;
- as antidepressants - e.g. alprazolam;
- as muscle relaxants - e.g. diazepam and tetrazepam;
- as anticonvulsants - e.g. diazepam, clobazam and clonazepam;
- as intravenous anesthetics - e.g. midazolam.

2. Effects of the Benzodiazepines

All benzodiazepine derivatives in clinical use possess anxiolytic, sedative, hypnotic, tranquilizing, anticonvulsant and muscle relaxant properties within their pharmacodynamic spectra. The prevalence of these properties, however, varies significantly between compounds. Therefore, individual drugs are selected for use in therapy on the basis of their relative potency in each of these categories as well as of the needs of the patient.

The action of benzodiazepines is based on binding to specific benzodiazepine receptors located mainly in the limbic system mediated by γ-aminobutyric acid (GABA) and a cyclic nucleotide [57,58]. The occurrence of endogenous benzodiazepines has been discussed in man and plants in low concentrations (1-32 ng/ml) [59,60], which may accumulate in subjects with hepatic impairment (e.g. hepatic encephalopathy) [61].

3. Development of Tolerance and Dependence, Potential for Abuse

Benzodiazepines are frequently abused. Tolerance may develop with repeated use leading to a progression to higher doses. In these situations doses used may be many times those recommended for therapeutic use. Consequently, blood concentrations can exceed those normally seen in therapeutic drug monitoring literature. Although metabolism is not known to be induced by long-term use of benzodiazepines, physical dependance on benzodiazepines

can lead to withdrawal symptoms including hyperactivity, anxiety, delirium, hallucinations, seizures and muscular twitches. The longer half-life benzodiazepines often cause more pronounced withdrawal symptoms on discontinuation of use, which may develop several days after cessation of drug use. Benzodiazepines are often used in conjunction with other drugs such as narcotics or alcohol. This will inevitably affect the severity of toxicity.

Concomitant use of benzodiazepines and alcohol as well as use of elevated doses of benzodiazepines can produce marked behavioural changes, including aggression, dissociation and disinhibition. Hangover effects of benzodiazepines, similar to those experienced after alcohol use are common. Anterograde amnesia is also common after use of high doses and following intravenous administration [11,12].

D. Disposition

1. Routes of Metabolism

Benzodiazepines are metabolised through a variety of hydroxylation (aliphatic and aromatic), desalkylation, reduction and acetylation reactions (phase I) followed in many cases by conjugation to glucuronic acid (phase II) prior to excretion. In most cases the phase I metabolites have some biological activity which may be greater or less than that of the parent, whereas the conjugates possess no significant activity. Several benzodiazepines may be considered pro-drugs. Detailed descriptions of the metabolism of the benzodiazepines can be found in references 62-65. The major metabolites of the benzodiazepines under international control in blood and urine are listed in Table III.2. The general routes of metabolism are shown in Figures III.1-6, based on the review by Huang and Moody [66] and are modified in order to include other benzodiazepines of interest in this manual.

Figure III.1 illustrates the common metabolic pathways for 1,4-benzodiazepines. Nordazepam and oxazepam are common metabolites for these drugs. The half-life of nordazepam is lengthy (40-99 hours). Therefore, all compounds which are metabolised to nordazepam are generally considered long-acting. Prazepam and halazepam are so rapidly metabolised to nordazepam and the respective 3-hydroxy metabolites that the parent compounds are not detectable in blood or urine. Similarly, medazepam is rapidly metabolised to normedazepam. Clorazepate is converted to nordazepam already in the stomach.

Figure III.2 illustrates the common metabolic pathways for the triazolo- and imidazo-benzodiazepines involving primarily hydroxylation at positions 1 and 4 as well as ring cleavage in the case of alprazolam to form the corresponding methylaminobenzophenone.

Figure III.3 illustrates common metabolic pathways for 7-nitrobenzodiazepines, i.e. flunitrazepam, nitrazepam, nimetazepam and clonazepam. Metabolism involves reduction of the nitro-group and subsequent acetylation. For example, flunitrazepam is reduced to 7-amino-flunitrazepam and then acetylated to form 7-acetamidoflunitrazepam. In addition, flunitrazepam also undergoes *N*-demethylation. The metabolism of nimetazepam is similar to that of flunitrazepam. In contrast to flunitrazepam, nitrazepam additionally undergoes ring cleavage to the corresponding aminobenzophenone.

Figures III.4-6 illustrate metabolic pathways for the other benzodiazepines. Flurazepam and ethyl loflazepate are so rapidly transformed to desalkylflurazepam that the parent drugs are unlikely to be seen in blood or urine.

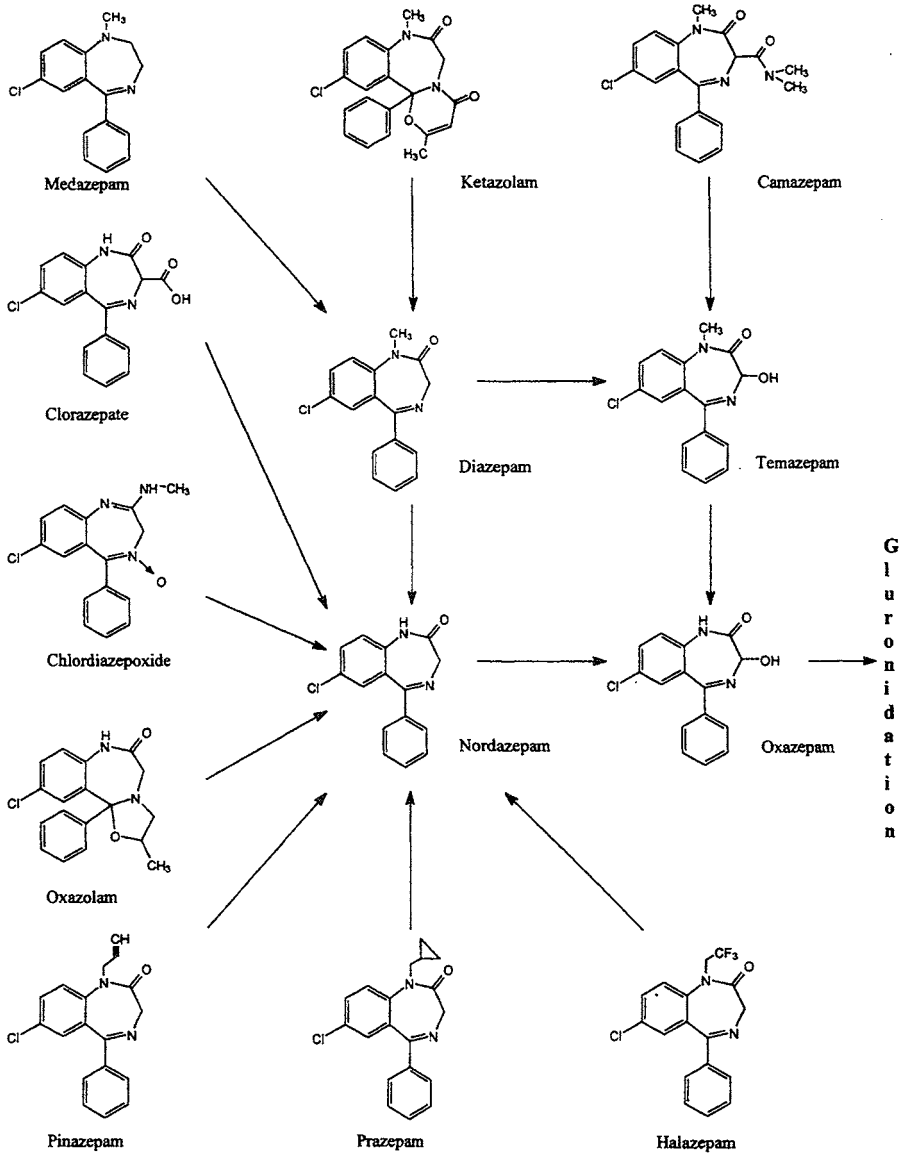
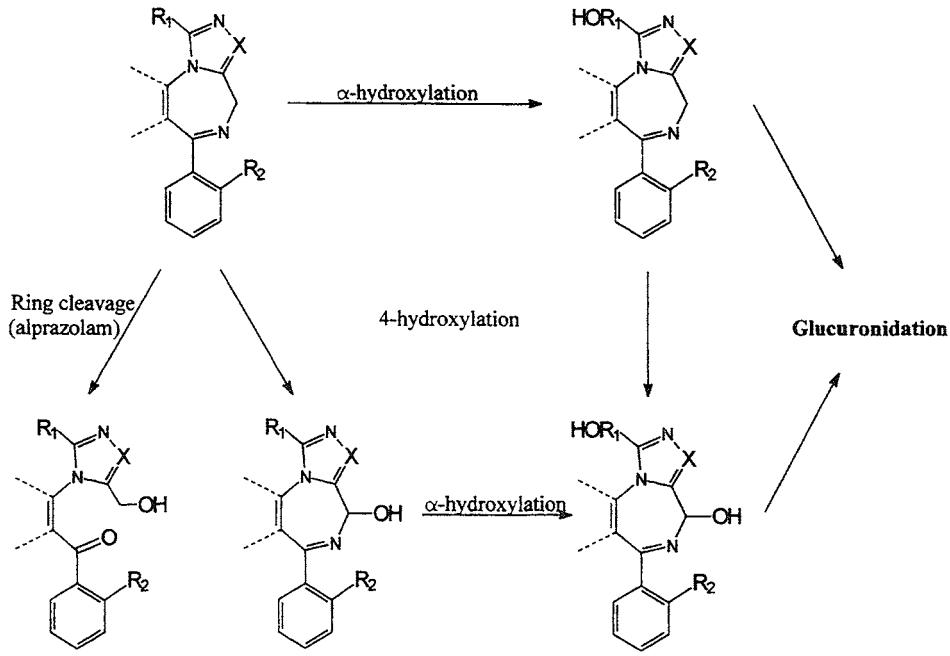
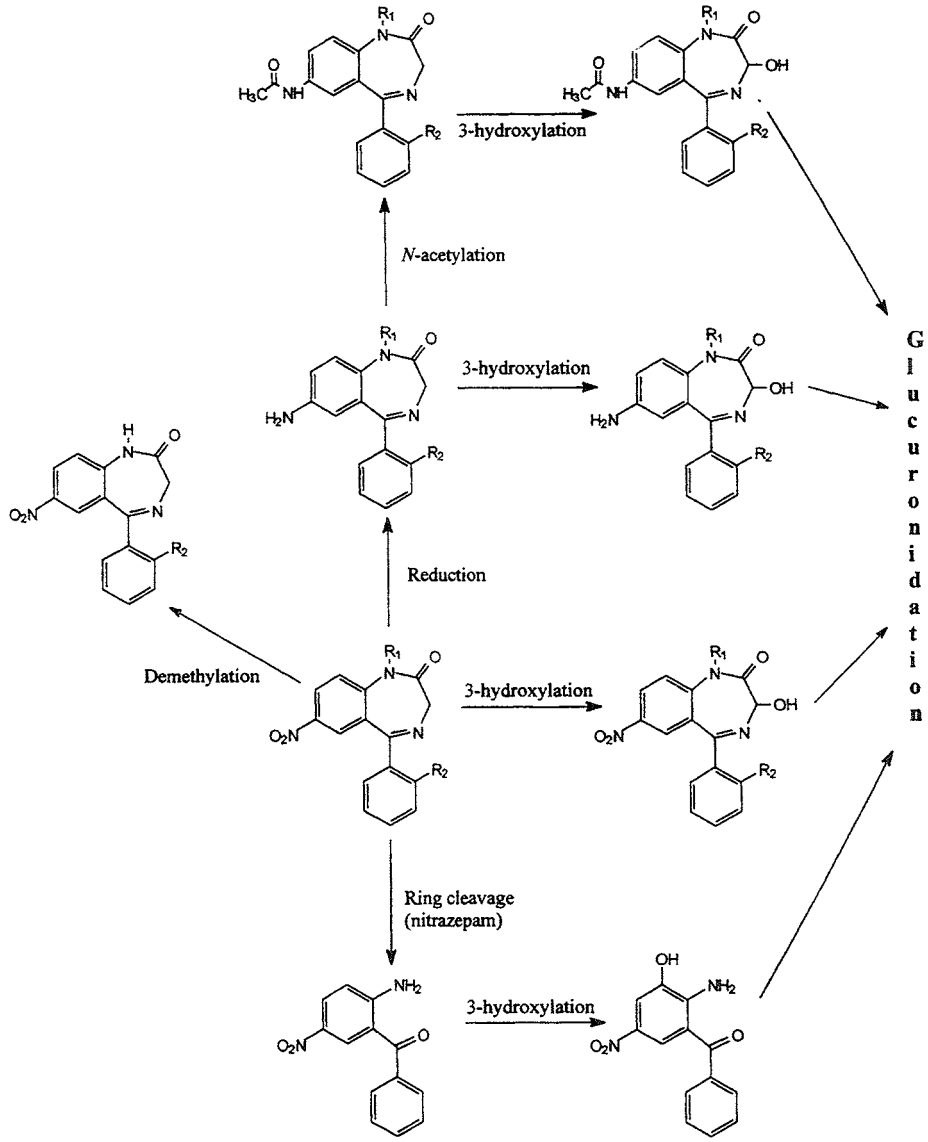


Figure III. General routes of metabolism for 1,4-benzodiazepines metabolised through nordazepam and oxazepam



Alprazolam	$R_1=CH_3$	$R_2=H$	X=N
Brotizolam	$R_1=CH_3$	$R_2=Cl$	X=N
Estazolam	$R_1=H$	$R_2=H$	X=N
Midazolam	$R_1=CH_3$	$R_2=F$	X=CH
Triazolam	$R_1=CH_3$	$R_2=Cl$	X=N

Figure III.2 General routes of metabolism for triazolo- and imidazo-benzodiazepines



Clonazepam R_1= H, R_2= Cl
Flunitrazepam R_1= CH_3, R_2= F
Nimetazepam R_1= CH_3, R_2= H
Nitrazepam R_1=H, R_2= H

Figure III.3 General routes of metabolism for nitro-benzodiazepines

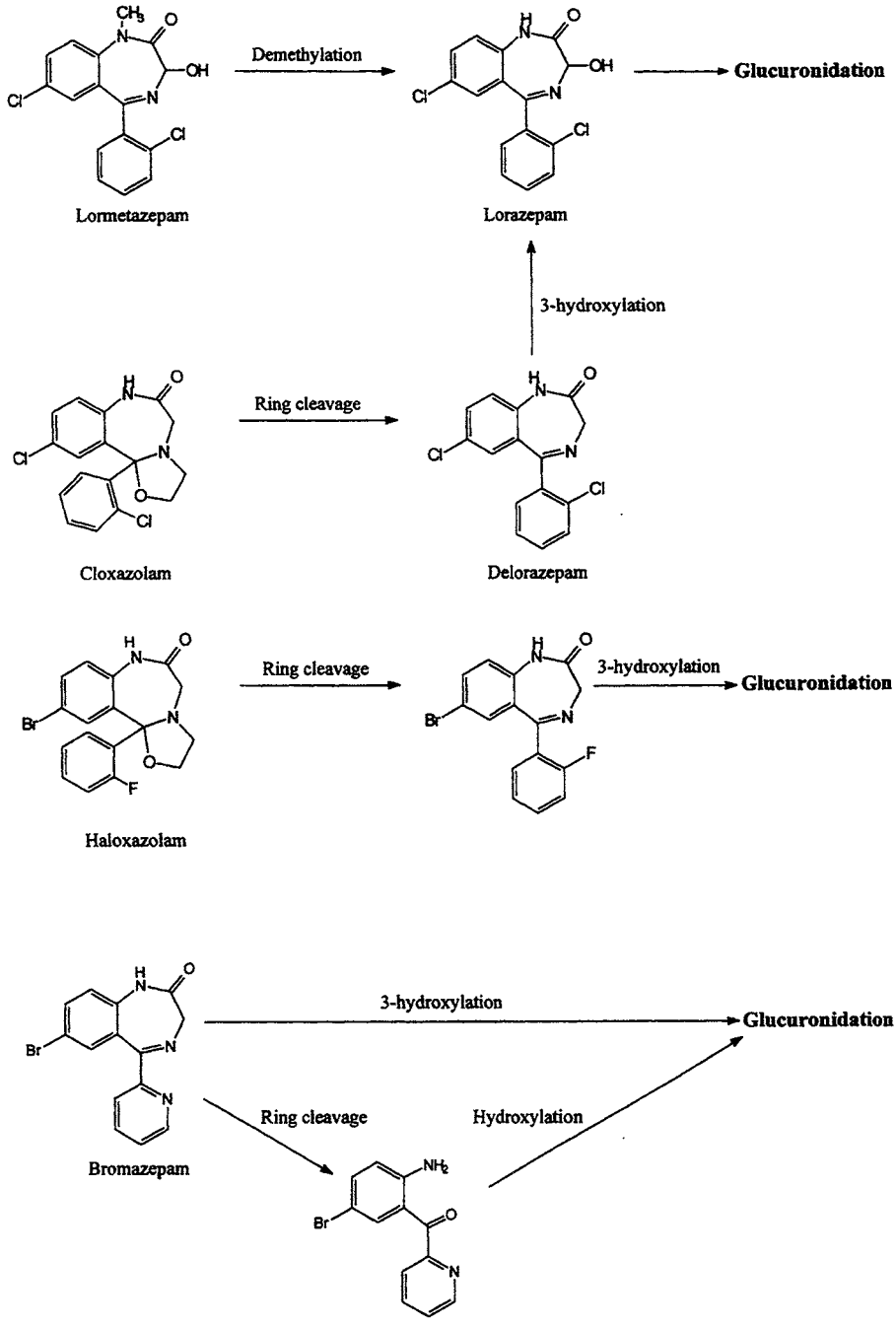


Figure III.4 General routes of metabolism for other benzodiazepines, part I

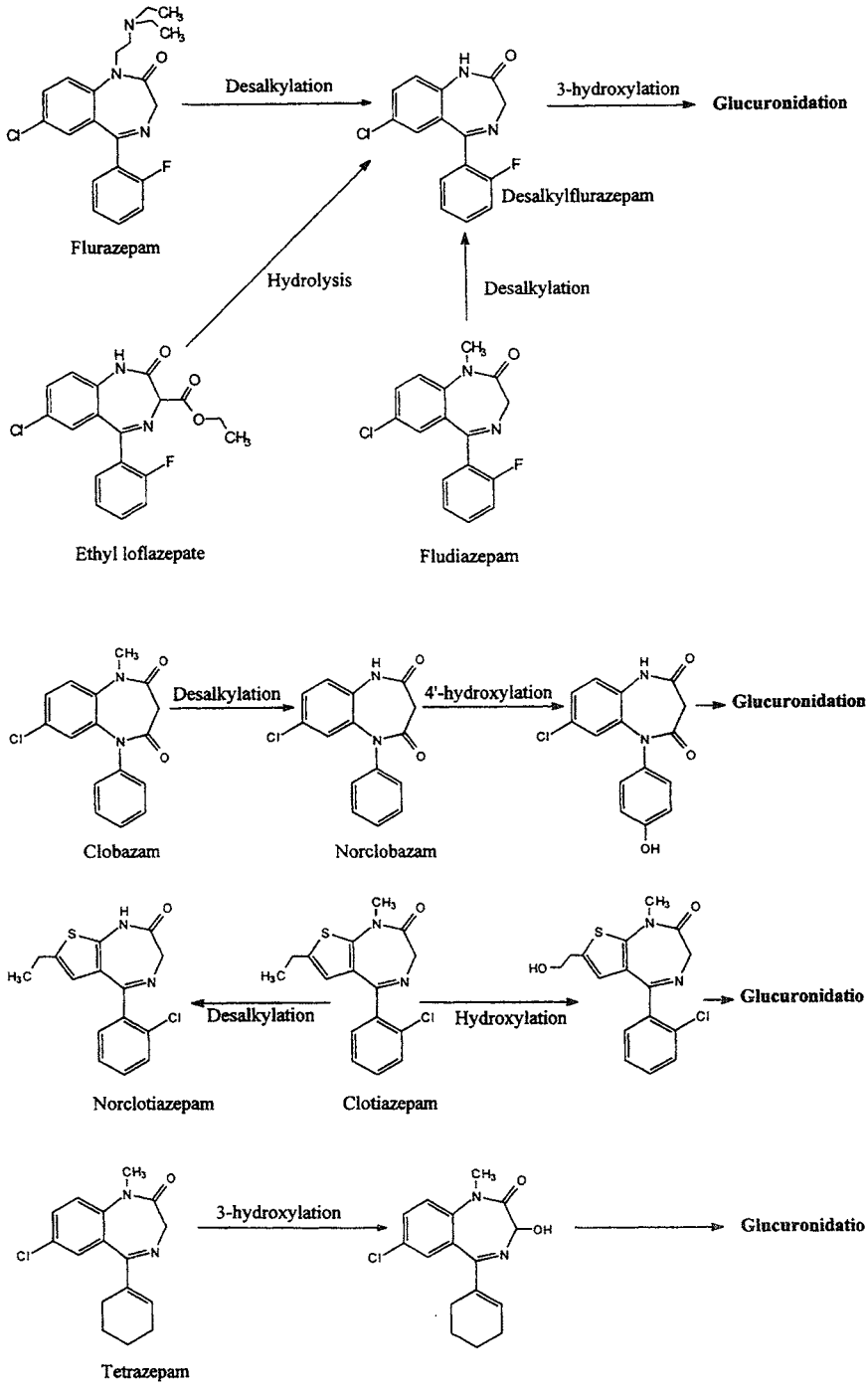


Figure III.5 General routes of metabolism for other benzodiazepines, part II

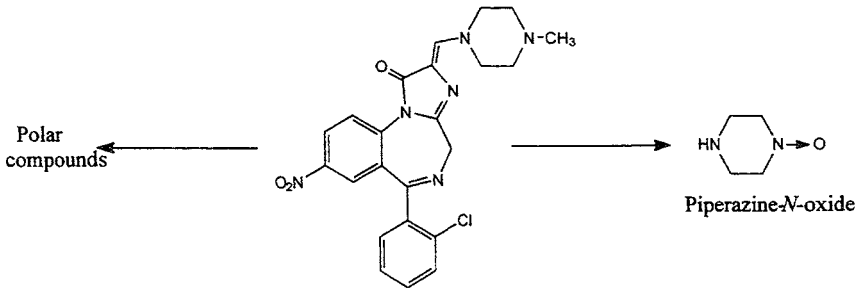


Figure III.6 General routes of metabolism for loprazolam

2. Urinary Excretion and Half-Life

The benzodiazepines can be classified as short-acting (half-life less than 10 hours), intermediate-acting (10-24 hours) and long-acting (greater than 24 hours). They are listed as such in Table III.2 below. The duration of action of a certain benzodiazepine depends not only on the half-life of the drug itself, but also on the possible formation of active metabolites. Medazepam, for example, has a half-life of one to two hours. But as significant amounts of an active metabolite of similar potency, nordazepam, are produced, medazepam is considered a long-acting benzodiazepine.

Differences in the classification of the duration of action of benzodiazepines can be observed. Some texts refer to ultrashort-acting rather than short-acting and short-acting rather than intermediate-acting.

A summary of the main metabolites and some relevant pharmacokinetic data is given in Table III.2. Data derives largely from references cited in the Table as well as references 21,23,67.

Table III.2 Metabolism, half-lives and excretion data for benzodiazepines

Benzodiazepine	**Parent compounds and metabolites**	**Half-lives (h)**	**% excreted**
Short-acting benzodiazepines (half-life < 10h)			
Alprazolam [68]	- alprazolam - α-hydroxyalprazolam - 5-chlorobenzophenone - 4-hydroxyalprazolam - 2-(3-hydroxymethyl-5-methyl-triazolyl)-5-chlorobenzo-phenone	9-30 1-2	12-20 15-17 17 trace trace

Benzodiazepine	Parent compounds and metabolites	Half-lives (h)	% excreted
Brotizolam	- brotizolam - 4-hydroxybrotizolam - α-hydroxybrotizolam - α,4-dihydroxybrotizolam	4-10	< 1
Clotiazepam	- clotiazepam - 7-(hydroxyalkyl) metabolite - norclotiazepam - 4-hydroxynorclotiazepam	3-18	
Loprazolam [68]	- loprazolam - piperazine-*N*-oxide	4-7	
Lorazepam [68]	- lorazepam - quinolone metabolite	8-25	75
Lormetazepam	- lormetazepam - lorazepam	10 8-25	80 6
Midazolam [68]	- midazolam - α-hydroxymidazolam - 4-hydroxymidazolam - α,4-dihydroxymidazolam	1-5 1 1	< 1 60-80
Oxazepam [69,70]	- oxazepam	5-15 (8)	70-80
Temazepam	- temazepam - oxazepam	3-38 (10) 5-15	80
Triazolam [68]	- triazolam - α-hydroxytriazolam - 4-hydroxytriazolam - α,4-dihydroxytriazolam	1-4 4 4	< 1 60-80 11
Intermediate-acting benzodiazepines (half-life 10-24h)			
Bromazepam	- bromazepam - 3-hydroxybromazepam - (2-amino-5-bromobenzoyl)-pyridine	9-19 (12)	2 28 39
Clonazepam	- clonazepam - 7-aminoclonazepam - 7-acetamidoclonazepam	10-50	< 1 major major
Delorazepam	- delorazepam - lorazepam	8-22	75
Estazolam	- estazolam - 4-hydroxyestazolam - 1-oxoestazolam	12-18	

Benzodiazepine	Parent compounds and metabolites	Half-lives (h)	% excreted
Flunitrazepam	- flunitrazepam - 7-aminoflunitrazepam - 7-acetamidoflunitrazepam - desmethylflunitrazepam - 3-hydroxyflunitrazepam	11-25	< 0.2 10 26 3.5
Tetrazepam [71]	- tetrazepam - 3-hydroxytetrazepam - α-hydroxytetrazepam - α,3-dihydroxytetrazepam	13-44 (22)	
Long-acting benzodiazepines (half-life > 24h)			
Chlordiazepoxide	- chlordiazepoxide - desmethylchlordiazepoxide - demoxepam (nordazepam-*N*-oxide) - nordazepam - oxazepam	5-30 (15) 50-99 5-15	 6 major
Clobazam	- clobazam - norclobazam - 4'-hydroxyclobazam - 4'-hydroxynorclobazam	10-50 (25) 40	
Clorazepate	- clorazepate - nordazepam - oxazepam	2 50-99 5-15	2-6 1 major
Cloxazolam	- delorazepam - lorazepam	72	
Diazepam [72]	- diazepam - nordazepam - oxazepam	20-50 50-99 5-15	trace trace 33
Ethyl loflazepate	- desalkylflurazepam - *N*-(1-hydroxyethyl)flurazepam	50-100	
Flurazepam	- flurazepam - desalkylflurazepam - *N*-(1-hydroxyethyl)flurazepam	2-3 50-100	trace trace 29-85
Halazepam	- halazepam - nordazepam - 3-hydroxyhalazepam	14 50-99	< 1
Ketazolam	- ketazolam - desmethylketazolam - diazepam - nordazepam - oxazepam	1.5 20-50 50-99 5-15	 56

Benzodiazepine	Parent compounds and metabolites	Half-lives (h)	% excreted
Medazepam	- medazepam - diazepam - nordazepam - oxazepam - temazepam - normedazepam	1-2 22-50 50-99 5-15 3-38	 detected 2-3 detected
Nitrazepam	- nitrazepam - 7-aminonitrazepam - 7-acetamidonitrazepam - 2-amino-5-nitrobenzophenone	18-38	 5 5-10 5
Nordazepam	- nordazepam - oxazepam	50-99 5-15	
Pinazepam	- nordazepam* - oxazepam*	50-99 5-15	
Prazepam	- prazepam - nordazepam - 3-hydroxyprazepam - oxazepam	3 50-99 5-15	
Oxazolam	- oxazolam - nordazepam	 50-99	
No information at present			
Camazepam	- temazepam - oxazepam - nordazepam - aminocarboxytemazepam*		
Fludiazepam	- fludiazepam - desalkylflurazepam		
Haloxazolam	- 7-bromo analogue of desalkylflurazepam		
Nimetazepam	- nimetazepam - 7-aminonimetazepam - nitrazepam		

* probable metabolite

3. Target Analytes

Based on the above considerations, a list of recommended target analytes in urine and blood is presented in Table III.3. This list is intended to reflect those parent drugs and

metabolites present in greatest quantities in blood and urine for each of the benzodiazepines under international control. The chemical properties of these compounds will dictate to a large degree the type of analytical procedures needed for their analysis as described in Chapter III.F on methods of analysis.

Table III.3 Target analytes in blood and urine

Benzodiazepine	Blood	Urine
Alprazolam	- parent	- parent - α-hydroxyalprazolam
Bromazepam	- parent	- 3-hydroxybromazepam
Brotizolam	- parent	- α-hydroxybrotizolam
Camazepam	- temazepam	- oxazepam - nordazepam
Chlordiazepoxide	- demoxepam - nordazepam	- oxazepam - nordazepam
Clobazam	- norclobazam	- 4'-hydroxynorclobazam
Clonazepam	- parent - 7-aminoclonazepam	- 7-aminoclonazepam
Clorazepate	- nordazepam	- oxazepam - nordazepam
Clotiazepam	- parent - norclotiazepam	- norclotiazepam - 7-(hydroxyalkyl) metabolite
Cloxazolam	- delorazepam - lorazepam	- delorazepam - lorazepam
Delorazepam	- parent - lorazepam	- lorazepam
Diazepam	- diazepam - nordazepam	- oxazepam - nordazepam
Estazolam	- parent	- 4-hydroxyestazolam
Ethyl loflazepate	- desalkylflurazepam	- *N*-(1-hydroxyethyl)flurazepam
Flurazepam	- desalkylflurazepam	- *N*-(1-hydroxyethyl)flurazepam
Fludiazepam	- parent - desalkylflurazepam	- desalkylflurazepam
Flunitrazepam	- parent - 7-aminoflunitrazepam	- 7-aminoflunitrazepam
Halazepam	- nordazepam	- oxazepam - nordazepam

Benzodiazepine	Blood	Urine
Haloxazolam	- 7-bromo analogue of desalkylflurazepam	unknown
Ketazolam	- diazepam - nordazepam	- oxazepam - nordazepam
Lorazepam	- parent	- parent
Lormetazepam	- parent - lorazepam	- lorazepam
Medazepam	- normedazepam - nordazepam	- oxazepam - nordazepam
Midazolam	- parent	- α-hydroxymidazolam
Nimetazepam	- parent - 7-aminonimetazepam	- 7-aminonimetazepam
Nitrazepam	- parent - 7-aminonitrazepam	- 7-aminonitrazepam
Nordazepam	- parent	- oxazepam - parent
Oxazepam	- parent	- parent
Oxazolam	- nordazepam	- oxazepam - nordazepam
Pinazepam	- nordazepam	- oxazepam - nordazepam
Prazepam	- nordazepam	- oxazepam - nordazepam
Temazepam	- parent	- oxazepam
Tetrazepam	- parent	- 3-hydroxytetrazepam
Triazolam	- parent	- α-hydroxytriazolam

Note
In urine the hydroxy metabolites are present almost entirely as glucuronides.

E. Toxicology

Overdosage with benzodiazepines generally results in drowsiness, ataxia, muscular weakness and deep coma. For the treatment of benzodiazepine overdose administration of the specific benzodiazepine antagonist flumazenil has been used [73]. Intravenous dosages of up to 1 mg of flumazenil will reverse the coma and CNS depression associated with benzodiazepine toxicity. Flumazenil can also be taken orally.

Lethal intoxications resulting from the ingestion of benzodiazepines alone are infrequent, but possible [74,75]. This is particularly true when benzodiazepines with extremely long elimination half-lives are considered.

1. Blood Concentration

The concentrations presented in Table III.4 represent serum drug levels commonly achieved during benzodiazepine therapy [21,23,67,76,77]. Levels may exceed these during chronic therapy, in the elderly, in those with reduced liver function and in patients who increase their dose following the development of tolerance. The Table also includes concentrations above which toxic symptoms have been reported. Note that daily doses may vary based on the indication for which the drug is prescribed and on the patients history. It should be emphasized that significant overlap between therapeutic and potentially toxic ranges can exist and that toxic symptoms may develop at lower concentrations in susceptible individuals. Values in this Table should be used as a guide only and interpretation made with caution, based on all available clinical, pathological and toxicological information.

The data in the Table derive from Uges [76] and other references [12,23,67,81].

Table III.4 Therapeutic and toxic plasma concentrations for benzodiazepines and their metabolites

Benzodiazepine	**Maximum therapeutic level (mg/l)**	**Minimum level for toxicity (mg/l)**
Alprazolam	0.07	0.10
Bromazepam	0.17	0.25
Brotizolam	0.02	
Camazepam	0.60	2.00
Chlordiazepoxide as Demoxepam	2.00 4.00	3.50
Clobazam as Norclobazam	0.40 4.00	
Clonazepam as 7-aminoclonazepam	0.06 0.10	0.10 0.10
Clorazepate as Nordazepam	2.0	2.0
Diazepam as Nordazepam	2.0 2.0	2.0 2.0
Estazolam	0.10	
Ethyl loflazepate as Desalkylflurazepam	0.15	0.2

Benzodiazepine	Maximum therapeutic level (mg/l)	Minimum level for toxicity (mg/l)
Flunitrazepam as 7-aminoflunitrazepam	0.02 0.02	0.05 0.2
Flurazepam as Desalkylflurazepam	0.01 0.15	0.15 0.2
Ketazolam as Nordazepam	0.02 2.0	2.0
Loprazolam	0.01	
Lorazepam	0.25	0.30
Lormetazepam	0.02	
Medazepam as Nordazepam	0.10 2.0	0.60 2.0
Midazolam	0.25	1.0
Nitrazepam as 7-aminonitrazepam	0.15 0.2	0.2 0.4
Nordazepam	2.0	2.0
Oxazepam	2.0	2.0
Oxazolam as Nordazepam	2.0	2.0
Pinazepam as Nordazepam	2.0	2.0
Prazepam as Nordazepam	2.0	2.0
Temazepam	2.0	2.0
Tetrazepam	1.0	
Triazolam	0.02	0.01

The interaction of benzodiazepines with other centrally acting compounds, e.g. alcohol, opiates, tricyclic antidepressants, phenothiazines or barbiturates, may lead to fatal intoxications at lower concentrations. Information about typical dosage regimens can be found in national pharmacopoeias (see also reference 67).

Benzodiazepines have the potential to affect cognitive and psychomotor performance, and thus impact divided attention tasks such as driving [78]. These impairing effects can be potentiated by alcohol.

2. Detection Time Limits in Urine

The time interval over which benzodiazepines can be detected in urine is highly variable. Several factors contribute to this variability including the dose of the drug, differences in the metabolism and excretion of various benzodiazepines, chronic versus acute administration, co-ingestion of other drugs which may impair or enhance metabolism of the drug and finally the analytical methodology used. In general, benzodiazepines can be detected in the urine for approximately 24-48 hours after use for short-acting benzodiazepines to up to seven days or longer for intermediate- and long-acting drugs [12,23,67].

3.Interpretation of Results

(a) Urine

Urine concentrations of benzodiazepines and their metabolites cannot be related to a particular drug dose or time since last dose since urine concentration depends on the volume of liquid excreted, creatinine clearance (kidney function) and time since last dose. Individuals also vary in their ability to metabolise drugs.

Oxazepam is a common metabolite for many benzodiazepines (see Table III.2 and Figures III.1-6). Therefore, unless other, specific metabolites are formed it will not be possible to determine which benzodiazepine was ingested. Measurement of blood for the presence of the parent drug may be useful in this context. The triazolo- and the nitro-benzodiazepines, for example, do form specific metabolites (see Table III.2 and Figures III.2-3).

It is not possible to infer a likely degree of intoxication from urine concentrations.

(b) Blood, Serum and Plasma

Blood (or serum or plasma) can be used to obtain an estimate of the degree of drug use. Table III.4 illustrates the usual concentrations associated with therapeutic use and those possibly associated with the development of toxic symptoms.

There is, however, overlap between the ranges depending on the treatment modality, tolerance, the presence of any natural disease or the concomitant use of other drugs which depress the CNS (e.g. alcohol, narcotics).

(c) Interferences

1. Immunoassay screening tests may not necessarily lead to the detection of benzodiazepines because of low cross-reactivities or because the target compound is only present at a level below the cut-off or detection limit. Similarly, positive immunoassay tests may not be found positive by the confirmatory method if the target compound is present at a level below the detection limit of the confirmatory method. This can be avoided if the limit of detection for the confirmatory method is lower than the one for the screening method.
2. False positive results with benzodiazepine immunoassays have also been reported with the non-steroidal anti-inflammatory drug oxaprozin [79] and with the serotonin re-uptake inhibitor sertraline [80].

F. Methods of Analysis

1. Introduction

The following chapters concern different aspects of methods for the analysis of benzodiazepines in biological specimens. The specimens covered are urine, the recommended specimen for detecting drug use and abuse [3], and blood, serum and plasma, which are used for therapeutic drug monitoring and detection of drug use in the clinical setting, the assessment of drug-induced impairment in, for example, the traffic safety context, and for assessment of the cause of death in drug-related fatalities in forensic medicine.

The procedures described in this manual are intended to guide the laboratory in the appropriate selection of suitable assay procedures. To improve the readability of the text, plasma and serum are treated as synonymous although they are hematologically distinct.

As mentioned before, benzodiazepines are metabolised through a variety of hydroxylation, desalkylation and reduction reactions followed in many cases by glucuronidation prior to excretion. As a result, the choice of analytical method is often not straightforward. When a complete method for the analysis of one or more benzodiazepines is required for a particular biological specimen such as urine or blood, appropriate selections should be made, as required, from the information provided in subsequent chapters. Alternatives have been provided to assist the analyst, who may not have access to all of the materials and equipment necessary for any complete method published in the literature. Each of these individual components of a method are recommended as being suitable for their specific purpose and can be depended upon to work reliably.

However, as stated in Chapter I., the ultimate choice of methodology depends inter alia on the type of specimen to be analyzed, the context and purpose of the analysis (clinical or forensic), the facilities available in the laboratory and the experience of the laboratory staff in this field of analytical chemistry. The analyst has the final decision on the choice of which is the most appropriate method, for only the analyst is in possession of all the relevant facts concerning the specimen which is to be analyzed.

Since benzodiazepines are present often at very low concentrations in blood, blood is not the optimum sample for benzodiazepine screening. Urine is a preferable sample for this purpose and numerous immunological and chromatographic methods have been described. Note that - as mentioned in Chapter I.F. - screening methods, although they can be highly selective and sensitive, should always be confirmed by an independent chemical method to ensure veracity.

Above all, it is imperative that any method, constructed from components listed below, is evaluated by the analyst testing it with standards made up in the same sample matrix as the specimens to be analyzed and establishing if the method is suitable for the intended purpose.

A method may contain some or all of the components listed below. The decision on whether or not they should be included in a method depends on the type of specimen (urine or blood), the type of analysis (qualitative or quantitative), the purpose of the analysis (screening or confirmatory) and the type of equipment which will be used in the analysis (immunoassay, TLC, GC, GC-MS or HPLC). The latter (analytical equipment) determines the sensitivity and specificity of the analysis and, as a corollary, the amount of analyte which must be available at the end step. This consequently determines the volume of specimen required for the analysis.

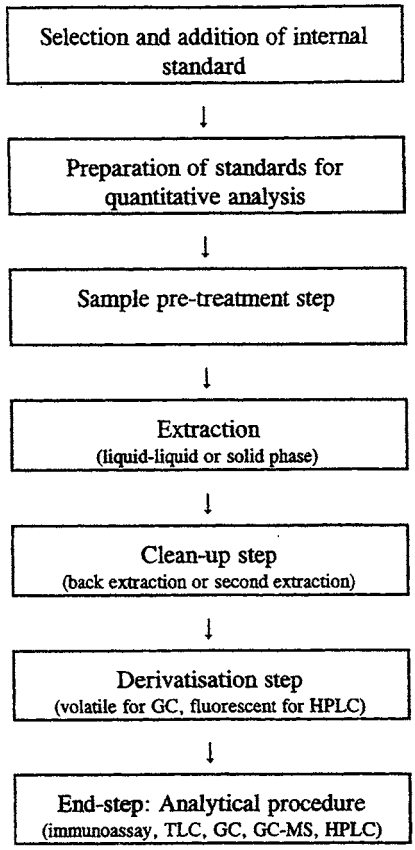


Figure III.7 Flow diagramme for the analysis of benzodiazepines in biological specimens

2. Choice of Internal Standard

The extraction procedure normally includes the addition of an internal standard when using a chromatographic procedure. Suitable internal standards must take into account the method of extraction and the end-step procedure for detection, confirmation and quantitation. Suitable internal standards in all cases are benzodiazepine derivatives which are unlikely to be found in specimens because they are not used as drugs or are no longer prescribed in that country.

Solid-Phase Extraction (SPE) methods must use an internal standard with similar chemical properties. This may not be true for solvent extraction unless a back extraction step is included. For GC-MS, deutero-labelled standards are suitable for quantitation by selected ion monitoring.

3. Enzymatic Hydrolysis

The target analyte in blood is frequently the parent drug or a desalkylated metabolite (see Table III.3). These compounds can be readily extracted as described later in a variety of solvent systems. Most benzodiazepines are further metabolised to hydroxy-benzodiazepines, e.g. oxazepam (see Table III.2 and Figures III.1-6). These hydroxy metabolites are excreted in urine almost entirely as glucuronides. The glucuronides should be hydrolyzed in order to obtain the free metabolites necessary for binding with an antibody in immunoassay and for allowing efficient extraction. This hydrolysis is done enzymatically since extreme acidic or basic conditions can cause cleavage or rearrangement of the benzodiazepine structure itself.

The following method is recommended.

ENZYMATIC HYDROLYSIS METHOD FOR GLUCURONIDES

after Meatherall, 1994 [82]

Materials and Reagents

1. 2M sodium acetate buffer, pH 4.5.
2. *Helix pomatia* β-glucuronidase (100,000 U/ml).

Method

1. Add 50 µl of 2M sodium acetate buffer, pH 4.5 to 1 ml of urine.
2. Add 50 µl of *Helix pomatia* β-glucuronidase (100,000 U/ml).
3. Heat the mixture at 56°C for 2 hours.

Note
Significant hydrolysis takes place in the first 20-30 minutes, but 2 hours is usually optimal although shorter incubation times may be used if necessary [83].

Note that the enzymatic activity of glucuronidase preparations can vary from batch to batch. Therefore, it is important to validate the activity of a new batch of this reagent. Acceptable validation methods would be to either include a hydrolysis control with each batch of samples analyzed using a commercially available benzodiazepine glucuronide. Alternatively, the activity of a new batch of reagent could be assessed using any available glucuronide metabolite for which the laboratory has a method for analysis.

4. Extraction Procedures

Benzodiazepines and their non-conjugated metabolites can be readily extracted into a variety of organic solvents by both liquid-liquid and solid-phase extraction techniques and the extracts applied to suitable sensitive analytical instrumentation. As noted above urine samples should be hydrolyzed prior to extraction.

(a) 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].

Solvents used for the isolation of benzodiazepines include *n*-butyl chloride [84], dichloromethane [85], hexane - dichloromethane (70:30, v/v) [86], *n*-butyl chloride - ethyl acetate (1:4, v/v) [87], ethyl ether [88] and ethyl acetate [89]. Table III.5 summarizes the range of extraction recoveries seen with common benzodiazepines.

Table III.5 Extraction effiencies of benzodiazepines

Solvent	Recovery (%)
n-butyl chloride	50-100
hexane - dichloromethane (70:30)	79-90
n-butyl chloride - ethyl acetate (1:4)	91-107

The following method is recommended.

LIQUID-LIQUID EXTRACTION METHOD

after Drummer et al., 1994/95 [90,91]

Method

1. 1 ml of sample is transferred to a glass extraction tube of 15 ml capacity.
2. The sample is spiked with an appropriate internal standard.
3. 1 ml of 2M Tris buffer, pH 9.0 is added, followed by 8 ml of *n*-butyl chloride.
4. The mixture is rotated gently for 15 min, then gently centrifuged.
5. The *n*-butyl chloride layer (upper layer) is transferred to a clean extraction tube and evaporated to dryness.
6. The residue is reconstituted in a solvent, e.g. methanol, appropriate for the analytical purpose.

Notes

1. The method can be modified for use with lower volumes of sample by reducing the volume of Tris buffer accordingly.
2. The method was validated for nitrazepam, clonazepam and flunitrazepam and their 7-amino-metabolites and for many other benzodiazepines including diazepam, oxazepam, temazepam.

(b) Solid-Phase Extraction

(i) Urine

Extraction methods using sorbent materials such as diatomaceous earth have been reported for a variety of biological fluids [92]. The use of bonded phase silica materials is generally reported for the preparation of extracts of hydrolyzed urine samples [93].

The following method is recommended.

SOLID-PHASE EXTRACTION METHOD FOR URINE
USING MIXED-PHASE COLUMNS

after Moore et al., 1994 [93]

Materials and Reagents

1. Clean Screen DAU columns (United Chemical Technologies).
2. SPE vacuum manifold system.
3. 100 mM phosphate buffer, pH 6.0.

Method

1. Hydrolyze 5 ml of urine with β-glucuronidase (see Chapter III.F.3).
2. Spike the specimen with an appropriate internal standard.
3. Precondition Clean Screen DAU column with 3 ml of methanol followed by 3 ml of water and 1 ml of 100mM phosphate buffer, pH 6.0.
4. Transfer urine specimen to a column and elute at 1-2 ml/min (set vacuum manifold at 10-17 kPa).
5. Wash the column with 2 ml of water followed by 2 ml of 20% acetonitrile in 100mM phosphate buffer, pH 6.0.
6. Dry the column by leaving on full vacuum for approx. 5 min.
7. Wash column with 2 ml of hexane.
8. Elute substances off with 3 ml of ethyl acetate.
9. Evaporate the eluate to dryness and reconstitute residue with 100 μl of ethyl acetate.

Notes

1. Recoveries of benzodiazepines range from 85 to 94%.
2. The method was validated for desalkylflurazepam, nordazepam, oxazepam, diazepam, lorazepam, nitrazepam, temazepam, clonazepam, α-hydroxy-alprazolam and α-hydroxytriazolam.

Other methods recommended are:

Solid-phase extraction methods described by Casas et al. [95] and Lin and Beck [97] using ethyl (C_2) modified silica columns.

(ii) Blood

Solid-phase extraction methods for the analysis of whole blood, serum and plasma include the use of diatomaceous earth [92] as well as of a variety of non-polar bonded phases [94-96].

The following method is recommended.

SOLID-PHASE EXTRACTION METHOD FOR BLOOD USING OCTADECYL SILICA COLUMNS

after Mußhoff et al., 1992 [96]

Materials and Reagents

1. Clean Up C_{18} columns, 100 mg (Worldwide Monitoring Corporation).
2. SPE vacuum manifold system.
3. Borate buffer, pH 9.

Method

1. Spike 2 ml of serum, antemortem or postmortem blood with an appropriate internal standard.
2. Add 2 ml of acetone, mix and centrifuge briefly.
3. Evaporate the supernatant to dryness and dissolve the residue in 2 ml of borate buffer, pH 9.
4. Precondition Clean Up C_{18} column with 2 ml of methanol followed by 2 ml of water and 1 ml of borate buffer, pH 9.
5. Transfer the pretreated specimen to the column and elute at a flow rate of approx. 1 ml/min.
6. Wash the column with 1 ml of water followed by 1 ml of 15% methanol in water.
7. Dry the column by centrifugation, then elute the benzodiazepines with 1 ml of methanol into a collection vial.
8. Evaporate the eluate to dryness and reconstitute the residue in 20 μl of methanol.

Notes

1. Recoveries of benzodiazepines range from 75 to 94%.
2. Borate buffer, pH 9 is prepared by mixing 835 ml of solution A (12.37 g boric acid in 100 ml of 1 M sodium hydroxide with 0.05 M sodium tetraborate to 1 l) and 165 ml of solution B (0.1 M hydrochloric acid).
3. The method was validated for bromazepam, diazepam, oxazepam, nordazepam, flunitrazepam, midazolam, tetrazepam and triazolam.

Other methods recommended are:

1. Solid-phase extraction method using diatomaceous earth (Extrelut, Merck) described by Zweipfennig [92].
2. Solid-phase extraction method using ethyl (C_2) modified silica described by Casas et al. [95].

5. Screening Methods

(a) Immunoassay Methods

A number of different immunoassay methods are available for benzodiazepines, including enzyme immunoassays (EIA) and fluorescent immunoassays (FPIA). These assays have proven their usefulness as screening procedures, but their shortcomings must be taken into account: False negative results can be caused by presence of conjugated metabolites as antibodies in most currently available assays do not react significantly with conjugated metabolites. Note that the degree of cross-reactivity for a given assay varies markedly from drug to drug and even between batches of antibodies from the same manufacturer. It should also be noted that the degree of cross-reactivity for an antibody can be affected by the nature of the sample matrix [98]. Immunoassays should only be used for the matrix recommended by manufacturer or on a matrix for which the assay has been validated [99,100].

Cut-off levels of 200-300 ng/ml using nordazepam or oxazepam as calibration compounds are common. Due, however, to the great variation in dosage, metabolism and cross-reactivity of the antibody unacceptably high rates of false negatives can occur for some important analytes. Therefore, it is important to know the degree of cross-reactivity for a given assay for a particular target analyte. If cross-reactivity is particularly low, the immunoassay may cause a false negative result even in the case of intoxications. For example, there is little cross-reactivity of most commercial assays to flunitrazepam or its metabolites. Specific assays for this compound are under development.

The benzodiazepine antagonist flumazenil is known to have no cross-reactivity with FPIA and EIA [101]. Table III.6 contains cross-reactivity data for a variety of benzodiazepines on some of the most commonly available immunoassay systems. The degree of cross-reactivity is also dependent on the concentration. Note that the TDx method is calibrated for nordazepam while some EIA methods are calibrated for oxazepam.

Table III.5 Cross-reactivities of selected immunoassays

Benzodiazepine	[c]	C. (%)	[c]	C. (%)	[c]	C. (%)	[c]	C. (%)	[c]	C. (%)	[c]	C. (%)
KIT	TDx		EMIT		ONTRAK		ONLINE		CEDIA		DPC	
Alprazolam	232	116	100	300	188	53	112	89	100	206	14	354
Bromazepam	83	41	550	54			135	74	300	58	312	16
Chlordiazepoxide	45	22	1400	21	375	27	175	58	773	24	833	6
Clobazam	273	27	270	111					100	249		
Clonazepam					188	53	167	60	11500	1.7	2500	2
Demoxepam	67	33	1000	30	375	27	128	78	250	73	53	94
Diazepam	246	123	80	375	170	59	118	85	75	332	16	302
Desalkylflurazepam	118	59	180	167	250	40	174	57	150	164		
Estazolam	132	132							75	321		
Flunitrazepam	140	70	230	130	125	80	182	57	201	106	116	43
Flurazepam	150	75	130	230	375	27	164	61	125	204	1667	3
Halazepam			160	187	500	20			196	106	357	14
Ketazolam			100	300								
Lorazepam	100	50	1300	23	250	40	169	59	7000	3.4	1250	4
Lormetazepam			280	107					2125	11		
Medazepam	198	99	140	214	375	27	345	29	153	130	1250	4
Midazolam	197	98	180	167	250	40	130	77			238	21
Nitrazepam	178	89	260	115	150	67	133	75	190	96	52	96
Nordazepam	200	100	100	300			100	100	100	252	250	20
Oxazepam	185	92	300	100	200	50	139	72	200	100		
Pinazepam					225	44	127	79				
Prazepam	237	119	100	300	250	44	139	72	125	199	625	8
Temazepam	198	99	190	157	188	53			166	142	14	352
Tetrazepam			100	300			~300	~33				
α-hydroxytriazolam			140	214	188	53	114	88	1353	12		
Triazolam	165	83	100	273			127	79	1214	16	833	6

[c] = concentration at which cross-reactivity was tested
C.(%) = cross-reactivity; degree of immunoreactivity at tested concentration

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 which 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 which may cross-react with the antibody reaction. For example, oxaprozin immunoreacts with EMIT [81] as well as high concentrations of sertraline or its metabolites may cause a positive result with CEDIA. Therefore, the analyst should always consider the possibility of interfering substances in an analysis. See Chapter I.F.1. of this manual for more information.

(b) Thin Layer Chromatography

Thin-layer chromatography (TLC) is a very useful method for screening for benzodiazepines and their metabolites [64,65,102,103].

Method A describes a sensitive procedure involving hydrolysis of the benzodiazepines to yield the corresponding primary aminobenzophenone derivatives which are then extracted, separated by TLC and detected by diazotization and with Bratton-Marshall reagent. The resulting azo-dyes have a distinctive violet colour. The procedure is designed for urine since the strong acid hydrolysis makes it unsuitable for blood or serum. In some reports, the procedure includes a photolytic desalkylation step following acid hydrolysis. However, there will generally be sufficient desalkyl metabolites to allow omission of this desalkylation step, except in circumstances of massive acute ingestion.

Method A does not work with benzodiazepine metabolites which cannot be hydrolyzed to the primary aminobenzophenones required to react with Bratton-Marshall reagent. Specifically, this applies to the triazolo-benzodiazepines, e.g. alprazolam, estazolam, triazolam, as well as to the nitro-benzodiazepines, e.g. nitrazepam, flunitrazepam, clonazepam.

Method B describes a procedure for the 7-amino metabolites of nitro-benzodiazepines. These are primary amines and directly react with Bratton-Marshall reagent. Therefore, hydrolysis is not required. The developing solvent is different from that used in Method A as toluene is not appropriate for 7-aminonitrazepam and 7-aminoclonazepam. Other TLC procedures for nitro-benzodiazepines described in literature are not recommended in this manual since they are generally less sensitive for the typically low concentrations of metabolites present in biological specimens.

The following methods are recommended.

TLC METHOD A

Plates

Activated silica gel G on glass plates; no fluorescence indicator.

Developing Solvent

Toluene

Preparation of sample solution to be applied to the TLC plate

1. Place 3 ml of urine in a glass tube and add 3 ml of concentrated (10 M) hydrochloric acid.
2. Heat the mixture at 100°C for 30 min.
3. After cooling, add 10 ml of water, apply the mixture to a diatomaceous earth column (16 ml capacity) and wait 3 min.
4. Elute with 2 × 15 ml of ethyl ether (petroleum ether can also be used) and collect in suitable containers.
5. Evaporate the eluate to dryness.
6. Reconstitute the residue in 50 μl of ethanol and apply to the TLC plate.

Visualization

The plates are air dried prior to visualization.

1. Overspray with 1.9 M sulfuric acid, then with freshly prepared 1% aqueous sodium nitrite solution.
2. Overspray with 5% aqueous solution of ammonium amidosulfonate ($H_2NSO_3NH_4$).
3. Overspray with Bratton-Marshall reagent.

   Bratton-Marshall reagent

   Dissolve 1 g of *N*-(1-naphthyl)ethylenediamine in water - acetone (8.7:2).

Notes

1. Sulfuric acid and sodium nitrite (visualization step 1) convert the aminobenzophenones to the respective diazo-compounds necessary to react with Bratton-Marshall reagent.
2. This procedure is very selective for benzodiazepines which form primary aminobenzophenones and is more sensitive than other TLC procedures which analyze the intact drugs.
3. The colours of the spots and their hR_f values are listed in Table III.7.

TLC METHOD B
FOR NITRO-BENZODIAZEPINES

Plates

Activated silica gel G on glass plates; no fluorescence indicator.

Developing Solvent

Ethyl acetate	85
Methanol	10
25% ammonia	5

Preparation of sample solution to be applied to the TLC plate

Extract the samples using *n*-butyl chloride according to the procedures outlined in Chapter III.F.4.a.

Visualization

Perform visualization with Bratton-Marshall reagent as described in Method A.

Note
The hR_f values of the spots are listed in Table III.8.

Table III.7 Benzophenones of benzodiazepines and relevant TLC data using TLC Method A [64,65,102,103]

Benzodiazepine	Benzophenone	Colour	hR_f ($R_f \times 100$)
Alprazolam	no useful benzophenone formed	-	-
Bromazepam	(2-amino-5-bromobenzoyl)pyridine (ABP, ABBP)	red-violet	2
Brotizolam	no useful benzophenone likely to be formed	-	-
Camazepam	2-methylamino-5-chlorobenzophenone (MACB)	negative (yellow)[1]	52
Chlordiazepoxide	2-amino-5-chlorobenzophenone (ACB)	violet	27
Clobazam	1-amino-2-(phenylamino)-4-chlorobenzene[2]	violet	n/a
Clonazepam	2-amino-5-nitro-2'-chlorobenzophenone (ANCB)	red-violet	16
7-aminoclonazepam	2,5-diamino-2'-chlorobenzophenone (DACB)	blue-violet	0
Clorazepate	2-amino-5-chlorobenzophenone (ACB)	violet	27

Benzodiazepine	Benzophenone	Colour	hR_f ($R_f \times 100$)
Cloxazolam	2-amino-5,2'-dichlorobenzophenone (ADB)	violet	33
Delorazepam	2-amino-5,2'-dichlorobenzophenone (ADB)	violet	33
Diazepam	2-methylamino-5-chlorobenzophenone (MACB)	negative (yellow)[1]	52
Estazolam	no useful benzophenone formed	-	-
Ethyl loflazepate	2-amino-5-chloro-2'-fluorobenzophenone (ACFB)	violet	31
Fludiazepam	2-methylamino-5-chloro-2'-fluorobenzophenone (MCFB, CFMB) 2-amino-5-chloro-2'-fluorobenzophenone (ACFB)[3]	negative violet	55 31
Flunitrazepam	2-methylamino-5-nitro-2'-fluorobenzophenone (MNFB)	negative (yellow)[1]	29
Flurazepam	2-amino-5-chloro-2'-fluorobenzophenone (ACFB)[4]	violet	31
Halazepam	2-(2,2,2-trifluoroethyl)amino-5-chlorobenzophenone (TCB)	negative (yellow)[1]	74
Haloxazolam	2-amino-5-bromo-2'-fluorobenzophenone (ABFB)	violet	32
Ketazolam	2-methylamino-5-chlorobenzophenone (MACB)	negative (yellow)[1]	52
Loprazolam	no useful benzophenone likely to be formed	-	-
Lorazepam	2-amino-5,2'-dichlorobenzophenone (ADB)	violet	33
Lormetazepam	2-methylamino-2',5-dichlorobenzophenone (MDB)	negative (yellow)[1]	57
Medazepam	2-methylamino-5-chlorobenzophenone	negative (yellow)[1]	52
Midazolam	2-amino-5-chloro-2'-fluorobenzophenone (ACFB)	violet	31
Nimetazepam	2-methylamino-5-nitrobenzophenone (MNB)	negative (yellow)[1]	29
Nitrazepam	2-amino-5-nitrobenzophenone (ANB)	red-violet	15
7-aminonitrazepam	2,5-diaminobenzophenone (DAB)	violet	0
Nordazepam	2-amino-5-chlorobenzophenone (ACB)	violet	27
Oxazepam	2-amino-5-chlorobenzophenone (ACB)	violet	27
Oxazolam	2-amino-5-chlorobenzophenone (ACB)[5]	violet	27
Pinazepam	2-(2-propinylamino)-5-chlorobenzophenone (CPB, PCB)	negative (yellow)[1]	57
Prazepam	2-(cyclopropylmethyl)amino-5-chlorobenzophenone (CCB, CMCB)	negative (yellow)[1]	68
Temazepam	2-methylamino-5-chlorobenzophenone (MACB)	negative (yellow)[1]	52
Tetrazepam	no data available	-	-

Benzodiazepine	Benzophenone	Colour	hR_f ($R_f \times 100$)
Triazolam	no benzophenone formed	-	-

[1] Spot is negative (yellow), but turns purple after photolytic degradation.
[2] Benzophenone of metabolite norclobazam.
[3] Benzophenone of metabolite *N*-desmethylfludiazepam.
[4] Benzophenone of metabolite desalkylflurazepam.
[5] Benzophenone of metabolites nordazepam and oxazepam.

Table III.8 hR_f data for 7-aminobenzodiazepine metabolites (TLC Method B)

Nitro-Benzodiazepine	Metabolite	hR_f ($R_f \times 100$)
Flunitrazepam	7-aminoflunitrazepam	63
Nitrazepam	7-aminonitrazepam	54
Clonazepam	7-aminoclonazepam	56

6. Confirmatory Methods

(a) Gas Chromatography

Gas chromatography is a suitable technique for the analysis of benzodiazepines. Both, packed and capillary column methods are available. The low doses of many of the benzodiazepines makes capillary gas chromatography the preferable technique of the two.

The chemistry of many of the analytes, specifically the polarity if the hydroxy- and amino-substituents, can limit the efficiency of gas chromatography. Therefore, derivatization techniques are used. The formation of silyl, acyl and alkyl derivatives have been described. High concentrations of analytes (injection of 1-2 μg) can be detected without derivatization.

Trimethyl silyl derivatives (*N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) [105]) and methyl *tert*-butyl silyl derivatives (*N*-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) [106]) are used. Silyl derivatives have the advantage that they give stable, high molecular weight ions for mass spectrometry (see Chapter III.F.6.b). However, silylated extracts from biological materials tend to be dirty and are, therefore, not suitable for Nitrogen-Phosphorous Detectors (NPD). Silylation does not enhance the response of Electron Capture Detectors (ECD).

Other approaches are to acylate and alkylate the hydroxy- and amino-substituents with propionyl chloride and propyl iodide, respectively [107]. Acylation/alkylation has the advantage that the derivatives can be subjected to further clean-up procedures prior to analysis.

(i) Packed Column Technique

The selection of stationary phases available for the gas chromatography of benzodiazepines on packed columns is large. Any moderately non-polar packing such as

dimethyl silicone (OV-1) or 50% phenyl 50% methyl silicone (OV-17) is suitable. While certain phases are recommended in this manual, this does not necessarily mean that other phases are not suitable. This comment also applies to column dimensions. The length and internal diameter, while affecting the retention characteristics of substances, can still be varied from those recommended, providing the chromatography conditions are well established by the analyst and the final procedure validated with respect to specificity and reproducibility and other critical performance indicators for the method at hand.

Packed column gas chromatography has limited sensitivity and will, therefore, only be suitable for those analytes present at high concentrations in hydrolyzed urine samples.

The following methods are recommended.

PACKED COLUMN METHODS

after Peel et al., 1980 [108]

Operating Conditions

METHOD A

Column:	0.91 m × 4 mm ID glass column, packed with 10% OV-1 on Chromosorb G-HP, 80-100 mesh
Carrier gas:	Argon/methane (95:5) at 50 ml/min*
Column temperature:	230°C
Injector temperature:	230°C
Detector temperature:	300°C

METHOD B

Column:	1.83 m × 2 mm ID glass column, packed with 3% OV-17 on Chromosorb W-HP, 80-100 mesh
Carrier gas:	Argon/methane (95:5) at 30 ml/min*
Column temperature:	260°C
Injector temperature:	230°C
Detector temperature:	300°C

Detector

ECD (Ni^{63})

Preparation of sample solutions

Urine samples should be hydrolyzed prior to extraction according to the procedure described in Chapter III.F.3..
The recommended extraction procedures described in Chapter III.F.4. should be used.

* Nitrogen is also suitable as carrier gas for ECDs.

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.

(ii) Capillary Column Technique

Similar to packed column gas chromatography, the selection of capillary columns with respect to stationary phase, film thickness, column dimensions and type is large. The chromatography characteristics of each column must be established as being adequate for the analytical procedure. Most dimethyl silicone or 5% phenyl methyl silicone columns can be used for analysis of both derivatized and underivatized extracts.

The following method is recommended.

CAPILLARY COLUMN METHOD

after Drummer et al., 1994 [90]

Operating Conditions

Column:	12 m × 0.53 mm ID chemically bonded fused silica capillary column with 1.0 µm coating of 5% phenyl methyl silicone (BP-5 or equivalent)
Carrier gas:	Helium at 2-3 ml/min
Split ratio:	splitless mode
Column temperature:	start at 100°C for 2 min, then programmed at 7.5°C/min to 310°C, hold at 310°C for 10 min
Injector temperature:	250°C
Detector temperature:	300°C

Detector

NPD or ECD or NPD and ECD in parallel after split of column effluent.

Preparation of sample solutions

Urine samples should be hydrolyzed prior to extraction according to the procedure described in Chapter III.F.3..
The recommended extraction procedures described in Chapter III.F.4. should be used.

Table III.9 Relative retention times for benzodiazepines by capillary column GC

Benzodiazepine	Relative retention time*
Alprazolam	1.225
Bromazepam	1.079
Chlordiazepoxide (as demoxepam)	1.025
Clobazam	1.053
Clonazepam	1.186
7-aminoclonazepam	1.186
Diazepam	1.000
Flunitrazepam	1.086
7-aminoflunitrazepam	1.099
Flurazepam	1.136
Desalkylflurazepam	1.002
Lorazepam	0.986
Midazolam	1.069
Nitrazepam	1.159
7-aminonitrazepam	1.148
Nordazepam	1.034
Oxazepam	0.843
Prazepam	1.088
Temazepam	1.075
Triazolam	1.276

* relative to diazepam

(iii) Detectors

Suitable detectors for the analysis of benzodiazepines by GC include Electron Capture Detector (ECD) and Nitrogen Phosphorous Detector (NPD) (often termed a nitrogen-selective detector). Flame Ionization Detectors (FID) generally do not have sufficient sensitivity to be useful for the analysis of benzodiazepines.

Mass selective detectors such as mass spectrometers or ion traps are often used in combination with capillary GC and is the technique of choice for sensitivity and discriminating power. See Chapter III.F.6.b. on Gas Chromatography - Mass Spectrometry.

(b) Gas Chromatography - Mass Spectrometry

Gas chromatography-mass spectrometry is a highly specific confirmatory method for benzodiazepines.

When identifying benzodiazepines by GC-MS, spectra must be taken in the full scan mode. Identification of an analyte is obtained by comparing its retention time and at least three qualifier ions with those of reference standards. To ensure specificity, quantification should be carried out using reconstructed ion chromatogrammes, i.e. using ion chromatogrammes generated from full scan data, comparing the base peak ion with the representative area of the internal standard ion and the calibration curve. Ions below m/z 50 are unlikely to be of diagnostic value.

For GC-MS, deutero-labelled standards are suitable for quantitation by selected ion monitoring.

The following method is recommended.

CAPILLARY COLUMN GC-MS METHOD

after Mulé et al., 1989 [104]

Operating Conditions

Column:	a) 12.5 m × 0.20 mm ID chemically bonded fused silica capillary column with 0.33 μm coating of dimethyl silicone (e.g. HP-1) b) 25 m × 0.20 mm ID chemically bonded fused silica capillary column with 0.33 μm coating of 5% phenyl methyl silicone (e.g. HP-5)
Detector:	Electron impact mode at 70eV
Carrier gas:	Helium at 0.65 ml/min, linear velocity 34.7cm/s
Split ratio:	splitless mode
Column temperature:	start at 140°C for 1 min, then programmed at 30°C/min to 260°C, hold at 260°C for 5 min, then rise to 320°C at 50°C/min
Injector temperature:	250°C
Interface temperature:	280°C

Preparation of sample solutions

Urine samples should be hydrolyzed prior to extraction according to the procedure described in Chapter III.F.3..
The recommended extraction procedures described in Chapter III.F.4. should be used.

Derivatizing Conditions

The final extract is evaporated to dryness. 25 μl of BSTFA with 1% TMCS are added to the residue of each sample, mixed and heated at 60°C for 15 min. 1 μl is injected.

Table III.10 Mass spectral data (EI^+) for benzodiazepines*

Benzodiazepine	Base peak ion, m/z	Other ions, m/z (abundance)
Alprazolam	308	279, 204
α-hydroxyalprazolam-TMS	381	382(30), 396(40), 383 (39)
Bromazepam-TMS	388	374(16), 386(95), 387(96)
Chlordiazepoxide	282	283, 284
Clonazepam-TMS	387	352(87), 386(45)
7-aminoclonazepam-TMS	429	394(99), 414(30)
Diazepam	256	283(92), 284(67), 221
Flunitrazepam	312	286(85), 285(71)
7-aminoflunitrazepam-TMS	355	327(87), 326(36)
Flurazepam	86	99(9), 387(3)
Desalkylflurazepam-TMS	359	360(91), 341(62), 245
Halazepam	324	352, 289
Lorazepam-diTMS	429	430(43), 431(33)
Midazolam	310	297, 312
α-hydroxymidazolam-TMS	310	312(34), 399(10), 413(37)
Nitrazepam-TMS	352	353(65), 306(26)
7-aminonitrazepam-diTMS	394	395(87), 396(30)
Nordazepam-TMS	341	342(56), 343(45), 327
Oxazepam-diTMS	429	415(18), 401(20), 430
Pinazepam	308	280(99), 307(86)
Prazepam	269	295(83), 91(54), 241, 324
Temazepam-TMS	343	283, 257
Triazolam	313	238, 312, 314, 342
α-hydroxytriazolam-TMS	415	417(70), 430(50), 432(34)

* data after a variety of sources including references 104, 105.

(c) High Performance Liquid Chromatography

HPLC is a useful technique for the quantitative analysis of benzodiazepines. Its value as a screening tool is controversial as the reproducibility of retention data varies from column

to column and is dependant on a number of factors.

Reversed phase columns are used for the analysis of benzodiazepines in biological specimens. To protect the analytical column the use of a guard column with the same packing is recommended.

The mobile phases used are acidic, e.g. a mixture of phosphate buffer and acetonitrile and/or methanol. It is important to control the pH of the mobile phase as small differences can significantly affect the chromatography.

Gradient as well as isocratic analysis have been reported. For complex mixtures of benzodiazepines or for a general unknown, gradient analysis or a combination of isocratic systems (shich target specific benzodiazepines) are necessary.

Most benzodiazepines are detected by UV at 230 nm, nitrobenzodiazepines at 240 nm. For peak identification diode array detection is required.

(i) Blood

The method described below includes liquid-liquid extraction of the blood samples. It is recommended for the HPLC analysis of benzodiazepines administered at higher doses such as temazepam, oxazepam, diazepam and nordazepam. HPLC is usually not suitable for benzodiazepines given at lower doses such as triazolam, alprazolam, lorazepam and lormetazepam.

HPLC METHOD FOR BLOOD

after Robertson et al., 1995 [91]

Operating Conditions

Column:	Phenyl bonded silica (Waters Nova-Pak Phenyl or equivalent), 4 μm particle size 150 mm × 3.9 mm ID
Mobile phase:	Acetonitrile - 40 mM phosphate buffer, pH 3.75 (28:72, v/v)
Flow rate:	0.8 ml/min
Detector:	230-250 nm depending on the analytes of interest

Preparation of sample solutions

1. 0.5 ml of sample is transferred to a glass extraction tube.
2. 0.5 ml of 0.2 M carbonate buffer, pH 11.5 are added.
3. The mixture is extracted with 6 ml of *n*-butyl chloride.
4. The *n*-butyl chloride layer (upper layer) is transferred to a clean extraction tube and evaporated to dryness.
5. The residue is reconstituted with 200 μl of mobile phase.

(ii) Urine

As mentioned before, the target analytes in urine are often different from those in blood or plasma (see Table III.3). Enzymatic hydrolysis of the urine specimens is required prior to analysis (see Chapter III.F.3.). HPLC conditions for analysis of benzodiazepines in urine are similar to that described for blood and plasma, but optimized for the polar urinary metabolites.

The following methods are recommended.

HPLC METHOD A
FOR URINE

after Ferrara et al., 1992 [40]

Operating Conditions

Column:	Octyl silica (LiChrospher 100 RP8 or equivalent), 5 μm particle size, 250 mm × 4 mm ID, with a precolumn
Mobile phase:	a) gradient technique: 0.01M phosphate buffer, pH 3.5 containing 0.02 M methanesulfonic acid - acetonitrile (70:30, v/v) held for 1 min, then changed to 60:40 over 6 min. The final composition is held for 11 min. b) isocratic technique: 0.01 M phosphate buffer, pH 3.5 containing 0.02 M methanesulfonic acid - acetonitrile (55: 45, v/v)
Flow rate:	1 ml/min
Detector:	234 nm

Preparation of sample solutions

Urine samples should be hydrolyzed prior to extraction according to the procedure described in Chapter III.F.3..
The extraction procedures described in Chapter III.F.4. should be used, solid-phase extraction is particularly recommended.

HPLC METHOD B
FOR URINE

after Chopineau et al., 1994 [109]

Operating Conditions

Column:	Octadecyl silica (LiChrospher 100 RP18 or equivalent), 5 μm particle size, 125 mm × 4 mm ID, with a precolumn
Mobile phase:	0.01 M phosphate buffer, pH 5.6 - acetonitrile (60:40, v/v)
Flow rate:	1.6 ml/min
Detector:	254 nm

Preparation of sample solutions

Urine samples should be hydrolyzed prior to extraction according to the procedure described in Chapter III.F.3..
The extraction procedures described in Chapter III.F.4. should be used, solid-phase extraction is particularly recommended.

Note
The limit of detection is 5 ng/ml from 1 ml of urine.

HPLC METHOD C
FOR URINE

after Chiba et al., 1995 [85]

Operating Conditions

Column:	Octadecyl silica (LiChrospher 100 RP18 or equivalent), 5 μm particle size, 250 mm × 4 mm ID
Mobile phase:	Water - methanol - triethylamine (70:30:0.1, v/v/v), adjusted to pH 5.5 with phosphoric acid
Flow rate:	0.7 ml/min
Detector:	240 nm

Preparation of sample solutions

Urine samples should be hydrolyzed prior to extraction according to the procedure described in Chapter III.F.3..
The extraction procedures described in Chapter III.F.4. should be used, liquid-liquid extraction is particularly recommended.

Notes

1. The limit of detection is 2 ng/ml from 1 ml of urine.
2. The reproducibility was better than 6%.

References

1 Report of the Expert Group on Recommended Methods for Testing Cannabis and Amphetamine/Methamphetamine Analysis, E/CN.7/1987/8.

2 Report of the International Conference on Drug Abuse and Illicit Trafficking (United Nations Publication, Sales No. E.87.1.18), para 84.

3 Report of the Expert Group on Guidelines for the Establishment of National Testing Programmes and Laboratories for Drugs of Abuse in Body Fluids, E/CN.7/1988/CRP.5, para 42.

4 Commission on Narcotic Drugs, Report of the Tenth Special Session, E/1988/13-E/CN.7/1988/14, para 236 (b).

5 Recommended Guidelines for Quality Assurance and Good Laboratory Practices, Manual for Use by National Laboratories, ST/NAR/25, United Nations, 1995.

6 Recommended Methods for Testing Barbiturate Derivates under International Control, Manual for Use by National Narcotics Laboratories, ST/NAR/18, United Nations, 1989.

7 Recommended Methods for Testing Benzodiazepine Derivatives under International Control, Manual for Use by National Narcotics Laboratories, ST/NAR/16, United Nations, 1988.

8 Recommended Methods for the Detection and Assay of Heroin, Cannabinoids, Cocaine, Amphetamine, Methamphetamine and Ring-Substituted Amphetamine Derivatives in Biological Specimens, Manual for Use by National Laboratories, ST/NAR/27, United Nations, 1995.

9 E/INCB/1995/W.16, 59th Session, 30 October - 16 November 1995.

10 Multilingual Dictionary of Narcotic Drugs and Psychotropic Substances under International Control, ST/NAR/1/REV.1 (United Nations Publication, Sales No. E/F/S.93.XI.2).

11 Goodman and Gilman's the Pharmacological Basis of Therapeutics, 9th Edition, McGraw-Hill, New York, 1996.

12 Ellenhorn, M.J., Barceloux, D.G., Medical Toxicology, Elsevier Science Publishing Company, New York, 1988.

13 Fraser, D.B., Tomlinson, J., Satzger, R.D., Analysis of black pearl and other herbal preparations, Proceedings of the AAFS Meeting, Seattle, 1995, 208.

14 Tang, B.K., Ineba, T., Kalow, W., *N*-hydroxylation of pentobarbital in man, Drug Metab. Disp. 5, 71-74 (1977).

15 Baldeo, W.C., Gibert, J.N., Powell, J.W., Multidose studies in the human metabolism of pentobarbitone, Eur. J. Drug Metab. Pharmacokinet. 5, 75-80 (1980).

16 Therapeutic Drugs, Dollery, C. (Ed.), Churchill Livingstone, Edinburgh, 1991.

17 Whyte, M.P., Dekaban, A.S., Metabolic fate of phenobarbital: A quantitative study of *p*-hydroxy-phenobarbital elimination in man, Drug Metab. Dist. 5, 63-70 (1977).

18 Butler, T.C., Mahaffee, C., Waddell, W.J., Phenobarbital: Studies of elimination, accumulation, tolerance, and dosage schedules, J. Pharmacol. Exp. Therap. 111, 425-435 (1954).

19 Harvey, D.J., Glazener, L., Stratton, C. et al., Detection of a 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-metabolite of phenobarbital and mephobarbital in rat, guinea pig and human, Res. Comm. Chem. Pharm. 3, 557-565 (1972).

20 Tang, B.K., Kalow, W., Grey, A.A., Metabolic fate of phenobarbital in man: *N*-glucoside formation, Drug Metab. Disp. 7, 315-318 (1979).

21 Clarke's Isolation and Identification of Drugs, 2nd Edition, Moffat, A.C. (Ed.), The Pharmaceutical Press, London, 1986.

22 Stead, A.H., Moffat, A.C., A collection of therapeutic, toxic and fatal blood drug concentrations in man, Human Toxicol. 3, 437-464 (1983).

23 Disposition of Toxic Drugs and Chemicals in Man, 3rd Edition, Baselt, R.C., Cravey, R.H. (Ed.), Year Book Medical Publishers, New York, 1989.

24 The Bulletins of The International Association of Forensic Toxicologists.

25 Schramm, W., Smith, R.H., Craig, P., Kidwell, D.A., Drugs of abuse in saliva: A review, J. Anal. Toxicol. 16, 1-9 (1992).

26 Chan, E.M., Chan, S.C., Screening for acidic and neutral drugs by high performance liquid chromatography in postmortem blood, J. Anal. Toxicol. 8, 173-176 (1984).

27 Mulé, S.J., Casella, G.A., Confirmation and quantitation of barbiturates in human urine by gas chromatography/mass spectrometry, J. Anal. Toxicol. 13, 13-16 (1989).

28 Mangin, P., Lugnier, A.A., Chaumont, A.J., A polyvalent method using HPLC for screening and quantification of 12 common barbiturates in various biological materials, J. Anal. Toxicol. 11, 27-30 (1987).

29 Chen, X.-H., Franke, J.-P., Wijsbeek, J., de Zeeuw, R.A., Isolation of acidic, neutral, and basic drugs from whole blood using a single mixed-mode solid-phase extraction column, J. Anal. Toxicol. 16, 351-355 (1992).

30 Maurer, H., Identification and differentiation of barbiturates, other sedative-hynotics and their metabolites in urine integrated in a general screening procedure using computerized gas chromatography-mass spectrometry, J. Chromatogr. B 530, 307-326 (1990).

31 Bailey, D.N., Kelner, M., Extraction of acidic drugs from water and plasma: study of recovery with five different solvents, J. Anal. Toxicol. 8, 26-28 (1984).

32 Lillsunde, P., Michelson, L., Forsström, T., Korte, T., Schultz, E., Ariniemi, K., Portman, M., Sihvonen, M.-L., Seppälä, T., Comprehensive drug screening in blood for detecting abused drugs or drugs potentially hazardous for traffic safety, Forensic Sci. Int. 77, 191-210 (1996).

33 Baselt, R.C., Analytical Procedures for Therapeutic Drug Monitoring and Emergency Toxicology, PSG Publishing Co. Inc..

34 Wallace, F.E., Hall, L.R., Harris, S.C., Determination of pentobarbital and certain other barbiturates by capillary gas-liquid chromatography, J. Anal. Toxicol. 7, 178-180 (1983).

35 Budd, R.D., Mathis, D.F., GLC screening and confirmation of barbiturates in postmortem blood specimens, J. Anal. Toxicol. 6, 317-320 (1982).

36 Logan, B.K., Stafford, D.T., Liquid/solid extraction on diatomaceous earth for drug analysis in postmortem blood, J. Forensic Sci. 34, 553-64 (1989).

37 Liu, R.H., McKeehan, A.M., Edwards, C. et al., Improved gas chromatographic/mass spectrometry analysis of barbiturates in urine using centrifuge-based solid-phase extraction, methylation, with d_5-pentobarbital as internal standard, J. Forensic Sci. 39, 1504-1514 (1994).

38 Pocci, R., Dixit, V., Dixit, V.M., Solid phase extraction and GC/MS confirmation of barbiturates from human urine, J. Anal. Toxicol. 16, 45-47 (1992).

39 Anderson, W.H., Fuller, D.C., A simplified procedure for the isolation, characterisation, and identification of weak acid and neutral drugs from whole blood, J. Anal. Toxicol. 11, 198-204 (1987).

40 Ferrara, S.D., Tedeschi, L., Frison, G., Castagna, F., Solid-phase extraction and HPLC-UV confirmation of drugs of abuse in urine, J. Anal. Toxicol. 16, 217-222 (1992).

41 Armbruster, D.A., Schwarzhoff, R.H., Hubster, E.C., Liserio, M.K., Enzyme immunoassay, kinetic microparticle immunoassay, radioimmunoassay, and fluorescence polarization immunoassay compared for drugs-of-abuse screening, Clin. Chem. 39, 2137-2146 (1993).

42 McElwee, D.J., A new method for the detection of sedative drugs on thin-layer chromatograms using chlorine vapors and 2,7-dichlorofluorescein, J.Anal. Toxicol. 3, 266-268 (1979).

43 Thin-Layer Chromatographic R_f Values of Toxicologically Relevant Substances on Standardized Systems, DFG/TIAFT, VCH Verlagsgesellschaft (Weinheim), 1987.

44 A. Curry's Poison Detection in Human Organs, 3rd Edition, (Charles C. Thomas), 1976.

45 The Analysis of Drugs of Abuse, Gough, T. (Ed.), John Wiley & Sons, New York.

46 Jack, D.B., Drug Analysis by Gas Chromatography, Academic Press, Orlando, 1984.

47 Gambaro, V., Mariai, R., Marozzi, E., Flash alkylation for the identification of barbiturates in biological material, J. Anal. Toxicol. 6, 321-323 (1982).

48 Barbour, A., GC/MS analysis of propylated barbiturates, J. Anal. Toxicol. 15, 214-215 (1991).

49 Joern, W.A., Unexpected volatility of barbiturate derivatives: An extractive alkylation procedure for barbiturates and benzoylecgonine, J. Anal. Toxicol. 18, 423 (1994).

50 Gill, R., Stead, A.H., Moffat, A.C., Identification of drugs by the effective combination of gas-liquid, high-performance liquid and thin-layer chromatographic techniques, J. Chromatogr. 204, 275-284 (1981).

51 Coudoré, F., Alazard, J.-M., Paire, M., Andraud, G., Lavarenne, J., Rapid toxicological screening of barbiturates in plasma by wide-bore capillary gas chromatography and nitrogen-phosphorus detection, J. Anal. Toxicol. 17, 109-113 (1993).

52 Jupp, M., Gill, R., Osselton, D., Comparison of drug retention indices determined by packed, wide bore capillary and narrow bore capillary columns, J. Forensic Sci. 32, 1574-1586 (1987).

53 Gill, R., Moffat, A.C., Smith, R.M., Hurdley, T.G., A collaborative study to investigate the retention reproducibility of barbiturates in HPLC with a view to establishing retention databases for drug identification, J. Chromatogr. Sci. 24, 153-159 (1986).

54 Hagan, R.L., Clarification of benzodiazepine structural classes, J. Anal. Toxicol. 19, 58 (1995).

55 Hagan, R.L., Annelated benzodiazepines: A brief overview of their chemistry, disposition and analysis, California Association of Toxicologists Newsletter 1995, 22-33.

56 Klotz, U., Kangas, L., Kanto, J., Clinical Pharmacokinetics of Benzodiazepines, Gustav Fischer Verlag, Stuttgart, New York, 1980.

57 Haefely, W., The biological basis of benzodiazepine actions, J. Psychoactive Drugs 15, 9-39 (1983).

58 Haefely, W., Structure and function of the benzodiazepine receptor, Chimia 41, 389-396 (1987).

95 Casas, M., Berrueta, L.A., Gallo, B., Vicente, F., Solid phase extraction of 1,4-benzodiazepines from biological fluids, J. Pharm. Biomed. Anal. 11, 277-284 (1993).

96 Mußhoff, F., Daldrup, T., A rapid solid phase extraction and HPLC/DAD procedure for simultaneous determination and quantitation of different benzodiazepines in serum, blood and postmortem blood, Int. J. Legal Med. 105, 105-109 (1992).

97 Lin, Z., Beck, O., Procedure for verification of flunitrazepam and nitrazepam intake by gas chromatographic-mass spectrometric analysis of urine, J. Pharm. Biomed. Anal. 13, 719-722 (1995).

98 Spiehler, V., Hand, C., Cross reactivity in the DPC serum and urine benzodiazepine radioimmunoassays, California Association of Toxicologists Newsletter, 1991, 2-3.

99 Lewellen, L., McCurdy, H., A novel procedure for the analysis of drugs in whole blood by homogenous enzyme immunoassay (EMIT), J. Anal. Toxicol. 12, 260-264 (1988).

100 Asselin, W.M., Leslie, J.M., McKinley, B., Direct detection of drugs of abuse in whole hemolyzed blood using EMIT d.a.u. urine assays, J. Anal. Toxicol. 12, 207-215 (1988).

101 Kintz, P., Mangin, P., Plasma determination of flumazenil, a benzodiazepine antagonist, by immunotoxicology and by capillary gas chromatography/mass spectrometry, J. Anal. Toxicol. 15, 202-203, (1991).

102 Schütz, H., TLC data of hydrolysis products of 1,4- and 1,5-benzodiazepines, J. Anal. Toxicol. 2, 147-148 (1978).

103 TLC R_f Values of Toxicologically Relevant Substances on Stanardized Systems, (Report XVII of the DFG Commission for Clinical-Toxicological Analysis, Special Issue of the TIAFT Bulletin), VCH Verlagsgesellschaft (Weinheim), 1992.

104 Mulé, S.J., Casella, G.A., Quantitation and confirmation of the diazolo- and triazolobenzodiazepines in human urine by gas chromatography/mass spectrometry, J. Anal. Toxicol. 13, 179-184 (1989).

105 Black, D.A., Clark, G.D., Haver, V.M., Garbin, J.A., Saxon, A.J., Analysis of benzodiazepines using solid phase extraction and gas chromatography-mass spectrometry, J. Anal. Toxicol. 18, 185-188 (1994).

106 Dickson, P.H., Markus, W. McKernan, J., Nipper, H.C., Urinalysis of *alpha*-hydroxyalprazolam, *alpha*-hydroxytriazolam and other benzodiazepine compounds by GC/EIMS, J. Anal. Toxicol. 16, 67-71 (1992).

107 Meatherall, R., GC/MS confirmation of urinary benzodiazepine metabolites, J. Anal. Toxicol. 18, 369-381 (1994).

108 Peel, H.W., Perrigo, B.J., Toxicological analysis of benzodiazepine type compounds in post mortem blood by gas chromatography, J. Anal. Toxicol. 4, 105-113 (1980).

109 Chopineau, J., Rivault, F., Sautou, V., Sommier, M.F., Determination of temazepam and its active metabolite, oxazepam in plasma, urine and dialysate using solid-phase extraction followed by high performance liquid chromatography, J. Liq. Chromatogr. 17, 373-383 (1994).