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**DETECTION OF
DEPENDENCE-PRODUCING
DRUGS IN BODY FLUIDS**

**Report of a
WHO Meeting of Investigators**

WORLD HEALTH ORGANIZATION

GENEVA

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OF DEPENDENCE-PRODUCING DRUGS IN BODY FLUIDS

Geneva, 28 January - 1 February 1974

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DETECTION OF DEPENDENCE-PRODUCING DRUGS IN BODY FLUIDS

Report of a WHO Meeting of Investigators

1. INTRODUCTION

A WHO Meeting of Investigators on Tests for Detection of Dependence-producing Drugs in Body Fluids met in Geneva from 28 January to 1 February 1974. Dr T. A. Lambo, Deputy Director-General, opened the meeting on behalf of the Director-General and welcomed the participants and the representatives of the United Nations, the International Narcotics Control Board, the Council of Europe, the International Council on Alcohol and Addictions, the International Union of Pharmacology, and the Special Action Office for Drug Abuse Prevention, Washington D.C., USA. He noted that since its inception, the World Health Organization has been concerned with problems of drug dependence. Expert committees and scientific, study, and working groups have been convened to consider a variety of matters related to the use of dependence-producing drugs outside appropriate medical practice. Published reports cover such matters as the recognition, management, and prevention of public health and social problems resulting from drug use, the epidemiology of drug dependence, the use of cannabis, and problems of drug taking in youth. The detection of dependence-producing drugs in body fluids is an important adjunct to any programme related to drug dependence, whether preventive, therapeutic, or rehabilitative in its scope. Many methods have been described in recent years but not all have been adequately evaluated. It is therefore timely to review recent developments, particularly in the fields of gas phase analysis and immunoassay. In the field of public health there is a need for rapid, sensitive, simple, specific, and inexpensive tests for all dependence-producing drugs. Immunoassays, while lacking complete specificity, may have a useful function but additional facilities may be needed for the final identification of suspected drugs.

Dr Lambo invited the Meeting to discuss comparative studies on an international basis and to consider whether collaborating laboratories might be designated as reference centres where training and research facilities would also be provided. He also asked the participants to review research priorities, outline the most important research objectives, and indicate the public health requirements in both the developing and the developed countries.

2. GENERAL CONSIDERATIONS

2.1 Purposes of testing

Body fluids are usually tested for the presence of dependence-producing drugs for one or more of the following purposes : (1) to assist in establishing a clinical diagnosis ; (2) to help in monitoring the progress of treatment of drug-dependent persons ; (3) to help in determining the prevalence of drug use during the course of epidemiological and related studies ; (4) to further pharmacokinetic and metabolic research ; and (5) to contribute to the clarification of medicolegal problems. A given test may be utilized as an initial or screening procedure (see section 2.4, p. 11). It may be used (1) to establish the possible presence of one or more dependence-producing drugs without necessarily determining the chemical identity of such substances as may be detected or the quantity present, or (2) to assess the need for corroborative or more definitive testing. Some tests lend themselves to both initial and corroborative application, while others are more suitable for one of the other uses. In choosing a test or a combination of tests for use in a given laboratory in a particular geographical location, a number of factors have to be considered, among them the purpose to be achieved, the cost, the time needed to complete the test, the accuracy required, the technical difficulties involved, and the possible sources of error. Before considering these and related matters it will be useful first to indicate the way in which certain terms are used in this report and to describe, in broad terms, the types and characteristics of testing methods currently available.

2.2 Use of terms

The Meeting adopted the following definitions and usages of terms for the purposes of the report.

Drug. " Any substance that, when taken into the living organism, may modify one or more of its functions. " ¹

Drug dependence. " A state, psychic and sometimes also physical, resulting from the interaction between a living organism and a drug, characterized by behavioural and other responses that always include a compulsion to take the drug on a continuous or periodic basis in order to experience its psychic effects, and sometimes to avoid the discomfort of its absence.

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1969, No. 407, p. 6 (section 1.1). This definition is intentionally broader than that used in connexion with substances intended always to be of benefit to a patient ; see *Wld Hlth Org. techn. Rep. Ser.*, 1966, No. 341, p. 7 (section 2).

Tolerance may or may not be present. A person may be dependent on more than one drug.”¹

Sensitivity. The lowest concentration of a drug that a test will detect with reasonable assurance. Sensitivity might also be considered as an amount, not as a concentration, and thus a common statement might be “the test detects 10 µg” or “the sensitivity is 10 µg”. The statement means that the test detects 10 µg of the drug, but it does not give the volume of undiluted fluid that originally contained the 10 µg. For example, if the 10 µg of the drug were in 10 ml of fluid, the sensitivity would be 1 µg/ml.

Discriminating concentration. Because certain of the tests to be considered, e.g., immunoassays, yield quantitative results that are continuous variables, a value, the discriminating concentration, sometimes referred to as a “cut-off” is selected to distinguish arbitrarily between positive and negative results. The sensitivity and discriminating concentration are not necessarily the same.

Specificity. The ability of the method to distinguish unequivocally the compound or compounds to be measured. It is a fact of drug chemistry and pharmacology that many drugs are very closely related in their chemical structure but their actions and use may be quite dissimilar. For example, methadone and the commonly prescribed analgesic dextropropoxyphene are related in their chemical structures although they are prescribed under very different circumstances. Some tests may not differentiate between these two drugs. Clearly, if both drugs are detected by a test system, tests for methadone will have limited usefulness since many individuals taking dextropropoxyphene but not methadone will produce positive urine samples.

Non-specificity of a test may even be advantageous if the metabolites are measured with the original compound (morphine glucuronide and morphine, for example) thereby increasing the sensitivity; the results may then be expressed as drug equivalents (see p. 18).

2.3 Criteria for selection of methods

Following its administration, a drug is absorbed into the blood, distributed around the body and, in time, metabolized and excreted. Drug action may be correlated with the concentration of the drug or its metabolites or both in the blood. The selection of the analytical method must take into account the likely concentration of a particular drug or its meta-

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1969, No. 407, p. 6 (section 1.1).

bolite or metabolites in the volume of body fluids available after the required time interval following therapeutic dosage. The nature of the metabolic products must also be considered, especially with regard to the specificity of the detection method.

The availability of sensitive and specific tests for the presence of dependence-producing drugs in body fluids should not in itself be an indication of the relevance of, and need for, their application under any circumstances. Care should be taken to guard against the indiscriminate use of such tests, particularly since the interpretation may have far-reaching consequences for the individual.

On the other hand, it may not necessarily be a disadvantage if a test does not discriminate between a drug and its metabolites (between morphine and morphine glucuronide, for example, if the purpose is to test for suspected opiate use). Where a test is non specific, the evaluation and interpretation of test results must take into consideration the nature and circumstances of drug use as well as factors such as genetic predisposition ; exposure to other drugs, certain foodstuffs, or environmental contaminants ; and alterations in the elimination and metabolism processes.

The choice of method could be influenced by the nature and quantity of specimens potentially available as well as by existing facilities, available manpower, and economic considerations. The Meeting agreed unanimously that test specificity is an ideal that in practice can only be approached and considered the recognition and definition of experimental limitations to be of prime importance for the correct interpretation of results. Considering commercially available testing packs (kits) for the rapid detection of drugs in body fluids, the Meeting recognized the need for careful assessment of the manufacturer's specification. Other factors to be considered include reproducibility, simplicity, speed, and cost (including the capital outlay for, and maintenance costs of, equipment, the running costs of reagents, technicians' time, and the provision of appropriate supervision). Most of these factors are shown in Tables 1 and 2. The availability of local resources is an important factor, particularly in developing countries, and the following requirements have to be met.

(1) Technical personnel require manual dexterity and an ability to carry out instructions accurately. The extent of training required by these personnel will usually be related to the degree of elaboration of the methods selected.

(2) Supervisory staff must be scientifically competent in the use of existing techniques and able to assess new methods. Supervisors must be qualified to assess the validity of results and to recognize, and perhaps rectify, simple failures in the equipment.

(3) Clinical staff should be fully cognisant with the implications of results obtained by the laboratory staff, understanding the limits of sensitivity and the possibility of false positive results.

(4) Facilities for the maintenance and repair of equipment are essential if methods based on the use of gas chromatography, spectrophotometry, spectrofluorimetry, mass spectrometry, radioimmunoassay, or spin-label immunoassay are selected. Total disruption of the work may occur unless equipment faults are quickly diagnosed and the necessary repairs made. The availability of spare parts must be taken into account when equipment is selected for purchase. Ideally, equipment should be maintained and repaired by staff trained by the manufacturer.

2.4 Initial and corroborative testing

Testing procedures usually involve an initial test requiring confirmation by an independent test in the event of positive results being obtained. The choice of tests will vary according to the facilities available and the use to be made of the results. Thus, if results are required urgently in an emergency the knowledge that a specific drug (or drugs) is not present and thus does not account for the patient's clinical condition may be more important than confirmation several hours later of the presence of another drug.

Unless an unequivocal test is applied in the first instance, confirmation of a positive result is essential. The presence of a drug detected by thin-layer chromatography of a solvent extract can be confirmed in various ways including gas chromatography or microcrystal tests for example. Similarly, a positive finding in an immunological test will usually require further investigation.

3. METHODOLOGY OF DETECTING DEPENDENCE-PRODUCING DRUGS IN BODY FLUIDS ¹

3.1 Extraction methods

Body fluids contain a large number of compounds of both endogeneous and exogeneous origin. The drug to be investigated will be present as a minor component and separation and concentration is often required

¹ The group noted the extensive bibliographical work carried out by members of secretariat and expressed a wish to include an up-to-date bibliography as an annex to the report. For practical reasons, however, the bibliography is being issued as a separate publication: Chruściel, T. L. & Chruściel, M. *Selected bibliography on detection of dependence-producing drugs in body fluids*, Geneva, World Health Organization (in press).

before analysis. In general, the drug is extracted with an organic solvent, which is then evaporated to dryness; the drug in the residue may be further concentrated prior to analysis by thin-layer or gas chromatography or a combination of chromatographic methods. Drugs that are metabolized and excreted in the urine as glucuronides or sulfates are highly polar and are therefore unlikely to be extracted by the commonly used organic solvents. The polar compounds may be recovered by utilizing appropriate ionic or non-ionic resins. Although little used so far, the ion-pair extraction technique for the selective recovery of dependence-producing drugs is an attractive alternative (1).

3.2 Paper- and thin-layer chromatography

Paper chromatographic separations are time consuming and have mostly been superseded by the faster thin-layer systems of analysis. Thin-layer chromatography is a well tried technique available in the majority of laboratories concerned with drug detection. It is quick, inexpensive, and

TABLE 1. COMPARISON C

	Immunoassay			
	Free radical	Enzyme activation	Radio isotope	Haemagglutination inhibition
No. of tests per technician per 8-hour shift	150-350	200-225	200-400	200-350
Costs (US \$) ¹				
Materials per test	0.30-0.75	0.30-0.75	0.45-0.75	0.15-0.35
Labour per test	0.10-0.23	0.17 ⁵	0.07-0.58	0.10-0.18
Total cost per test	0.40-0.98	0.47-0.75	0.52-1.33	0.25-0.53
Cost of establishing facilities	27 000	6 000-8 000	8 000-15 000	200
Turn-around time ²	2-5 min	2-5 min	1-2 hours	1.5-2 hours
Equipment maintenance required	Tests depend on one major piece of equipment that could have virtually no time lost due to failures or as many as 25 inoperable days per year. Service contracts are available.			Negligible (no major equipment)
Test results expressed as:	Simple linear measurement or peak height	Difference in extinction	Counts per minute	Visual recognition of a pattern of agglutination or non-agglutination
Sensitivity ³				
total morphine	0.50	0.50	0.03-0.06	0.03-0.06
barbiturate	1.0-2.0	1.5-2.0	0.05-0.10	} $\mu\text{g/ml}$
amphetamine	1.0-2.0	2.0-2.5	0.10-0.15	
cocaine ⁴	0.5-1.0	1.0-1.5		
methadone	0.5-1.0	0.50		

* This table represents the best estimates and approximations as of January 1974 (131).

¹ Cost estimates for immunoassay materials are based on the purchase and use of currently available commercial versions of each assay. The labour cost is based on the work of an average technician earning US \$30 per day.

² This refers to the amount of time needed to complete the analysis of one sample, assuming

simple to perform. In many instances, thin-layer chromatography of extracts in several carefully selected systems in conjunction with the use of specific and sensitive reagents may be adequate for detecting drugs. However, when more than one drug and its metabolites could be present thin-layer chromatographic methods should not be relied on without corroborative tests. While many systems of thin-layer chromatography are well known and tested, it is essential to ensure that a drug is properly characterized before a conclusion is drawn about its identity.

A general comparison of sensitivity, costs, time required, etc., of various analytical methods is given in Table 1.

3.3 Spectrophotometric methods

3.3.1 Ultraviolet absorption

Ultraviolet absorption measurements have been used for the detection of various drugs such as barbiturates in extracts of body fluids, but these methods are limited by the comparatively poor light absorbing properties of

ANALYTICAL METHODS *

Automated spectrofluorimetry	Chromatography		Mass fragmentography
	Thin-layer	Gas	
10-500	60-100	30-50	30-50
10-1.00	0.30-0.50	0.02-0.16	0.50
05-0.08	0.58 ⁵	1.00 ⁵	1.00
15-1.08	0.88-0.50	1.02-0.16	1.50
100-25 000	300-500	5 000-10 000	25 000-50 000
5-1 hour	1-2 hours	1-2 hours	1-3 hours
Service contracts available	Negligible (no major equipment)	The GC and GC/MS are the tests most likely to suffer from equipment failures. Maintenance contracts can be negotiated	
Difference in peak heights	Intensity of spot on chromatogram	Peak height	Peak height and mass
20 µg/ml	0.5-1.5 } 1.5-2.0 } 1.0-2.0 } µg	0.5-3.0 } 1.0-3.0 } 1.0-2.0 } µg	0.0001-0.1 } 0.0001-0.1 } 0.0001-0.1 } µg
	1.0-2.0 } 1.0-3.0 }	0.1-1.0 }	0.0001-0.1 }

that all the necessary equipment and supplies are available (131).

³ Defined as the minimal concentration of a drug or metabolites that can be detected in biological fluid.

⁴ The principal metabolite of cocaine, benzoylecgonine, is measured.

⁵ Mean cost.

some drugs, methadone and amphetamine, for example, and the fact that other drugs and endogenous substances may interfere. For example, ultraviolet absorption measurements will not allow a distinction to be made between methaqualone and some of its metabolites when present in an extract if the metabolic change does not involve the ultraviolet absorbing moiety of the drug.

3.3.2 *Spectrofluorimetry*

Spectrofluorimetric procedures involve irradiation of a solution with light of a known wavelength and measurement of the fluorescence produced at a defined (longer) wavelength. Some drugs such as lysergide may be examined directly, but others have first to be converted into fluorescent derivatives; for example, morphine may be oxidized to form the highly fluorescent compound pseudomorphine.

3.4 **Gas chromatography**

It was accepted by the Meeting that not all drugs could be satisfactorily analysed directly by this technique. The preparation of more volatile derivatives (e.g., trimethylsilyl ethers) might be helpful but this is time consuming and may result in the partial loss of a substance. Adsorption on column-packing materials during gas chromatography may lead to significant losses of some substances, especially polar compounds. Improved sensitivity might be achieved by using glass capillary columns but these are comparatively untested with the drugs present in extracts of biological fluids.

3.5 **Gas chromatography-mass spectrometry and mass fragmentography**

3.5.1 *Gas chromatography-mass spectrometry*

Combining gas chromatography with mass spectrometry permits the direct identification of compounds. The advantage of this technique is that the good separating power of gas chromatography and the elegant means of identifying compounds by mass spectrometry facilitate studies of drug metabolism. The identity of a compound is established by comparing its mass spectrum with that of a reference substance. If an unknown metabolite is investigated, its mass spectrum usually, but not always, indicates the molecular weight of the compound. If the structure of the parent compound is known, a structure can then be proposed for the metabolite. Partial structures, even of completely unknown compounds, can often be deduced from the presence of key ions. By linking gas chromatography-mass spectrometry to an appropriate computer programme it becomes

possible to identify drugs and drug metabolites from a file of reference spectra. Some such systems for examining blood and other body fluids from patients have been in operation for several years and the equipment, reagents, and detailed instructions for carrying out the tests are now available commercially (2-7).

3.5.2 *Mass fragmentography*

Using ion-specific detection, the intensity (abundance) of the ions produced when the effluent from the gas chromatograph is ionized in the ion source, may be recorded as continuous curves (mass fragmentography). Depending on the equipment used, different numbers of fragments (ions) typical of the compound to be analysed may be detected simultaneously. A mass fragmentogram is similar in appearance to a gas chromatogram. Like the latter, it gives the following information: the retention time, which is characteristic of the compound, and the peak area or the peak height, which is proportional to the amount. In addition, the relative intensities of the peaks should be the same as the relative intensities of the ionic fragments in the mass spectrum of the compound—another criterion of identity.

Mass fragmentography is 100-1 000 times more sensitive than ordinary gas chromatography or gas chromatography-mass spectrometry. It requires the exclusive use of the spectrometer for the mass specific recording of preselected ions. A mass fragmentogram is obtained by on-line analogue processing of data, and requires that the mass spectrometer be focused beforehand on the masses at which ion abundance is to be measured. By contrast with mass fragmentography, mass chromatography is the analysis of data from repetitively scanned mass spectra, stored for the most part on magnetic tapes or discs, to permit the recovery of a graph of a specific ion abundance against time. No previous knowledge of the masses of interest is required. Any masses can be recalled. The sensitivity is considerably lower than that of mass fragmentography (8, 9).

3.6 **Immunoassay methods**

The immunoassay procedure was initially introduced as a means of measuring hormones in biological fluids but recently the technique has been used in the development of methods for determining drugs in body fluids. Morphine, for example, may be linked by a protein carrier, through the carbon atom in the 2 position, the substituents at the 3 and 6 positions, or the N-atom, at various sites of the molecule. The resulting conjugate (immunogen), often emulsified in Freund's adjuvant, may be used to immunize animals such as rabbits, sheep, or goats. Antisera obtained from these animals are used as reagents in the immunoassay.

Failure to detect a drug by immunoassay is very strong evidence that the drug in question is not present in excess of the discriminating (or "cut off") concentration utilized. Detection of the drug is presumptive evidence that the drug or its metabolites or both are present, and the greater the quantity reported the stronger the evidence. Cross-reactions do occur, however, and for a more definitive identification it is essential that the result be confirmed by a nonserological method.

The immunoassay is generally performed by incubating labelled (e.g., radioactive or spin-labelled)¹ drug and unlabelled drug (standard or unknown) in the presence of a limiting amount of antibody. When increasing amounts of unlabelled drug are added, the antibody binding sites are progressively occupied by unlabelled drug, and the diminished binding of labelled drug is evidence for the presence of unlabelled drug.

Another immunoassay uses for the indicator system an enzyme-drug complex.² This complex is enzymatically inactive in the presence of antibody reactive with the drug. Addition of the drug in solution (or in biological fluids) displaces the antibody and activates the enzyme. If the enzyme is lysozyme, its activation may be observed by the clearing action of the active enzyme on a suspension of cell walls from *Micrococcus lysodeikticus*.

The haemagglutination inhibition reaction (see Annex 1) is another example of an immunoassay. The procedure involves the attachment of morphine, for example, on the surface of the erythrocytes, which then agglutinate when morphine antibody is added. Any morphine present in a biological fluid will bind with the available sites on the antibody and thereby inhibit agglutination of the red blood cells.

Since immunoassays are inherently very sensitive (see Table 2), simple, and rapid (150-400 tests can be made in a day), the Meeting anticipated that there will be a proliferation of such methods.

3.7 Other methods

3.7.1 Liquid chromatography

Liquid chromatography, a new separation method, seems to be a powerful supplement to the other separation techniques. Its value is partly based on the fact that it can also be applied to polar compounds, the separation of which is difficult or even impossible with other methods, or demands the preparation of derivatives.

¹ Sometimes referred to as the free radical assay system or FRAT.

² Also known as EMIT.

TABLE 2. APPROXIMATE SENSITIVITY IN SOME ANALYTICAL SYSTEMS

Method	Concentration (mol/litre)	Amount (mol. (vol.))
Spectrophotometry	10^{-6}	10^{-10} (100 μ l)
Fluorimetry	10^{-9}	10^{-13} (100 μ l)
Gas chromatography		
flame ionization detector	10^{-4}	10^{-10} (1 μ l)
phosphor detector	10^{-7}	10^{-15} (1 μ l)
electron capture detector	10^{-7}	10^{-13} (1 μ l)
Gas chromatography-mass spectrometry	10^{-5}	10^{-10} (10 μ l)
Mass fragmentography	10^{-3}	10^{-14} (10 μ l)
Immunoassays	10^{-7} - 10^{-8}	10^{-11} - 10^{-12} (10-100 μ l)

3.7.2 Microcrystal tests

Microcrystal tests may be used to identify many drugs, including, morphine, codeine, and methylamphetamine, in eluates from thin-layer chromatograms (10, 11, 12).

3.7.3 Other physicochemical methods

Many other physicochemical methods of detection and assay may be appropriate but few have found a practical application, particularly where demands are only occasional.¹

3.7.4 Bioassay

Simple biological tests may be useful, at least on a preliminary basis, and can be as sensitive as some of the described above. Being simple, they can be carried out without elaborate equipment. Tests such as the Straub mouse-tail reaction (13) or contraction of the isolated guinea pig ileum (14) may be used to indicate the possible presence of an opiate. Positive results require confirmation by a definitive chemical test. Bioassays remain important in testing biological material to assess the possible contribution of substances other than the main biologically active component.

3.8 Current assessment of recent methods

In attempting to assess the suitability of available methods for studying particular problems, the Meeting concentrated on those introduced fairly

¹ References to these methods will be found in the bibliography (see footnote 1 on p. 11).

recently. It must be emphasized, however, that thin-layer chromatographic methods are still widely used for the qualitative detection of drugs in extracts of body fluids.

When available, preliminary tests employing immunological procedures are quick, sensitive, and simple to perform. At or near their maximum sensitivity, properly controlled immunological tests allow specimens that appear free from the drug to be excluded from further consideration.

Certain structural features of a drug molecule determine the specificity of antibodies used in immunoassays. These structural features may be common to the drug and its metabolites and to other compounds. Thus the specificity may be low. Positive immunoassay test results (the finding of drug equivalents) therefore require confirmation by nonserological methods.

Spectrophotometric methods, both absorption and fluorescence, offer adequate sensitivity and specificity for the routine analysis of some drugs.

Gas chromatography is more sensitive than paper- or thin-layer chromatography by a factor of approximately 10. Its application is restricted to volatile compounds or those for which volatile derivatives can be prepared. Selective detectors used with gas chromatography systems may increase the sensitivity as much as a thousand-fold.

A mass spectrometer can also be used as a gas chromatography detector (mass fragmentography); the order of sensitivity is similar to that of the electron capture detector. The ideal internal standard is the same compound containing a stable isotope (or isotopes). An important use of mass spectrometry or gas chromatography-mass spectrometry is to check the validity of other methods, although even a mass spectrum does not in all cases provide absolute proof of the presence of a specific chemical compound.

The high initial and running costs of a gas chromatograph-mass spectrometer are a disadvantage of the method, but it is feasible to carry out approximately 50 quantitative determinations in a working day.

4. PROSPECTS FOR MEANINGFUL DETECTION OF PARTICULAR DRUGS

4.1 Opiates and their synthetic alternates

4.1.1 *Heroin, morphine, and codeine : metabolism and elimination*

The metabolism and elimination of these drugs in man has been reviewed extensively (15, 16, 17) but their metabolic fate has not been completely established.

Heroin (diamorphine) is rapidly and extensively deacetylated to 6-monoacetyl morphine and morphine. According to Way & Adler (15), little or no heroin and only small amounts of 6-monoacetylmorphine are found in urine following parenteral administration. The ratio of morphine to morphine glucuronide is essentially the same as if morphine were the drug administered and varies in random urine specimens in relation to the time elapsed since the drug was taken. Codeine has been identified in the urine of heroin users (18).

Mo & Way (19) studied the total morphine excreted in the urine by 16 addicts whose daily intravenous dose of heroin hydrochloride had been stabilized; individual dosages ranged from 150 to 425 mg per day but were mostly between 225 and 300 mg per day. The mean proportion of the dose recovered from the urine in 24 hours was 68%.

Elliott and his associates (20) attempted to determine the maximum capacity of man to metabolize heroin. A mean total quantity of 70.5 mg was infused continuously in 4 volunteers over a period of 7 hours. It was possible to account for $45 \pm 5\%$ of the infused dose in urine collected for 40 hours after the start of the infusion. The proportions of urinary metabolites excreted over the 40 hours were as follows: morphine 4.2%, morphine glucuronide 38.3%, monoacetylmorphine 1.3%, heroin 0.13%. While Elliott et al. (20) were unable to detect any free or conjugated morphine in the blood of their subjects, Spector & Vessel (21), by radioimmunoassay, detected morphine equivalents in the serum of 10 volunteers following a single intravenous dose of 10 mg morphine sulfate per 70 kg of body weight. The serum concentration-time curve was complex; the first part described a half life of a few minutes, the second a half life of about 3 hours and then a late phase with a half life of several hours. Following intravenous doses of heroin at 2.5, 5, and 10 mg per 70 kg, Gorodetsky & Kullberg (22) detected morphine equivalents in the plasma collected 1-6 hours after drug administration. The immunoassay employed in these two studies did not distinguish between morphine or heroin and metabolites.

Conjugation of morphine to form morphine-3-glucuronide is the major metabolic pathway for morphine (15, 16, 17, 23). The observation by Elliot et al. (24) of $^{14}\text{CO}_2$ in the expired air in man receiving N- ^{14}C -methylmorphine was suggestive of N-demethylation and the expected metabolite, normorphine, has been identified recently in the urine of subjects receiving 60 mg of morphine per 70 kg of body weight (25). Conjugated normorphine (25), morphine ethereal sulfate (25), and codeine (18) are also found in urine in small quantities.

The major route of excretion of morphine and its metabolites is renal although biliary excretion also occurs. The relative amounts of morphine and morphine glucuronide excreted vary with the time elapsed since dosage,

more of the free drug being excreted initially following a drug dose. Way & Adler (15) reported that in the first 24 hours, the average amount of free morphine excreted in urine was 7% of the dose with a range of 1–14%. The average excretion of morphine glucuronide quoted in the older literature is approximately 30% with a range of 11–45%. More recent studies indicate that up to 62% of the daily dose is recovered as morphine glucuronide in urine from subjects stabilized on 60 mg of morphine per 70 kg of body weight (25).

Three studies performed prior to 1955 reported that 1–10% of the total dose is excreted into the gastrointestinal tract and can be recovered in the stools. Very small amounts can be detected in perspiration (15) and saliva (22). Elliott et al. (24) reported a range of 3.4–6.1% of the total dose excreted in expired air and 70–104% of the total administered dose recovered by all routes of excretion examined over a 4-day period.

The metabolic fate of codeine in man has been described by Adler et al. (26); some morphine is formed.

The published comparative studies of detection methods for heroin, morphine, and their metabolites are summarized in Annex 2.

4.1.2 *Pethidine and derivatives*

Approximately 65% of a 175 mg oral of pethidine (meperidine) may be recovered from urine during 24 hours: about 5% as the unchanged drug, 7% as norpethidine (pethidine intermediate B), 47% as free and conjugated pethidinic acid, and 17.5% as free and conjugated norpethidinic acid. The metabolites lack significant analgesic activity.

A gas-liquid chromatographic determination of pethidine in plasma was described by Sullivan et al. (27); Ramsay & Campbell (28) claimed a sensitivity of 2–5 µg/ml for a 2-ml specimen. Spectrophotometric methods lack specificity and are not sufficiently sensitive for monitoring therapeutic dosages. Thin-layer chromatography of extracts containing at least 5 µg of pethidine allows qualitative characterization to be made (29).

4.1.3 *Diphenylpropylamines*

Methadone is rapidly absorbed, widely distributed throughout the body, and extensively metabolized. The major metabolites are the products of mono-N-demethylation (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine) and di-N-demethylation (2-ethyl-2,3-dihydro-5-methyl-3,3-diphenyl-1H-pyrrole). Additional metabolites have also been identified (27, 28). Both of the major metabolites are found in sweat and urine (29–35), and have not yet been reported in plasma (34). Methadone, itself, is found in plasma (34, 35, 36), whole blood (34), urine (29, 31, 32, 33), and sweat (33). The

urinary excretion of methadone and metabolites is dependent on the dose, pH of the urine, and sex of the individual (33). For detailed discussions of pharmacokinetic data and detection techniques see Annex 3.

4.1.4 *Some other morphine-type drugs*

A number of methods for testing other morphine-type drugs have been described in the literature.¹ The Meeting discussed tests for two such drugs in which some interest has recently been shown.

4.1.4.1 *Dextropropoxyphene* is an analgesic drug that is sometimes used inappropriately but also has an application in the maintenance treatment of heroin-users. The pharmacokinetics of this substance have been reported by Wolen & Gruber (37) and the metabolic pathways established by McMahon et al. (38). Approximately 25% of a 65 mg oral dose of dextropropoxyphene hydrochloride is excreted in the urine, partly unchanged but mostly as metabolites. The drug and its metabolites may be recovered from urine for at least 48 hours. Analytical methods based on gas chromatography and thin-layer chromatography are available but the results need careful assessment. Cross-reaction of the morphine immunoassay reagents with dextropropoxyphene has been noted (38).

4.1.4.2 *Hydromorphone*. Fatalities due to an overdosage of hydromorphone have been reported in recent years. It is therefore prudent to consider testing methods. Both gas- and thin-layer chromatographic methods allow the separation of morphine and hydromorphone, although with difficulty (39, 40). In immunological assays there is cross-reaction between morphine reagents and hydromorphone (41).

4.2 Barbiturates

In general, barbiturates are rapidly absorbed and extensively metabolized, and the metabolites and unchanged drugs are excreted in the urine (see Annex 4).

In clinical situations, the blood levels following therapeutic dosage have in the past been measured by ultraviolet absorption methods. The lack of specificity resulting from the extraction and measurement of metabolites together with the unchanged drug stimulated the search for other methods. Gas chromatography and spectrofluorimetry have proved satisfactory for quantitative work; polarographic and colorimetric methods have been proposed but have not yet been applied in clinical laboratories. Thin-layer chromatography is suitable only for qualitative studies.

¹ See footnote 1 on p. 11.

Rapid methods for use in large-scale urine-testing programmes have been developed to meet public health demands. The immunological reagents are sufficiently sensitive for pharmacokinetic studies. Specificity may be achieved by using gas chromatography-mass spectrometry.

4.3 Other hypnotics and sedatives

4.3.1 Methaqualone

Methaqualone alone or in combination with diphenhydramine has been widely used since its introduction in 1958. An increase in self-administration of this drug has been noted in recent years and very often it is taken together with alcohol. Methaqualone has generally been stated in drug manuals to be a safe hypnotic drug that is rapidly absorbed and eliminated. This appears to be supported by the work of Morris et al. (42), who reported plasma concentrations of methaqualone in man after single oral therapeutic doses. When the period during which blood samples were taken was extended and mass fragmentographic analysis was used, the pharmacokinetics of methaqualone after single oral doses were shown to be different from those originally expected (43). The elimination of methaqualone is biphasic; the slower phase has a half life ranging from 19.6 to 41.5 hours. Steady-state plasma levels can be obtained when therapeutic doses are administered daily.

Among the other techniques proposed for the analysis of methaqualone, ultraviolet absorption spectrophotometry, fluorimetry, and gas chromatography have been checked against mass fragmentography. Gas chromatography can then be taken as the standard for other techniques. Ultraviolet absorption measurements after chloroform extraction give entirely erroneous results, hexane extraction is somewhat better but there is a wide scattering of results.

4.3.2 Glutethimide

A single oral dose of 1 g of glutethimide in man results in a peak blood level of approximately 7 µg/ml after 2 hours. Several gas chromatographic procedures are available (44, 45, 49). The procedure described by Widdop (45, 47) allows the detection of 0.3 µg of glutethimide per ml of plasma.

The colorimetric (48) and spectrophotometric methods (49) used in early studies lacked specificity since the metabolites as well as the unchanged drug are extracted by some solvents including chloroform. Gas chromatographic and spectrophotometric assays of hexane extracts are reported to give comparable results (50).

Urinary excretion products include 1-phenyl-2,6-piperidinedione and 1-ethyl-1-phenyl-2,6(1*H*,3*H*)-pyridinedione (51).

4.3.3 *Meprobamate*

In man, a peak blood level of approximately 5 µg of meprobamate per ml is observed approximately 2 hours after the ingestion of a single 400-mg dose. Blood levels of 15–20 µg/ml are observed when 400-mg doses of meprobamate are administered at 6-hourly intervals (52). About 20% of the dose is slowly excreted in the urine.

Gas chromatographic methods of quantitative analysis for meprobamate are satisfactory; the estimation of 2 µg/ml in a 3-ml specimen presents no difficulty. Colorimetric methods based on the procedure of Hoffman & Ludwig (53) have been widely used in hospital clinical laboratories (54, 55, 56).

4.3.4 *Benzodiazepines* (including chlordiazepoxide and diazepam)

Benzodiazepines are generally not excreted in the urine in a free unchanged form to any appreciable extent and it is necessary to analyse the urine for the presence of metabolites (57).

Determinations of benzodiazepines in physiological fluids have been achieved by means of ultraviolet spectrophotometry (58), colorimetry (59), fluorimetry (60), cathode ray polarography (61), and gas-liquid chromatography with electron capture detection (62). Schwartz et al. (63) found that the principal metabolites of diazepam excreted in the urine are N-demethylated diazepam, hydroxylated diazepam, and hydroxylated N-demethylated diazepam (oxazepam). These metabolites occur in the urine as conjugates with glucuronides and sulfates. De Silva et al. (58, 64), in their studies on blood levels of diazepam, applied strong acid hydrolysis to produce benzophenones.

Walkenstein et al. (65) found that (2-amino-5-chlorophenyl)phenylmethanone, the product of acid hydrolysis of oxazepam, can be detected by the Bratton-Marshall (66) reaction for primary aromatic amines. Kokoski et al. (67), utilizing this procedure suitably modified for thin-layer chromatographic application, devised a method for detecting metabolites of chlordiazepoxide, diazepam, and oxazepam. The sensitivity of this test, which relies on the detection of (2-amino-5-chlorophenyl) phenylmethanone, is 0.3 µg of oxazepam per ml in urine. Positive reactions have been found for as long as 8–9 days following a single 5-mg dose of diazepam.

4.4 Psychostimulants

4.4.1 Cocaine

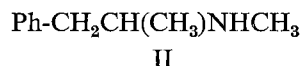
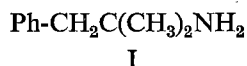
Metabolites excreted in urine may be detected by two methods (68, 69).

(1) The enzyme-multiplied immunoassay procedure; this is sensitive to 1 µg benzoylecgonine/ml but is less sensitive to ecgonine (9 : 1) and cocaine (400 : 1).

(2) Extraction of ecgonine and benzoylecgonine and thin-layer chromatography of the butyl esters visualized by iodoplatinate reagent; this technique allows the detection of 1 µg of both esters.

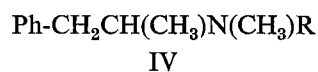
4.4.2 Amphetamine

The pharmacokinetics of amphetamine in man have been studied by many research groups. A recent drug metabolism study during amphetamine psychosis was described by Änggård et al. (70). Their report also includes a comprehensive bibliography. Sever et al. (71) have described the metabolism of amphetamine in dependent subjects. Although only a small proportion of an administered dose is excreted unchanged in the urine, available methods are sensitive enough to show the presence of amphetamine for several days following a single therapeutic dose. The gas chromatographic method originally devised by Beckett & Rowland (72) allows detection and confirmation by derivative formation or thin-layer chromatography of 0.1 µg of amphetamine per ml when a 5 ml sample of urine is analysed. Careful measurement of retention times is required because different components, phentermine (I) and amphetamine (II), for example, show rather similar R_f values.



Differentiation between these compounds is difficult, even if gas chromatography-mass spectrometry is applied, because the mass spectra of the compounds are nearly identical (73). Unambiguous differentiation is obtained by the application of gas capillary chromatography.¹

Attention must be drawn to the fact that compounds with the general structures (III) and (IV)

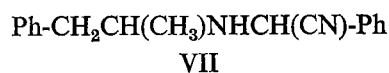


¹ Spitteller, C. et al., unpublished data, 1974.

are enzymatically cleaved in the body at the N-R bond to form amphetamine. Other compounds, similarly modified *in vivo*, include the anoretics benzphetamine (V) and furfenorex (VI)



and the stimulant (VII) known as AN1 (74).¹



A radioimmunoassay using ¹²⁵I has been developed for the detection of amphetamines in biological fluids. The sensitivity is around 100–125 ng/ml. Specificity appears similar to that for other immunoassays. The evaluation is not yet complete.

4.4.3 Ephedrine

Gas-liquid chromatographic separation of various optical isomers of ephedrine and some of its congeners has been investigated. For satisfactory analysis 50 µg of the ephedrine-like compound was required. The method is claimed to be suitable for metabolic studies. A 50 mg dose of ephedrine produced levels in the urine that could be detected with the aid of a nitrogen-specific alkaline flame detector and a high temperature gradient (74).

4.4.4 Khat (*Catha edulis* Forsk.)

One active principle of khat is (+)-norpseudoephedrine (“cathine”), which comprises 0.1–0.2% of the dried material. This substance is easily detected by thin-layer chromatography. Another substance with amphetamine-like pharmacological properties has been isolated from the fresh plant material but its chemical structure awaits final elucidation.

4.4.5 Methylphenidate

Administration of the ¹⁴C-labelled methylphenidate (methyl- α -phenyl-2-piperidineacetate) to human volunteers (77) resulted in the excretion of 92% of the radioactivity in the urine. Less than 1% of the drug is excreted unchanged; the major metabolite is ritalinic acid but 6-oxo- α -phenyl-2-piperidineacetic acid is found also. The lactam of the major metabolite

¹ The chemical name for this compound is α -[(1-methyl-2-phenylethyl)amino]benzeneacetonitrile.

may be extracted from urine (at pH 3) into benzene and characterized by thin-layer chromatography.

4.5 Hallucinogens

4.5.1 Lysergide

Methods are required for measuring lysergide in very small quantities, since the drug is taken in microgram amounts and is distributed widely throughout the body.

For quantitation of lysergide in body fluids spectrofluorimetry has been used (78). Although this method is sensitive it lacks specificity. Efforts are currently being made to develop an immunoassay method for lysergide. However, while immunoassays are very sensitive, there is a possibility that the antibody, which is generated against a synthetically derived indole, might cross-react with endogenous indoles such as tryptophan, tryptamine, 5-hydroxytryptamine, melatonin, or their metabolic products.

4.5.2 Mescaline

Mescaline may be detected by methods similar to those used for amphetamine-like compounds. The metabolism and excretion of mescaline in man was discussed by Patel (79). Methods for determining mescaline using gas chromatography-mass spectrometry have been described recently (80).

4.5.3 Other hallucinogens

Drugs such as dimethyltryptamine (DMT), psilocybin, psilocin, harmine, tetrahydroharmine, harmaline, and phencyclidine, may be detected by ultraviolet absorption, fluorimetric, thin-layer chromatographic, gas chromatographic, or gas chromatographic-mass spectrometric methods (81).

4.6 Cannabis, tetrahydrocannabinols, their metabolites and derivatives

Recent research shows that cannabis contains a large number of components including nitrogen-containing compounds and volatile terpenoid substances, many of which have not been elucidated structurally. There are considerable variations between samples as regards both the total and proportional content of even the known cannabinoids. Differences in the results obtained by groups studying the chemistry or pharmacology or psychological effects of cannabis might be due to differences between the samples studied. Every study should therefore be based on properly characterized and analysed material, especially when pure compounds are not

available. The United Nations Narcotics Laboratory in Geneva has prepared reference samples of cannabis plant and resin. The reference samples are available for study and analytical control tests are regularly performed on them.

The main active principle of cannabis is Δ^9 -tetrahydrocannabinol but other active constituents are known. The true contribution of the mono-oxygenated metabolites to the activity of cannabis in man is still under debate.

Most analytical effort has been directed towards the determination of Δ^9 -tetrahydrocannabinol in body fluids. Experiments show that tetrahydrocannabinol metabolites are excreted mainly in faeces and that minor amounts of metabolites appear in the urine (82-85). Agurell et al. (86), using mass fragmentography, detected plasma levels of Δ^9 -tetrahydrocannabinol. The high cost per sample limits the routine use of this method. Recently, gas chromatographic methods have been reported for quantitation of tetrahydrocannabinol (87, 88, 89).

Friedrich-Fiechtl et al. (70) claim to have detected tetrahydrocannabinol in saliva for at least 2 hours after the cessation of cannabis smoking by the fluorescence of its dansyl derivative, but this method requires proper evaluation. Attempts to demonstrate the presence of cannabinoids in breath have yielded equivocal results.

In the opinion of the Meeting, efforts to improve methods for detecting Δ^9 -tetrahydrocannabinol and its metabolites should be continued.

4.7 Antagonists and agonist-antagonists of the morphine-type drugs

4.7.1 Cyclazocine

The agonist-antagonist cyclazocine may be detected by thin-layer and gas-liquid chromatography (40, 91, 92); both techniques are sensitive to microgram quantities. Determinations in the nanogram range are possible with mass fragmentography (93).

4.7.2 Pentazocine

The elimination and metabolism of this drug have been studied and reviewed by Berkowitz (94). Thin-layer and gas chromatographic methods of analysis can be used (95, 96). The radioimmunoassay method developed by Williams & Pittman (97) is reported to be sensitive to nanogram quantities but has not yet been fully evaluated. More recently, mass fragmentography has been applied to the measurement of small quantities (ng/ml) in plasma and cerebrospinal fluid (93).

4.7.3 *Naloxone*

The metabolism of this drug was studied by Weinstein (98). It is conjugated, N-dealkylated, and the quinone is reduced to a 6-hydroxy metabolite. Naloxone may be detected by thin-layer and gas-liquid chromatography. This drug is short-acting and is rapidly excreted in the urine. Blood levels are difficult to detect by existing methods 4 hours after administration.

5. THE FUNCTIONS OF NATIONAL REFERENCE AND OTHER LABORATORIES

5.1 Functions

It is important to determine which drugs an individual is using, the amounts taken, and the duration and pattern of use. Since the patient may be unable or unwilling to provide this information, his family, friends, and various agencies may be approached.

Hospital laboratories have a vital role to play in the identification, assessment, and clinical management of a patient's drug problems but their full potential may not be realized without close collaboration and understanding between the clinical and laboratory staff. The identification of a drug and its metabolites in a body fluid sample may confirm a clinical suspicion of inappropriate drug use or show that a prescribed drug has been administered. In some instances it may be possible to confirm by quantitative analysis that a prescribed drug has been administered at the correct dosage but urgent research is warranted in this field for many drugs.

All laboratories undertaking drug identification in biological specimens must continually check, review, and up-date their analytical methods to improve specificity, sensitivity, and speed consistent with their work load and budget. At the same time, the results of urine analyses have no significance unless the collection of specimens is properly supervised and there is no opportunity for substitution—a factor not always appreciated by junior personnel.

The hospital laboratory should therefore provide factual data about the nature and amounts of drugs used by patients so that the clinician can assess objectively the progress of both inpatients and outpatients. When relevant data for plasma levels, protein-binding, urinary excretion, and other characteristics of a drug and its metabolites are not available in the literature research might reasonably be undertaken if the cooperation of patients can be obtained and the supervision is adequate.

5.2 Medico-legal issues

The selection of tests, the interpretation and confidentiality of the results, the purpose of the test and way in which the results are to be used, and questions about the need for the informed consent of patients for the collection and testing of body fluids, are often influenced by the statutes, regulations, official directives in force and the ethical standards prevailing in the locality where the testing is carried out. Ethical standards may vary substantially from one country or locality to another and different considerations apply, even in a single locality, with respect to the testing of persons (1) suspected of driving under the influence of a dependence-producing drug, including alcohol; (2) suspected of taking, or being in possession of, such drugs; (3) under some form of deprivation of liberty (e.g., civil commitment, probation, parole, in prison); or (4) not involved in any of these situations. Many of these considerations may be involved in the collection and disposal of body fluids and other tissues from deceased persons.

The Meeting was of the opinion that clinical, research, and other personnel involved directly or indirectly in various aspects of testing body fluids for the presence of dependence-producing drugs should familiarize themselves with the prevailing legal and other sociocultural controls affecting their work.

6. QUICK DIAGNOSTIC TECHNIQUES APPLICABLE IN ACUTE INTOXICATION

The Meeting noted that several types of pack (kits), mostly utilizing colorimetric methods, are available commercially for the detection of certain dependence-producing drugs. The packs contain the reagents and disposable items required for performing the test. Two types of pack can be obtained, (1) those suitable for detecting drugs in substance, and (2) those intended for testing urine for the presence of drugs. In principle both types can be used by comparatively unskilled personnel.

Since these tests may lack specificity, the packs should not be used for definitive testing but they may be useful in connexion with the detection of street drugs. Positive tests must be confirmed in the laboratory. Extreme caution should be observed in testing drug residues or gastric aspirates from an unconscious and possibly poisoned patient; substances still unabsorbed or already excreted are not necessarily the cause of the patient's condition.

7. SOME IMPORTANT RESEARCH AREAS

In the view of the Meeting, research is needed particularly in the following areas.

- (1) The development of suitable test procedures for demonstrating drugs in body fluids when no satisfactory method is currently available; such drugs include cannabis, lysergide, and naltrexone;
- (2) Investigation of the morphine-like substance reported to be excreted in the urine irregularly, and for a prolonged period, after the discontinuation of heroin use;
- (3) The correlation of drug use with impaired driving skill in relation to traffic problems;
- (4) The refinement of immunoassay methods, particularly the improvement of the antibody reagents;
- (5) Further investigations of the pharmacokinetics, drug-protein binding, and drug metabolism in relation to the development of tolerance to dependence-producing drugs.

8. INTERNATIONAL COOPERATION

The Meeting recognized the need for an international body with the following functions.

- (a) To coordinate the supply and distribution for reference purposes of (1) pure samples of drugs, (2) pure samples of drug metabolites; and (3) defined samples of biological fluids, possibly in a freeze-dried state, from drug users (but not "spiked" specimens);¹ the samples to be made available to laboratories undertaking tests for dependence-producing drugs.
- (b) To encourage the standardization of immunoassay reagents for drug detection.
- (c) To encourage the checking of the validity and reliability of simple and inexpensive methods.
- (d) To advise on, and coordinate, training facilities in order that appropriate practical experience can be provided for trainees.

¹ i.e., A sample of biological fluid, such as urine, with an added amount of standard substance to be tested.

(e) To encourage and coordinate arrangements for intensive practical courses of short duration ("workshops"), which would offer experienced staff opportunities to obtain practical experience of new techniques.

The Meeting believed that, in all of these areas, WHO could play a leading role.

9. CONCLUSIONS

(1) The proper application of suitable tests for dependence-producing drugs in body fluids is vital to the recognition of a drug problem in a population at risk.

(2) The management of problems of inappropriate drug use, whether in individuals or larger populations, should include the objective testing of specimens of a body fluid or fluids, though not to the exclusion of subjective assessment of symptoms and behaviour.

(3) The indiscriminate use of nonspecific testing procedures can not be accepted, particularly when the results of such tests might be used as the basis of legal action.

(4) There is a need for continued research, particularly in the areas defined in section 7.

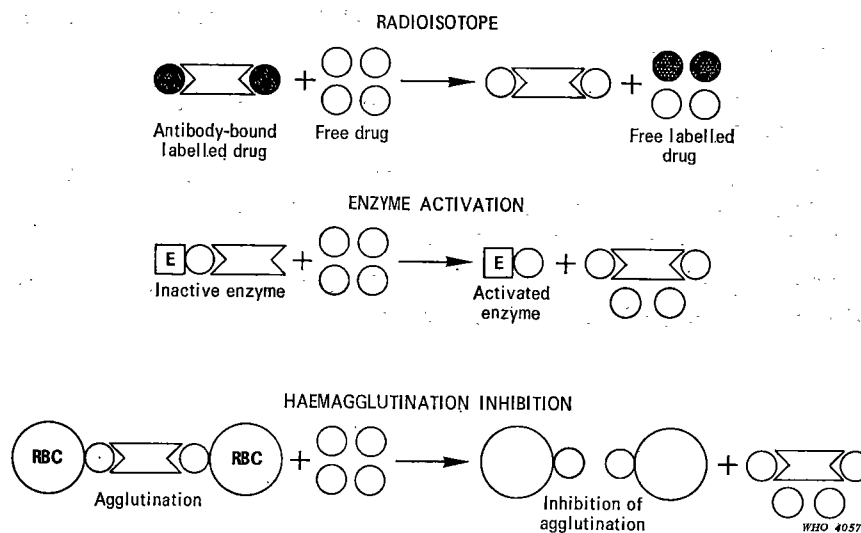
(5) International cooperation and collaboration is vital if public health and social problems arising from inappropriate drug use are to be recognized and progress in the management of such problems objectively monitored.

The Meeting expressed the hope that WHO would be able to undertake at least some of the activities involved.

HAEMAGGLUTINATION-INHIBITION (HI) TEST

This procedure is simple, sensitive, rapid, and inexpensive. Disposable glass and plastic items are needed but no permanent equipment other than a bench-type centrifuge. The principles on which the test is based are as follows. Erythrocytes, which serve as inert carriers, are coated with a drug such as morphine rendering them agglutinable by antibodies specific for the drug. If a limiting amount of antibody is used, agglutination is readily inhibited by adding free drug to the antibody solution prior to the addition of the coated erythrocytes (see Fig. 1). The minimum amount of morphine that can be detected by this test is determined by selecting appropriate dilutions of the antibody.

FIG. 1. SCHEMA FOR THREE IMMUNOASSAYS
(RADIOISOTOPE, ENZYME ACTIVATION, HAEMAGGLUTINATION INHIBITION)



In practice, this is carried out by means of a "chess-board" titration, in which serial dilutions of morphine in the range 0.4–100 ng/ml are incubated with several dilutions of the antiserum. The dilution of antiserum whose agglutinating activity is inhibited by morphine in concentrations of 3 ng/ml or greater can be used for urine analyses of specimens diluted

1 : 10, and morphine can be detected in urine if the drug is present in a concentration of at least 30 ng/ml.

The testing procedure is briefly as follows. A disposable haemagglutination tray is used for the titrations and drops of the sample and reagents are placed successively in the conical wells by means of disposable Pasteur pipettes : first, 1 drop (0.03 ml) of appropriately diluted antiserum, then 1 drop of urine diluted 1 : 10, and after 5 min 2 drops (0.06 ml) of the erythrocyte suspension. Urine specimens that contain more than the discriminating concentration (the cut-off quantity) of morphine will cause the red cells to settle in the same type of " button " that they would form in the absence of antibody. If the urine contains less than the cut-off amount of morphine, the cells agglutinate and settle in a distinct film like pattern. With incubation at ambient temperatures sedimentation is complete in 90–120 minutes.

Kits for detecting methadone and morphine are available commercially. These kits contain all the disposable supplies and reagents, required but economies can be made by purchasing antiserum and the protein–drug conjugates needed for coating the erythrocytes. Coated erythrocytes can be prepared with the aid of bis-diazobenzidine and are stable for 1 week at 4°C. The bis-diazobenzidine reagent is stable at –20°C for at least 2 months. Occasionally, lots of sheep erythrocytes fail to yield suitable cells. The preparation of coated indicator cells requires a degree of experience most readily obtained in a laboratory where the technique has become established. The following flow-sheet indicates the steps required. Data on various practical features of the HI test are given in Tables 1 and 2.

- (1) Sheep blood (citrated) is centrifuged to obtain the red cells.
- (2) The erythrocytes are washed 3 times with buffered saline¹ and a 5% red cell suspension in buffered saline is prepared.
- (3) The protein–drug conjugate and the red cell suspension are mixed, the bis-diazobenzidine reagent is added, and the reaction is allowed to proceed for 15 min at room temperature.
- (4) The coated cells are spun down in a centrifuge, washed twice in buffered saline containing 1% normal rabbit serum, and resuspended to form a 0.5% suspension in this medium. Thorough resuspension of the cells by pipetting at each step is essential.
- (5) If the coated cells are to be used on the days following their preparation, they are centrifuged at the start of the day's work and resuspended in fresh buffered saline containing 1% normal rabbit serum.

¹ Equal parts of 0.14 M sodium chloride and 0.15 M phosphate buffer.

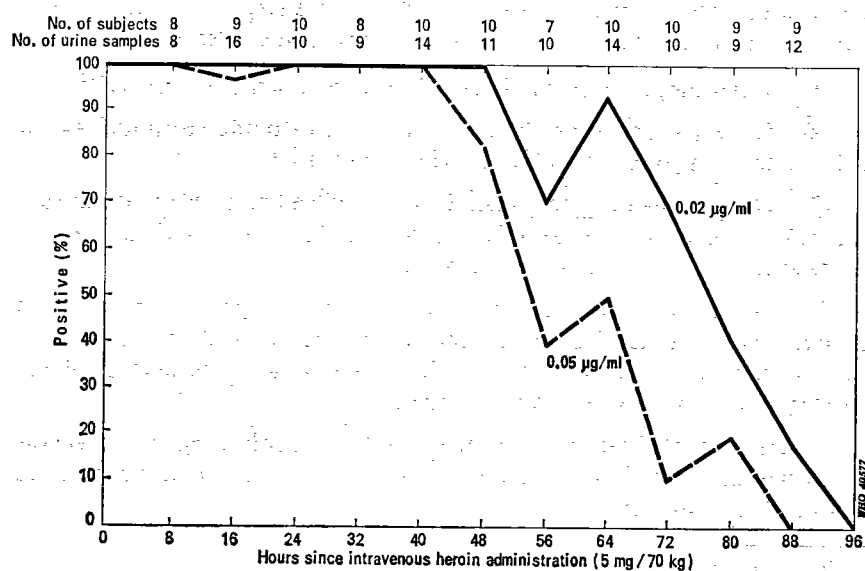
COMPARATIVE STUDIES OF METHODS FOR DETECTION OF HEROIN USE

An evaluation of the various parameters in one assay procedure and a comparison of several other methods have recently been undertaken in connexion with clinical studies on drug use. Some authors have tested random samples of different body fluids for drugs by various methods and compared the proportion of positive results, the ease with which the test is performed, and the time required (99, 100). However, the most important aspect of any test, and the most difficult to assess, is the reliability of the results in providing evidence of the use of a particular drug.

Studies of heroin users and former heroin users

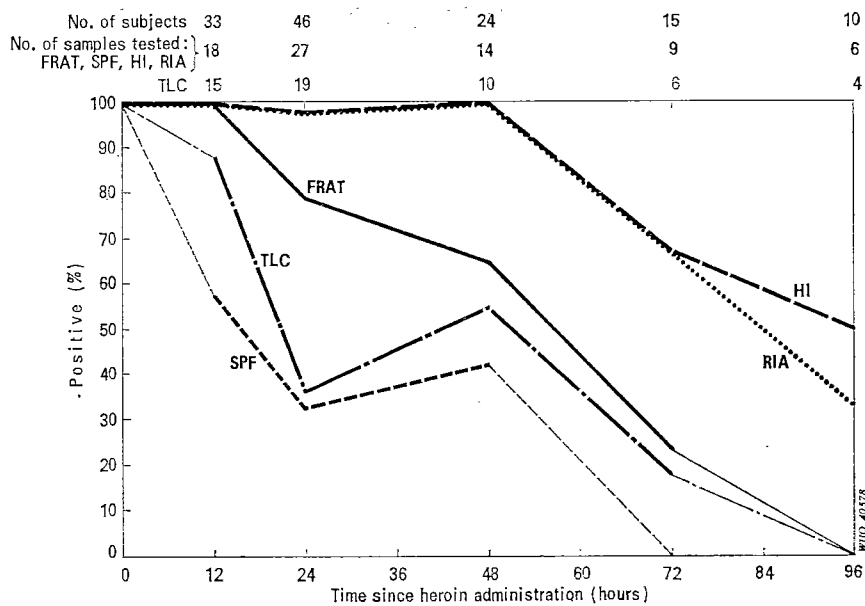
Healthy adult male former narcotic users received 2 single intravenous doses of heroin hydrochloride of 2.5 and 5.0 mg per 70 kg at weekly intervals

FIG. 2. DETECTION OF MORPHINE IN URINE BY MEANS OF RADIOIMMUNOASSAY WITH 0.02 $\mu\text{g}/\text{ml}$ AND 0.05 $\mu\text{g}/\text{ml}$ CUT-OFF LEVELS DURING THE FIRST 88 HOURS FOLLOWING THE ADMINISTRATION OF 5 mg OF HEROIN PER 70 kg OF BODY WEIGHT



in random order (101, 102). All urine produced by the volunteer subjects was collected for 1 week, each subject urinating whenever he wished and every eight hours. Each urine sample was analysed by several different methods. Radioimmunoassay gave 92-100% positive results for all periods up to 32 hours following the low heroin dose and 48 hours following the high dose. At 40 and 48 hours following the low dose and 56 and 64 hours following the high dose there were approximately 50% of positive results. The effect of utilizing different cut-off levels is shown in Fig. 2. With a 0.02 µg/ml cut-off, the detection time is extended by about 20 hours by comparison with a 0.05 µg/ml cut-off. In contrast, the same samples analysed by thin-layer chromatography gave positive results for a shorter time. Following either the 2.5 or the 5.0 mg heroin/70 kg dose, less than 50% of positive results were observed after 24 hours despite preliminary acid hydrolysis of the specimen. Gorodetzky (103) studied the excretion of morphine and metabolites following the intravenous administration of 6 and 12 mg of morphine per kg of body weight and obtained similar data.

FIG. 3. PERCENTAGE OF POSITIVE URINES AS A FUNCTION OF TIME SINCE THE LAST ADMITTED USE OF HEROIN. THE PERCENTAGE IS DETERMINED FOR EACH 12- OR 24-HOUR INTERVAL AND PLOTTED AT THE END OF THE INTERVAL ^a



^a HI, haemagglutination inhibition; RIA, radioimmunoassay; FRAT, free radical assay technique; TLC, thin-layer chromatography; SPF, automated spectrophotofluorometry. Reprinted by kind permission of the American Society of Clinical Pathologists from the *American Journal of Clinical Pathology*, vol. 60, p. 719 (1973).

Studies by Catlin (104) and Catlin et al. (105, 106) were concerned with the analysis of samples obtained from a population of heroin addicts. Each urine sample was divided into 5 subsamples and the 5 different methods of analysis shown in the following tabulation were employed (104). The minimum detectable concentration of morphine or morphine equivalents (sensitivity) is shown for each of the quantitative assays. Values greater or less than the sensitivity were considered positive or negative, respectively, in this study.

<i>Method</i>	<i>Sensitivity</i>
Haemagglutination inhibition	25 ng morphine equivalents/ml urine
Radioimmunoassay	25 ng morphine equivalents/ml urine
Free radical assay technique	500 ng morphine equivalents/ml urine
Automated spectrofluorimetry	200 ng morphine/ml urine

TABLE 3. THE PROPORTION OF URINE SAMPLES POSITIVE FOR MORPHINE OR MORPHINE EQUIVALENTS AS A FUNCTION OF THE ASSAY EMPLOYED, THE SENSITIVITY OF THE ASSAY, AND THE NUMBER OF HOURS ELAPSED SINCE HEROIN WAS USED *

Assay ^a	Sensitivity (µg/ml)	Percentage of positive samples for the following time intervals: ^b			
		0-12 hours (18)	13-24 hours (27)	25-48 hours (14)	49-96 hours (15)
RIA	0.025 ^c	100	96	100	53
	0.10	100	93	57	40
	0.50	89	78	43	0
	1.00	78	70	43	0
HI	0.025 ^c	100	96	100	60
	0.10	100	93	64	27
	0.50	94	85	50	13
	1.00	94	74	43	0
	0.50 ^c	100	78	64	13
TLC ^d	— ^e	87 (15) ^f	35 (20) ^f	50 (10) ^f	8 (12) ^f

* One sample was obtained from each of 74 known chronic heroin users. Hours elapsed were assessed by questioning the patient.

^a RIA, radioimmunoassay; HI, haemagglutination inhibition; TLC, thin-layer chromatography.

^b The number of individuals studied and samples obtained and analysed during each time interval is shown in parentheses.

^c Lowest recommended sensitivity.

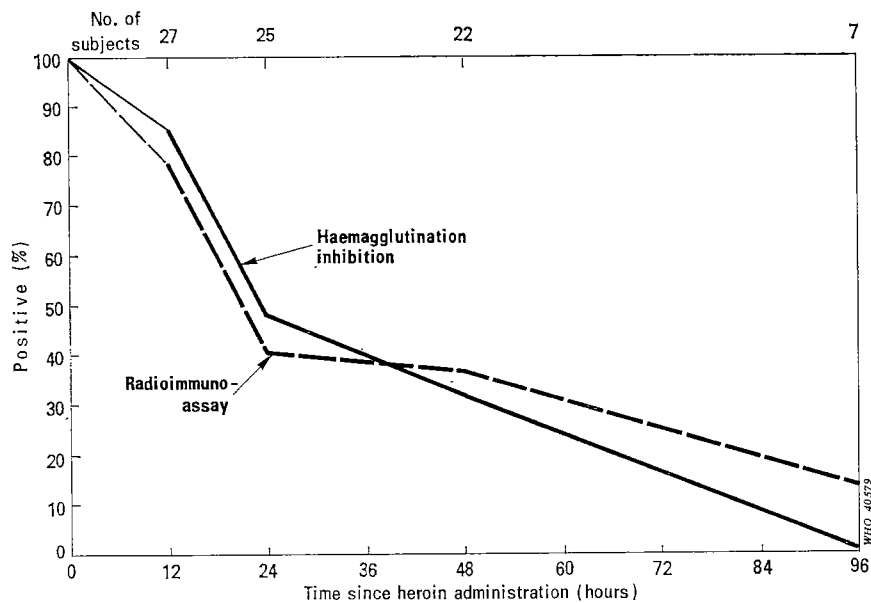
^d The TLC procedure included an organic extraction but not a hydrolysis.

^e The TLC sensitivity is estimated to be 0.50-1.9 µg/ml on the basis of a 30 ml specimen.

^f The number of samples analysed by TLC is shown in parentheses; the number is less than that for other assays on account of inadequate sample volumes.

Fig. 3 shows the proportion of urine specimens containing morphine equivalents as a function of the reported number of hours since the last dose of heroin. Fewer samples were tested by thin-layer chromatography than by the other methods because the volume of the sample was too small. The three immunoassays gave positive results for all samples obtained from patients who reported using heroin during a period of up to 12 hours before the sample was obtained, and one sample tested by thin-layer chromatography and several by spectrofluorimetry gave negative results. During the period 0-48 hours after administration of heroin the radioimmunoassay and haemagglutination inhibition tests gave positive results for all but one sample; after 12 hours, the proportion of urines reacting positively to the other methods declined. The two assays that detected free morphine only (thin-layer chromatography and spectrofluorimetry) did not detect morphine in a number of specimens obtained during the 0-24 hour period after heroin administration.

FIG. 4. PERCENTAGE OF POSITIVE SERA AS A FUNCTION OF TIME SINCE THE LAST ADMITTED USE OF HEROIN. THE PERCENTAGE IS DETERMINED FOR EACH 12- OR 24-HOUR INTERVAL AND PLOTTED AT THE END OF THE INTERVAL ^a



^a Sensitivity: haemagglutination-inhibition, 10 ng morphine equivalents/ml; radioimmunoassay, 10 ng morphine equivalents/ml.

The results from the analysis of one urine sample obtained from each of 74 individuals are summarized in Table 3. For all assays the proportion of positive results decreases as a function of time elapsed since administration and is dependent on the cut-off level. However, at the 0.025 $\mu\text{g/ml}$ cut-off level the proportion of positive results by radioimmunoassay and HI test is 96-100% during the initial 48 hours, but it subsequently declines sharply. In contrast, the proportion of positive results by spin-labelled immunoassay and thin-layer chromatography fall to 78% and 35% respectively, during the 13-24 hour period.

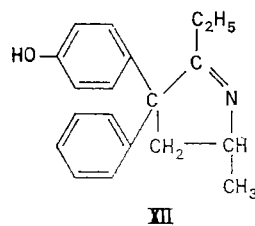
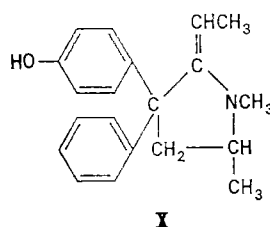
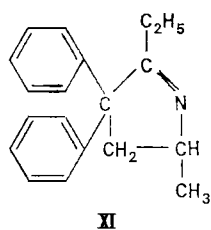
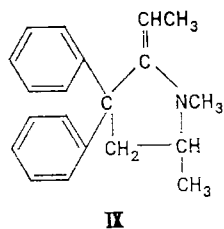
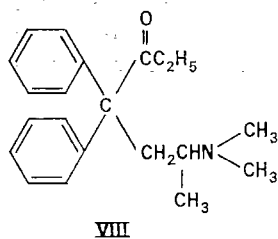
The effect of altering the sensitivity of the radioimmunoassay and HI tests are shown in Table 2 (104). For the population of addicts studied, a cut-off level of 0.025 $\mu\text{g/ml}$ was required in order to achieve 95% of positive results for a 48-hour period. A cut-off level of 0.10 $\mu\text{g/ml}$ still gave 90% of positive results.

The radioimmunoassay and haemagglutination inhibition assays were used also to quantify morphine equivalents in serum obtained from the addicts at the same time as the urine was collected. Fig. 4 shows the proportion of positive results for serum samples as a function of the probable elapsed time since heroin administration. In contrast to the urine data, the proportion of samples giving positive results is significantly less during the 0-12 hours period and continues to decline. For detecting recent heroin abuse urine analysis is superior to serum analysis.

METHADONE

Metabolism and elimination

Methadone (VIII) is extensively metabolized in man. The products of mono-N-demethylation (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (IX) and di-N-demethylation (2-ethyl-2,3-dihydro-5-methyl-3,3-diphenyl-1*H*-pyrrole) (X) have been identified in human urine (29-35). In 1972 the product of N-oxidation, (107) and *p*-hydroxy pyrrolidine (XI) and *p*-hydroxy pyrrole derivatives (XII) (107) were found in urine obtained from methadone-treated subjects. The relative predominance in subjects receiving 80 mg of methadone daily was : IX > XII > VIII > XI > X (108). The



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same group also found a small amount of a compound with chromatographic and mass fragmentation patterns identical with a known sample of α -[2-(dimethylamino) propyl]- α -phenylbenzeneacetic acid. Substances IX and X have been tested in the rat tail-jerk assay and found to be devoid of pharmacological activity (30). None of the other metabolites have been tested for pharmacological activity.

Several qualitative and quantitative investigations of the metabolism and excretion of methadone in man have recently been made. There are no reports of metabolites in plasma or blood (34). However, a metabolite has been found in blood taken at autopsy (109). Sweat and urine were collected immediately before a 70 mg dose of methadone was administered to 5 subjects who had been maintained on 70 mg daily for 3–5 months (110). Methadone was found in the sweat of all 5 subjects (maximum, 2.6 μ g/ml) and was present in sweat at higher concentrations than in urine in 3 out of the 5. Substance IX was present in 4 sweat samples at concentrations equal to, or lower than, those in urine. The ratio of substance X to methadone in urine ranged from 1 to 5.5. Substance X was present in the sweat and urine of 3 subjects (110). Despite the lack of a clear pattern of excretion in sweat, the quantity excreted in this manner may be considerable.

The renal excretion of methadone and substance IX was examined in subjects maintained on a wide range of doses for at least 3 weeks prior to study (33). The samples were collected immediately before the daily drug dose. The proportion of the total dose recovered in 24 hours and the urine concentrations of methadone and substance IX were dose-dependent. At doses of 50 mg approximately 50% of the drugs can be recovered as methadone and substance IX, while at doses above 100–120 mg virtually 100% recovery is achieved (33). (Other investigators found only 13% recovery in 1 subject receiving 80 mg daily (34).) The ratio of substance IX to methadone was relatively independent of dose but was correlated with the urine pH and sex of the subject (33), the ratio increasing with higher urinary pH in both males and females. For the same increment in pH the increase in ratio was considerably greater for females than for males. The same investigators (33) found that in a naive male subject receiving a 5 mg oral dose of methadone the rate of excretion was pH dependent for methadone and flow dependent for substance IX. Acidification of the urine (by the oral administration of ammonium chloride) resulted in a 4-fold increase in methadone excretion and little change in the excretion of substance IX. The dependence of methadone excretion on urinary pH has been confirmed in tolerant (111) and naive (112) subjects. The urinary substance IX : methadone ratio may be dependent on the route of administration in naive subjects. Intramuscular administration was found to result in ratios of 0.72, 0.21, and 0.22 (35), and after oral administration

ratios of 1.0–2.2 have been reported (111). Studies of subjects chronically maintained on oral methadone indicate urinary substance IX : methadone ratios in excess of 1.0 (33, 35, 110) except that one subject had a ratio of 0.31 (34). There is no consistent pattern in the excretion of substance X and the other metabolites have not been examined systematically.

Plasma levels

Plasma levels of methadone have been determined in naive (35, 112) and methadone-maintained subjects (34, 36, 113, 114). Inturrisi & Verebely (35) administered 10 mg of methadone intramuscularly in 3 subjects and noted the peak plasma levels—namely, 0.060, 0.065, and 0.095 $\mu\text{g/ml}$, at 1 hour and estimated half life values of 7.6, 6.7, and 7.5 hours from semi-logarithmic plots (the last sample was collected 6–10 hours after the administration of methadone). In a subsequent study (112) 5 naive subjects received 15 mg orally and plasma samples were obtained for the next 24 hours. The main peak plasma level of 0.074 $\mu\text{g/ml}$ occurred at 4 hours and declined to a mean level of 0.029 $\mu\text{g/ml}$ at 24 hours. The apparent half life was determined for each subject by means of a method for experimental curve analysis and showed considerable variation : 10, 12, 16, 18, and 18 hours.

Inturrisi & Verebely (113) determined plasma methadone levels in 5 subjects maintained on single daily oral doses of 100–120 mg for 6 weeks to 1 year. One sample was obtained from each subject at several points in time. Considerable variation in the range of plasma levels was found : pre-dose levels ranged from 0.20 to 0.39 $\mu\text{g/ml}$, peak levels from 0.51 to 1.08 $\mu\text{g/ml}$, and 24-hour (pre-dose) from 0.28 to 0.39 $\mu\text{g/ml}$. The peak level occurred between 2 and 4 hours after administration. The mean apparent half life determined by a least squares method for nonlinear regression was 25 hours, with a range of 13–47 hours. Similar plasma levels were found in one subject studied by Sullivan & Blake (34). Dole & Kreek (114) studied 9 subjects who had been receiving methadone for 1–5 years and who received 100 mg per day for the 8 weeks of study. At 2-weekly intervals the plasma levels were determined 24 hours after the last dose and 2 and 6 hours after the subsequent one. The 24-hour level for all subjects averaged 0.58 $\mu\text{g/ml}$ and analysis of variance showed no difference in the level in each subject over 8 weeks, and no difference in the values obtained at 2 and 6 hours. The average difference between the 24-hour level and the higher of the 2 and 6-hour levels was 0.33 $\mu\text{g/ml}$. From this data the plasma half life was estimated to be 24 hours. Verebely & Kutt (36) found daily variations of $\pm 8\%$ of the mean pre-dose plasma methadone level in 3 reliable methadone patients. If a single daily dose was omitted the plasma level (probably 48 hours after the last dose) declined by 35–85% of the mean level (36).

Methods of analysis

Methadone may be estimated by spectrofluorimetric techniques (15) although, compared with other methods, spectrophotometric methods lack sensitivity and specificity. Routine qualitative tests for methadone are most commonly performed by thin-layer chromatography (39), which may also be used to demonstrate methadone metabolites. Gas chromatography provides a more sensitive and specific estimation of methadone and its metabolites (30, 112). Methadone and its metabolites can be estimated easily by the most specific procedure—namely, gas chromatography-mass spectrometry. Recently an immunoassay was described for methadone (115).

BARBITURATES

The duration of pharmacological action of the barbiturates may be correlated with their rate of metabolism. Thus phenobarbital, whose action is of long duration is more slowly and less extensively metabolized than the shorter acting secobarbital, which is rapidly oxidized and excreted. The metabolic pathways include oxidation of the larger of the alkyl substituents at carbon atom 5, yielding alcohols, ketones, or acids; the urinary excretion of glucuronic acid conjugates and the free metabolites as well as the unchanged drug may follow administration. N-alkylated derivatives of barbituric acid may undergo N-dealkylation; ring fission is not a significant metabolic pathway.

The following data for specific substances relate to adult volunteer subjects.

Plasma levels and urinary excretion data

Phenobarbital. The biological half life of phenobarbital in plasma was reported as 3½ days by Ravn-Jensen et al. (116). Up to 50% of the dose may be recovered from the urine, about two-thirds being excreted as 4'-hydroxyphenobarbital.¹ Excretion may be detected for at least 6 weeks after large doses of the drug.

Amobarbital. A single 200 mg oral dose of amobarbital was found (117) to have a plasma half life of 16.4 ± 3.1 hours when a gas chromatographic assay technique was used with a mass spectrometer as detector. This value correlated well with the 14.9 ± 4.6 hours as determined with a conventional flame ionization detector. After 20 hours the plasma contained approximately 3 µg amobarbital and 0.5 µg 3'-hydroxyamobarbital²/ml.

Five volunteers each given a 200 mg oral dose (118) excreted approximately 14% of the dose as 3'-hydroxyamobarbital in the urine during 24 hours and about 40% during 6 days. Less than 1.5% of the dose was excreted in the urine in 24 hours and a total of up to 3.5% only during 6 days.

Pentobarbital. Intravenous infusion of 50 mg of pentobarbital during 5 min gave plasma levels of 1.2 µg/ml initially, falling during 1½ hours to about 0.5 µg/ml (119). The plasma half life after the 4-hour distribution

¹ 5-ethyl-5-(4-hydroxyphenyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione.

² 5-ethyl-5-(3-hydroxy-3-methylbutyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione.

period was about 50 hours. The same dose administered orally to the same patients, fasted, resulted in peak plasma levels of about 0.7 µg/ml after 1 hour.

Urinary excretion of the drug occurs mostly as hydroxyderivatives, 40–50% of a 500 mg oral dose being excreted in 48 hours and up to 80% during 96 hours (120).

Secobarbital. Maynert (121) reported the urinary excretion of 50% of administered secobarbital as secodiol and 5% as unchanged secobarbital during 48 hours following an oral dose of 2 g.

Nealbarbital. A single 300 mg dose of nealbarbital administered to volunteers resulted in the urinary excretion of 5-(2,3-dihydroxypropyl)-5-(2,2-dimethylpropyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione (nealbarbitone diol) which accounted for 30–40% of the dose (122). Peak excretion of this metabolite occurred at about 36 hours but continued for at least 4 days. Unchanged drug was not detected and none of these metabolites containing carboxylic acid groups were present at concentrations greater than 1.5 mg per 100 ml of urine.

THIN-LAYER CHROMATOGRAPHY OF DEPENDENCE-PRODUCING DRUGS

The thin-layer chromatographic technique, originally described by Stahl (123), is the most common and widely used method for the detection of drugs and metabolites in biological materials. Essentially, this technique is applied after extraction of the drug or drugs from biological materials either by solvent-solvent extraction, ion-exchange, or XAD-2 resin adsorption followed by solvent elution (124-130).

The following typical method may be used to analyse urine for drugs of dependence. Apply 25 ml of urine (usually) to columns containing XAD-2 resin. The flow-rate of urine through the columns should not exceed 20-25 min. Columns should be transferred to 50-ml tubes containing 1 ml of 0.1 N HCl and eluted with 10 ml of isopropyl ether. Shake the tubes; the two phases separate on standing. Remove the upper ether phases, transfer to clean tubes, and evaporate to dryness. The residues contain primarily acidic and neutral drugs. The XAD-2 columns are now be placed on the *original* tubes containing the aqueous acidic layers and eluted with 20 ml of chloroform-isopropanol mixture (3 : 1) in two equal 10 ml portions. Add 1 ml of 0.12 M borax solution to each tube (pH 8-9.5), shake, and remove the upper aqueous phases. Add 0.2 ml of 6 N HCl (in methanol) to each tube and evaporate to dryness. The residues contain basic and some neutral drugs.

The extracts containing the *acidic* and neutral drugs may be dissolved in methanol (50 μ l) applied to 0.25 mm "polygram" silica gel thin-layer sheets. These sheets should be developed in chloroform-methanol-ammonia mixture (90 : 10 : 1 v/v). Dry the sheets and then spray with 0.25% mercuric sulfate in 4 N sulfuric acid followed by 0.1% diphenylcarbazone in chloroform (to detect barbiturates). Air-dry the sheets and spray with 100% furfural in ethanol (to detect carbamates).

The extracts containing the *basic* and neutral drugs may be dissolved in 50 μ l of methanol and applied to 0.25-mm silica gel thin-layer plates and developed in ethyl acetate-methanol-water-ammonia (85 : 10 : 3 : 1 v/v). Dry the plates and spray with (1) 0.3% ninhydrin in 0.17 N acetic acid, then heat to 100°C for 5 min in an oven (for secondary amines); (2) 5% sulfuric acid and heat in hot air for 5 min (for phenothiazines and intensified quinine fluorescence); (3) iodoplatinate reagent (for alkaloids and narcotic analgesics, and organic bases in general); and (4) *p*-nitroaniline (prepared by

mixing 2.5 g of *p*-nitroaniline in 250 ml of 1 N HCl and diluting to 500 ml with ethanol. Before use, mix 20 ml of 5% sodium nitrate with 100 ml of *p*-nitroaniline ; cool and use when cold) ; spray heavily with 25% alcoholic sodium hydroxide (for primary amines, i.e., amphetamine).

The use of these techniques should result in the recovery of 80-100% of acidic and basic drugs from biological fluids.

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