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THE NECESSITY OF ELUTION AND IDENTIFICATION OF DRUGS INDICATED BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The existence of drugs found in human urine by extraction and thin-layer chromatography require further verification, not only because the great number of drugs now in use complicates identification from R_F values but also because the R_F values obtained from the crude extracts that are chromatographed are inexact and inconsist. Thus chromatography is essential for separation but insufficient for identification. The procedure for extraction and thin-layer chromatography, as well as the procedure for elution, is given. Elution from the thin-layer plate after spraying has previously posed some difficulties, but a simple shake-out from a basic slurry has now been found very satisfactory. Tests for final identification are then given. Color and microcrystal tests, aided when necessary by UV readings, are discussed for morphine, quinine, methadone, propoxyphene, chlorpromazine, nicotine, trimethobenzamide and procaine, on the basis of actual findings in urine samples.

INTRODUCTION

In recent years the necessity for analysis of drugs in human urine has expanded enormously. The most popular method of distinguishing the extracted basic drugs is thin-layer chromatography (TLC); however, the outstanding feature of this method is that the drug's identity requires verification.

First, hundreds of basic drugs cannot possibly be distinguished, one from other, simply by the positions (R_F) of their spots on the chromatogram in a short space of 10 cm, or even in a much longer distance. Only about 20 different positions really can be distinguished from each other on the usual thin-layer plate. The habit of reporting R_F values to hundreds, as 0.63 for example, conceals this fact, in part, only too well; but even if such R_F values were exact, they would account for only 100 positions. The overstated precision leads to laxity in interpreting R_F positions. The appearance of the spots helps somewhat, but not enough for any certainty; the spots, sprayed with platinic iodide, yield different colors for different substances, to a certain extent, which provides additional distinctions between the values. However, the colors are chiefly due to the color of the reagent and mostly have only a limited gamut. Therefor the identity at a particular R_F position cannot be settled decisively.

Secondly, because the crude extracts are actually chromatographed, the R_F value of a particular substance varies considerably in different cases, as does the color of its spot. Some analysts, even when searching chiefly for morphine, extract all the bases together without any real purification, and then spot them together with extractable impurities on one plate. Even with better extractions, the analyst soon finds much variability in the chromatographic results. He therefore begins regarding almost any spot similar to the right color and in the vicinity of the proper R_F value as representing what he is looking for. This assumption is unharmful if a real identification is made thereafter, but not if a mistake goes unnoticed perhaps until the analyst is called to account for his work.

The great drawback is the practice of accepting any sort of "positive" result as an affirmation of the existence of the substance that one is expecting or looking for. When an additional or "confirmatory" test is made, it must be very specific, not a reaction that will elicit a "positive" response from hundreds of compounds — for example, a chemical reduction reaction for the "confirmation" of morphine. Most confirmatory tests made by spraying with a different chromatographic reagent are in this general category. An additional disadvantage is that a response may be due to two or more substances in the same spot. Multiple spraying, in itself a confession of the fallacy of identification made after spraying once, may enhance the guesswork but not provide a real identification. Sometimes chromatographic results are reported only as "positive" and not as R_F values that can be confirmed.

In 1954, MANNERING *et al.*[4] reported on their careful identification of morphine in urine samples by paper chromatography, verified by color and microcrystal tests. From 1103 urine samples run, morphine was identified beyond doubt in 412. Of 601 morphine-negative samples, 36 gave spots at the R_F position for morphine, but most of these spots were from the wrong extract or were off-color or very feeble. The eluates of five morphine-positive spots verified, after derivation from the proper extract, from the R_F value and by the color of the iodoplatinate spot, did not give positive color tests with H_2SO_4 reagents and were not morphine. The authors conclude that while this error represents but a small percentage of the total determinations, "it points out the error that could result from drawing final conclusions from the chromatographic data alone". TLC is not likely to be more accurate now considering the great increase in the number of drugs since 1954 and sometimes the lack of a separate extraction before chromatography.

TLC or some other form of chromatography is usually quite indispensable for separation of constituents in a mixture and for good indication of what substances are present, but the results obtained from TLC of crude extracts should not be mistaken for real identification of the substances present. The examination of human urine for addictive drugs is not primarily a statistical matter but one of individual concern, and chemists should make completely certain that no mistakes can be attributed to the chemistry used.

EXPERIMENTAL

Extraction and TLC

Urine is extracted first with ether from acid, then with chloroform from an alkaline solution, then reacidified, hydrolyzed, adjusted to pH 8.5, and re-extracted

with chloroform–isopropanol (primarily for morphine). The two basic extracts from pH 11.0 and pH 8.5 are chromatographed separately. They are spotted, together with appropriate standards, on commercial thin-layer plates, 250-μ thick, and developed with a solvent of ethyl acetate–ammonium hydroxide–methanol (85:5:10) or ethanol–acetic acid–water (240:120:40), air dried and then sprayed lightly with 10% acetic acid, placed under UV light to check for fluorescent areas which are outlined with a pencil. The plate is then air dried to remove excess acetic acid and subsequently sprayed with platinic iodide solution. The sprayed plates are air dried and allowed to stand for 1 h or more, and a spot to be further investigated or verified for a particular substance is scraped into a beaker. Weak morphine spots have been observed to develop even after 4–18 h at room temperature.

The details of the foregoing procedure may be varied. The present article is concerned primarily with the elution and its relation to verification. We do recommend, however, that the three separate extractions be made carefully, and attempts to simplify the procedure too much will only cause difficulties and doubts later.

Elution

In a previous article[1] a simple methanol elution of a thin-layer spot was given. Microcrystal tests were then applied to the eluates. This has been useful (particularly for verifying morphine) but some objectionable features were soon recognized. Methanol elution is somewhat tricky for recovery of the drug and, in any case, methanol dissolves iodide from the spray which interferes with many of the best color and crystal tests. From paper chromatograms, elutions are made with a small volume of borax–Na_2SO_3 solution and 25 ml of chloroform which recovers the free base. This is far more satisfactory. Various attempts have been made to elute similarly the thin-layer spots, but the results seem to be unsatisfactory.

Recently, prolonged trouble with the eluates of methadone spots, even with the control spots, prompted a new attack on this problem. The results were so successful that it is somewhat difficult to understand exactly what the obstacles were. The methanol elution is not really as simple as it seems. The scraped material is digested with only 1 or 2 ml of methanol for a few moments while warming or heating in the steam bath; then the methanol is filtered into a 5-ml beaker. This treatment is usually repeated twice, and the filter is finally washed down with a little methanol. The separatory funnel extraction now proposed is not more difficult, even for routine, if a sufficient supply of clean, small separatories is kept at hand and is not particularly novel chemically. However, we feel that others should be advised of the successful elutions from TLC plates and reminded of the importance of verification of TLC spots.

The difference in the eluates is enormous. If the substance causing the spot is uncertain, the eluate may be first run in quite dilute HCl, or in alcohol, on the UV spectrophotometer, and the solution then again evaporated. If the substance is suspected, chemical tests may be applied without using an intervening UV procedure. Both color and crystal rests may be used, if the amount (judged by size and intensity of the spot) is more than minimal. If it is nearly minimal, the one best crystal test is generally sufficient verification of the identity, when successful, if the previous chromatographic indication was good.

Elution procedure

As a routine procedure in our laboratory, we transfer the scrapings of an out-

lined TLC spot into a 5- or 10-ml beaker, digest it with 5 ml of ammoniacal solution A or B, and transfer the suspension into a 120-ml separatory funnel. Scrapings remaining in the beaker are resuspended with an additional 5 ml of ammoniacal solution A or B and combined with the previous suspension. A second beaker, preferably an 8-ml "hollow stopper", polyethylene beaker properly labeled (case number, pH of extraction and R_F) is filled with the solvent of choice (ether, chloroform or chloroform–isopropanol) and poured into the separatory funnel. Contents are shaken for about 1 min. The organic layer is filtered into the properly labeled polyethylene beaker and evaporated to dryness. Specimens are now suitable for UV spectrophotometry and chemical color and microcrystal tests.

Reagents

Ammoniacal solution A is used primarily in association with TLC spots suspected of being morphine, morphine-like drugs, or weak bases. It is prepared by adding 0.82 ml of conc. ammonium hydroxide A.R. (58 %) to 400 ml of distilled water. One gram of sodium carbonate is added and brought to a volume of 500 ml. pH of solution should be checked and verified to be 8.5 to 8.8

Ammoniacal solution B is used for other basic drugs. It is prepared by adding 10 ml of conc. ammonium hydroxide A.R. (58 %) to 500 ml of distilled water. pH should be checked and verified to be 11.0.

Ether (ethyl ether anhydrous A.R.) is the solvent of choice for methadone and quinine. Chloroform (ACS) is a general solvent for all basic compounds. Chloroform–isopropanol (3:1) is used for morphine.

Identification (verification tests)

With the base reseparated by elution with solvent extraction, any test sufficiently sensitive may be applied. For numerous basic drugs, suitable crystal and color tests have already been given[2,3]. The best tests for some of the most important drugs will be reviewed here. The eluate is dissolved in about 0.02 ml of 2 % acetic acid. Little droplets of the solution are taken for tests.

To test for morphine, a little droplet of solution is evaporated on a spot plate and tested with the Ferreira reagent (conc. molybdate in $H_2SO_4 \cdot SO_3$) (ref. 2). With morphine, this gives an intense dark purple color which fades after a few minutes. This is exceedingly sensitive, and if the purple color is not produced, morphine is not present. If the test is positive, final proof of morphine is obtained by testing another droplet of the solution (or dilution of it, if the presence of much morphine is indicated) with aq. K_2HgI_4, observing the result microscopically[1,2]. K_2CdI_4 may also be used. Other substances have been found in spots at the R_F position of morphine, particularly one temporarily designated as Unknown N.J. No. 1, the amount of which may be fairly large. With I–KI reagent M-2, it gives sizable dark red isotropic grains. Sometimes morphine is present along with the other substance; sometimes not. With the methanol elution, the convenient color test could not be used. So far, we have identified morphine with certainty in more than a 100 specimens of urine collected from addicts.

Quinine is one of the most obvious substances on the chromatogram, since its spots are fluorescent. If an ammoniacal solvent has been used, the dried plate may first be sprayed with diluted acetic acid, the fluorescence observed and the plate dried

again, then sprayed with platinic iodide solution. This is not as objectional as spraying with diluted sulfuric acid, which dries down to concentrated acid capable of causing many changes in substances. However, these are certainly pitfalls in assuming a fluorescent spot, anywhere in the neighborhood, to be quinine. It might be quinidine, which in general will react just like quinine, except to the microcrystal tests; in fact what was supposed to be quinine in toxicologic cases has several times been found to be quinidine at the Chief Medical Examiner's in New York City. The quinine is most readily proved with H_2PtBr_6 in (4 + 1) HBr. More subject to interference with impurities, but even more sensitive, is the I–KI reagent Q-6. If the amount is not too small, I–KI reagent C-3 may be used for herapathite crystals[2]. This is certainly more proof than necessary.

Methadone travels very near the solvent front with the chromatographic solvent mixture that has been chiefly used here. Numerous impurities and several common drugs collect at the solvent front. Addicts admitted to the Drug Abuse Clinic here are receiving methadone. This makes methadone a logical suspect for a spot near the solvent front, but it has been possible to prove it only in a minority of cases of noticeable spots. One of the "impurities" may be a metabolite of methadone, but in several cases, the spot is definitely due to a different drug; there has been confusion with propoxyphene and chlorpromazine. In two other cases, a definite test was obtained, but the substance was not identified. The eluted methadone may be identified by testing the dry substance (the deposit on a microscope slide left by evaporation of a droplet of solution) with I–KI reagent Q-6 (ref. 1). The X-crystals are highly characteristic. If the amount is not too small, a good color test can be obtained with Mandelin reagent (vanadate in H_2SO_4) (ref. 2).

The base propoxyphene also travels near the solvent front with the chromatographic solvent used most here, and has been confused with methadone although the colors of the spots of the pure bases are different. Crystal tests for propoxyphene are difficult unless it is very pure or present in substantial quantity. It may be proved by three very sensitive reactions having different colors: Ferreira reagent, black; H_2SO_4 reagent C-2, purple-violet changing to black; and M/20 reagent (Marquis reagent diluted 20 times with H_2SO_4), purple[2].

Propoxyphene sometimes occurs at a lower R_F. The principal metabolic change is the loss of one methyl group from the nitrogen, thus probably not affecting the color tests which depend on another part of the molecule. However, a large spot of R_F 0.65, when rechromatographed, went up to R_F 0.93, showing that it was actually unchanged propoxyphene. The eluate gave the proper color tests. It is obvious that a spot due to propoxyphene may be taken for something else if identification is based chiefly on the R_F value or position of the spot. In any case, the substance should not be called dextropropoxyphene (Darvon) unless it is actually proved to be the dextro isomer, since levopropoxyphene (Novrad) is on the market. An isomer can be distinguished by microcrystal tests if recovered pure enough and if controls are available to form the racemate[2]. The method is due to the work of CLARKE.

One supposed methadone spot was identified as certainly a phenothiazine and almost certainly chlorpromazine by $HAuCl_4$ in HOAc-4 (1 + 1) H_2SO_4 added with coverglass to the dry substance. This test could not have been used on an iodide-containing methanol eluate. With the eluted substance, and likewise with known chlorpromazine, it gave a red color and dichroic crystals, colorless to pink. A few

other phenothiazine drugs are somewhat similar, but a good chlorpromazine result can be distinguished. In the particular case, there was insufficient sample for an additional test.

Nicotine is a common substance in the urine of smokers, often in surprisingly large amounts. The nicotine spots are usually not of interest except that the analyst must be reasonably sure that they are due to nicotine and not to a drug that may appear at the same position, with or without nicotine. Therefore, at times, the nicotine should be identified beyond doubt. Nicotine may be tested by volatilizing it either into a reagent drop or into a drop of dilute HCl, which is thereafter tested[2]. However, if elution is made with solvent extraction, the eluate may be advantageously tested directly and the tests will show if a spot is entirely or chiefly something else (but some degree of impurity of the nicotine is to be expected). The eluate is dissolved in a small drop of (2 + 1) HOAc, a droplet is transferred to a plain slide, and two successive dilutions are made on the slide with (2 + 1) HOAc. To the most dilute droplet, a droplet of $HAuBr_4$ in HOAc–(2 + 3)H_2SO_4 is added, and is allowed to stand without a coverglass. The nicotine bromaurate crystals are unmistakable[2]. If there is not any satisfactory result, the next stronger solution or finally the most concentrated of the three droplets is tested. If the most dilute solution shows the nicotine, the dry deposits of the next may be tested with I–KI reagent M-2, with a coverglass, and a fairly concentrated deposit with I–KI reagent N-2 or $HAuBr_4$ in (2 + 1) HOAc[2].

Trimethobenzamide is a drug recently identified here for the first time in a urine sample. In this case, we were told that amongst 3 or 4 substances mentioned as previously taken, a newly admitted addict was said to have been using Tigan (trimethobenzamide). The UV curve of the eluted thin-layer spot was seen to correspond to this substance. This finding was confirmed by 3 different color tests: with conc. molybdate in H_2SO_3, purple changing to violet; with nitrite in H_2SO_4, persistent purple; with conc. HNO_3, quick development of a purple color which changes to brown, then to yellow. Molybdate in fuming H_2SO_4 gives still a fourth distinct color test. No microcrystal test has yet been found (Reagents, 2).

The eluate of a completely unknown spot was recognized as procaine by a test with $HAuBr_4$ in HOAc-3 (2 + 3) H_2SO_4, added with coverglass to the dry substance, which gave large serrate red blades. The round forms in the aqueous test with H_2PtCl_6 were also obtained. These tests probably would not even have been tried on the methanol eluate because both these reagents react with iodide. Procaine has since been found in three other cases, and the UV curve has also been used in the identification. The subjects were newly admitted to the Drug Abuse Clinic. Procaine is the major adulterant of cocaine and a minor adulterant of heroin, but in these cases, its origin is not known to us. It is, of course, sometimes injected by a dentist. The case history, in one case, suggests the possibility that addicts were sold procaine and told it was Numorphan.

REFERENCES

1 M. Ono, B. Engelke and C. Fulton, *Bulletin on Narcotics*, 21, No. 2 (1969) 31.
2 C. C. Fulton, *Modern Microcrystal Tests for Drugs*, Wiley, New York, 1969.
3 E. G. C. Clarke (Editor) *Isolation and Identification of Drugs*, Pharmaceutical Press, London, 1969.
4 G. J. Mannering, A. C. Dixon, N. V. Carroll and O. B. Cope, *J. Lab. Clin. Med.*, 44 (1954) 292.