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IMPROVED COST EFFECTIVE THIN-LAYER DETECTION TECHNIQUES FOR ROUTINE SURVEILLANCE OF COMMONLY ABUSED DRUGS IN DRUG ABUSE URINE SCREENING AND PROFICIENCY TESTING PROGRAMS WITH BUILT-IN QUALITY ASSURANCE

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SUMMARY

Improved, sensitive, reliable and cost effective thin-layer detection procedures for poly-drug usage (12–15 drug evaluations per specimen) are presented. Extraction procedures using ion-exchange resin loaded paper and liquid–liquid extraction with the built-in quality assurance program are reported. The combined use of ninhydrin–fluorescamine detection reagent for the identification of various central nervous system stimulants is only one of the several modifications and improvements made during the past 14 years. Laboratories, participating in proficiency testing programs in drug abuse toxicology monitoring are encouraged the use of proposed extraction and identification techniques.

INTRODUCTION

We have been strong proponents of using reliable, inexpensive and sensitive thin-layer detection techniques for routine monitoring of urines of individuals attending drug abuse prevention and treatment programs. Extraction and detection procedures currently used in the field of drug abuse toxicology were reviewed by Kaistha[1,2] in depth elsewhere. Thin-layer identification procedures were reported for the detection of benzoylecgonine (a cocaine metabolite); pentazocine (Talwin) and tripelennamine (T's and Blue's); phencylidene (PCP); propoxyphene (Darvon), benzodiazepine type drugs and marijuana[3–6]. Proposed extraction and identification procedures are the modifications of the procedures reported earlier by Kaistha and Jaffe[7,8] and Kaistha *et al.*[9]. These procedures have been improved constantly over the past 13 years. Reeve Angel SA-2 cation-exchange resin loaded paper* used for the absorption of major drugs of abuse needs to be shaken for about 20–30 min at a medium speed of 140 strokes per min (Eberbach table model shaker) followed by a soaking period of about 30 min. The volume of urine needed is about 35 ml achieving

* H. Reeve Angel, currently supplied by Whatman (Clifton, NJ, U.S.A.).

a sensitivity level 0.3 μg of free morphine, 1.0 μg of each of central nervous system stimulants and sedative-hypnotics per ml of urine. Liquid–liquid extraction procedure needs about 20 ml of urine to achieve the comparable sensitivity levels obtained by 25–30 ml of urine using ion-exchange resin loaded paper, however residue obtained after the extraction of ion-exchange resin loaded paper yield much purer extracts with least interference from natural occurring body metabolites.

An improved detection procedure employing the combined use of fluorescamine (Fluram) and ninhydrin is proposed for the simultaneous detection of amphetamine(Benzedrine,Dexedrine),methamphetamine(Desoxyn),phenmetrazine(Preludin), phenylpropanolamine and methylphenidate (Ritalin). The combined use of ninhydrin and fluorescamine enhanced both the sensitivity and specificity by the formation of highly fluorescent chromophore with fluorescamine and a colored complex with ninhydrin. The methods reported using fluorescamine reagent alone[10,11] for the detection of primary amines generate a highly fluorescent chromophore which is examined on thin-layer plate under long-wavelength UV light. However, we noticed that the use of this reagent alone gives many false positives on the urines of clients ingesting multiple street drugs and/or legally prescribed medications. A built in quality control assurance program is also described.

A SINGLE-STEP EXTRACTION PROCEDURE FOR 14–17 MAJOR DRUGS OF ABUSE* (POLY-DRUGS) USING ION-EXCHANGE RESIN LOADED PAPER

The procedure is the same as described by Kaistha *et al.*[9] with the following modifications.

(i) A 6 × 6 cm piece of H. Reeve Angel, SA-2 cation-exchange resin loaded paper (marked with patient's identity number, drop day, month and program's name with a lead pencil) is soaked in 30 ml of fresh undiluted urine (20–50 ml range with a minimum cut-off volume of 20 ml), shaken for about 30 min (20–30 min) at a medium speed of about 140 strokes per min (Eberbach table model shaker), followed by a soaking period of about the same length of time to achieve a sensitivity level of 0.5 μg for methamphetamine, phenmetrazine, codeine and free morphine per ml of urine and 1.0 μg for amphetamine, methylphenidate and barbiturates per ml of urine. In the case of free morphine, the sensitivity level can be increased to a range of 0.15–0.2 μg per ml of urine by increasing the soaking period to 45–60 min after 30 min shaking on the shaker. In a field situation at a treatment facility, the ion paper may be soaked for a minimum period of 2 h with intermittent shaking or shaken for 10 min and then kept overnight or kept overnight without shaking after intermittent shaking during the first 30 min.

(ii) The single or pooled ion-exchange papers (ion-exchange papers representing different drops of urines of the same client during 1 week) are transferred to a 4-oz. wide mouth glass jar (rinsing with 10–20 ml of water is no longer recommended as it has been attributed to be the possible cause for the low recoveries of methadone and/or its metabolite).

* Major drugs of abuse included are morphine, codeine, methadone, quinine (heroin adulterant), antihistamine, propoxyphene and nor-propoxyphene, PCP, cocaine (unchanged if any), methylphenidate, phenmetrazine, methamphetamine, amphetamine, phenylpropanolamine, phenothiazines and sedatives-hypnotics (barbiturates, glutethimide, phenytoin).

(iii) Using an automatic pipetting machine (Brewer automatic pipetting machine or Filamatic filling unit, Model AB-5, single nozzle filler; National Instrument Co., Baltimore, MD, U.S.A.), 12 ml of pre-diluted ammonium chloride–ammonia buffer (saturated solution of ammonium chloride, 3000 ml, adjusted to a pH of 10.1 $\pm$ 0.1 with concentrated ammonium solution, about 3000 ml, and diluted with 5 l of deionized water) are added followed by 15 ml of chloroform–isopropanol (5:2) (automatic 15-ml repeating glass pipet or prepipetter is used to pour the extraction solvent).

(iv) The contents are shaken for 20 min at a medium speed of about 140 strokes per min (Eberbach table model shaker).

(v) Using a pipet filler, the lower organic phase (a period of about 5–10 min is needed to allow the separation of the aqueous phase from the organic phase) is then pipetted out into a 15-ml *plain*, conical centrifuge-tube containing two to four drops (50–100 μl) of 0.5 % sulfuric acid in methanol.

(vi) The test-tube racks (24 tubes are placed in alternate rows in a rack of four rows, each tube is separated by a piece of paper towel or any suitable absorbent paper to avoid any contamination of specimens due to bumping of the solvent) are then placed in an oven maintained at about 70°C with a horizontal air flow. The organic solvent is allowed to evaporate to about 50 % of its original volume and then the test-tube racks are transferred to another oven maintained at about 85°C to remove the solvent completely. Alternatively the test-tube racks may be left overnight in the oven maintained at 70°C to avoid the loss of work time for which a technician had to wait if the specimens are dried during the day.

(vii) The residue along the sides of the tube is washed with 0.5–1 ml of methanol, vortexed and the sides are again washed with a few drops of methanol. The methanol is evaporated to dryness as above in the oven maintained at 85–90°C.

LIQUID–LIQUID EXTRACTION PROCEDURE

A 20–25-ml volume of urine (cut-off minimum volume of urine is 15 ml) are transferred to a 4-oz. wide-mouth glass jar, 3 ml of undiluted ammonium chloride–ammonium buffer (pH 10.1 $\pm$ 0.1 prepared as described above) and 15 ml of chloroform–isopropanol (5:2) are added. Then the procedure as described above is followed from steps iv to vii.

BUILT-IN QUALITY CONTROL ASSURANCE PROGRAM

The following built-in quality control assurance steps have been incorporated to enforce high quality standards concomitant with the high-level productivity goals.

(i) Ion papers placed in extraction bottles are checked randomly by the supervisor for the correctness of their identification numbers.

(ii) At least 10 % of spiked controls are inserted (at least one control per ten or eleven specimens) between the specimens to monitor the correctness of the technicians' work and also to validate the accuracy of the various development solvents and chromatographic sprays used for the identification of various drugs of abuse. Each spiked control consists of one to three drugs each used at a concentration level of the cut-off limit for that drug equivalent to its free base.

(iii) All the recorded toxicology reports are submitted to the supervisor along with the processed TLC plates. Supervisor with extensive experience reviews each recorded result for the correct interpretation, transpositions and other possible mistakes, such as not recording the results although appeared on the TLC plate. All changes made are initialled by the supervisor.

(iv) After the results are transmitted by phone and also by mail, the counselor and/or the clinical coordinator can challenge the transmitted data by asking for a recheck for the particular drug. The test-tubes containing the left over residue after initial spotting are kept for 2 weeks to give sufficient time for the counselor to challenge the result(s) if he/she is not satisfied with the clients interview. The particular tube is taken out, rewashed with methanol and the residue is respotted using different solvent system. A specific spray is applied so that the challenged drug can be tested more specifically. The results of a recheck are transmitted back within a week.

(v) The urines of the clients who are on federal and/or state parole and the clients who are placed with treatment programs by the magistrates under the program called "Treatment Alternatives for Street Crime" are treated differently. The clinics having such clients have been advised to collect at least 2–3 oz. of urine and divide it into two approximate equal aliquots. One ion paper is then added to each aliquot of urine labeling one paper as A and the second paper as B. Each paper is placed in a separate plastic bag and both plastic bags are stapled together. Upon the receipt of these specimens, paper A is processed and paper B is saved and stored in a refrigerator.

If paper A gives positive results for any of the drugs of abuse, paper B is then taken out and is carried through the entire testing procedure to validate the result shown by paper A. Thus no positive result is transmitted for such clients' urines prior to revalidation.

PROFICIENCY TESTING PROGRAMS

Laboratories participating in any proficiency programs sponsored by state or federal agencies or licensing organizations for drug abuse toxicology monitoring are advised to use the proposed extraction and detection procedures. A 35-ml aliquot of urine using ion-exchange resin loaded paper is recommended and the urine left after taking the ion paper out should be saved for confirmation purposes. Residues should be first spotted for the detection of morphine, codeine, methadone and/or its metabolite, propoxyphene and/or nor-propoxyphene, phencyclidine, unchanged cocaine if any, amphetamine, methamphetamine, phenmetrazine and barbiturates. Every plate must have two spiked controls containing free morphine at 0.2 μg/ml of urine, codeine 0.5 μg/ml and other drugs each at 1.0 μg/ml (equivalent to their free bases). A two stage thin-layer development system first using solvents E or C and then F should be used. The confirmation is performed by washing the residues with methanol in the tubes left after first spotting, evaporating methanol, respotting the dried residue and developing in solvent D. This will enable the confirmation of methadone and its metabolite, phencyclidine, barbiturates, propoxyphene and nor-propoxyphene. The plates need to be sprayed only with diphenylcarbazone (DPC), silver acetate and mercuric sulfate for barbiturates, then dried in the oven for 5–7 min and resprayed with iodoplatinate followed by iodine–potassium iodide (for methadone, phency-

clidine, propoxyphene and its metabolite). If further confirmation is needed, the urines saved after taking the ion papers out may be extracted with undiluted ammonium chloride–ammonium buffer (3 ml) and 15 ml of chloroform–isopropanol (5:2) in the same 4-oz. wide mouth glass bottles (see section Liquid–liquid extraction procedure). The residue of each specimen obtained after drying the extraction solvent may be spotted using solvents E and F or D depending on the type of confirmation needed.

Benzoylecgonine although picked up by ion paper cannot be extracted as above, therefore the procedure reported for the detection of benzoylecgonine may be used[3] [5–10-ml aliquots of urine may be used depending on the cut-off limit desired to be tested, Center for Disease Control (CDC) established 4 μg/ml, in that case 5 ml of urine) using the procedure reported elsewhere[6]. The State of Illinois Dangerous Drugs Commission's Toxicology Laboratory has been participating in the quarterly drug abuse toxicology surveys conducted by the CDC from 1973 to January 1981 until this program was abolished. This laboratory has always earned 100% grade except on two occasions, on one occasion this laboratory was penalized for reporting 0.2 μg/ml of free morphine and another time a false methamphetamine was reported due to very high spiked concentration of phenylpropanolamine. Since last year, the Dangerous Drugs Commission has made suitable arrangements with a renowned forensic toxicologist to ship proficiency specimens to this laboratory and the laboratory has been earning 100% using this proposed method.

THIN-LAYER CHROMATOGRAPHY (TLC)

Gelman precoated silica gel glass micro fiber sheets, 20 × 20 cm (ITLC Type SA), with a layer thickness of 250 μm are used in this laboratory. These sheets are preferred because of the convenience with which they can be handled —can be cut into any desired size such as 10 × 20 cm and can be subjected to varying heat treatments for selective detection of certain drugs. Solvents and detection reagents penetrate the medium from both sides allowing rapid sample migration and more distinct visualization. Spraying detection reagents on both sides increase the sensitivity of the test and sometimes the spots are more distinct on the uncoated side of the sheet; in addition the plates are easy to store and the information can be recorded directly on the ITLC sheet. Urine specimens to be tested for opiates only (morphine, codeine, quinine, methadone and nor-propoxyphene) are spotted on 10 × 20 cm piece, while specimens tested for poly-drugs (15–17 drugs) are spotted on a 20 × 20 cm sheet.

Solvent systems

The solvent systems C–F given below are the same as the corresponding solvents used by Kaistha and Jaffe[7] and Kaistha *et al.*[9].

C: ethyl acetate–cyclohexane–concentrated ammonia–methanol–water (70:15:2:8:0.5)

D: ethyl acetate–cyclohexane–methanol–concentrated ammonia (56:40:0.8:0.4)

E: ethyl acetate–cyclohexane–methanol–concentrated ammonia (70:15:2:8:0.5)

F: ethyl acetate–cyclohexane–concentrated ammonia (50:40:0.1)

G: ethyl acetate–cyclohexane (50:60). This solvent will separate glutethimide from seconal and phenytoin from phenobarbital

It is recommended that solvents D and F be used fresh or within 24 h. Solvents C and E should preferably be used after storage overnight; they both keep well for 3–4 weeks.

Detection reagents

The following detection reagents except ninhydrin–fluorescamine were used, each as described by Kaistha and Jaffe[7] and Kaistha *et al.*[9].

(a) Ninhydrin–fluorescamine: 0.5% (w/v) ninhydrin and 0.02% (w/v) fluorescamine solution in butanol. (It takes about 10–20 min of vigorous shaking to dissolve ninhydrin–fluorescamine; the lumps formed may be broken with the aid of glass rod.) Fluorescamine is a fluorogenic reagent and is sold under the name of Fluram by Roche Diagnostics (Nutley, NJ, U.S.A.).

(b) diphenylcarbazone (DPC): 0.01% (w/v) in equal parts of acetone and water

(c) silver acetate: 1% (w/v) solution in water

(d) mercuric sulfate solution

(e) sulfuric acid: 0.5% (v/v) solution in water

(f) iodoplatinate

(g) iodine–potassium iodide reagent solution is the same as reported earlier, except that methanol was substituted for 95% ethanol due to difficulties in obtaining ethanol

Procedure

The procedure is essentially the same as described earlier[9] except the following modifications.

Volume of developing solvents. The quality of the ITLC plates supplied by Gelman (Ann Arbor, MI, U.S.A.) has improved considerably since the publication of the TLC procedure for the simultaneous detection of a wide variety of commonly abused drugs by Kaistha *et al.*[9]. It is recommended that the volumes of two-stage thin-layer development systems using solvent E or C during the first stage and then solvent F during second stage must be verified for each lot of TLC plates as a part of quality assurance program. Currently 50–60 ml of solvent E or 90–100 ml of solvent C and 110–130 ml of solvent F (per two plates, 20 × 20 cm each per TLC tank) for a two-stage solvent system have proved satisfactory. Two 10 × 20 cm plates used for the detection of opiates need 100 ml of solvent C.

The plates must be heated for 5–7 min at 85–90°C prior to placing in the developing solvents. The volume of solvent D used for the confirmation of barbiturates and also for the separation of pentazocine from pyribenzamine, propoxyphene, phenyclidine and methadone[4] varies from 100 to 150 ml per two plates 20 × 20 cm each per TLC tank. Currently 130 ml were found to be suitable for the separation of mixture of these five drugs (solvent D must be fresh). The volume of solvent G recommended for the separation of glutethimide from seconal and phenytoin from phenobarbital is about 100–140 ml.

Detection techniques. Detection reagents a–f are applied in succession to the specified areas of the same plate as described earlier by Kaistha *et al.*[9]. However, the following changes are proposed after the application of ninhydrin–fluorescamine reagent (detection reagent a).

(i) The plate is left exposed to air for 5–15 min. (The lower 4.5 cm and upper 5.0 cm area of the plate are covered with glass plates and the middle portion is sprayed with the ninhydrin–fluorescamine reagent.) The air exposure will visualize phenylpropanolamine as a purple color spot or a streak. A concentration of about 1 μg/ml of urine will take approximately 10 min of air exposure. High concentrations take 1–3 min, while weaker concentrations could take up to 20 min.

(ii) After air exposure, the plate is examined under long-wavelength UV light (Chromatovue table model) for green fluorescence at the level of the amphetamine standard. The spots showing green fluorescence at the level of the amphetamine standard are circled, then the plate is irradiated for 7 min under long-wavelength UV light. The amphetamine positives will appear as purple spots. (Only the spots at the level of amphetamine standard and the spots which previously showed green fluorescence as well, are considered as positives.)

(iii) The plate is then heated in the oven at 85–90°C for 5–7 min. Methamphetamine appears as a purple spot; pseudoephedrine sold as a look alike drug will also appear at this stage as a streak.

(iv) Re-irradiation under short-wave light for 7 min as described in the procedure reported earlier is not needed, however re-irradiation under long-wavelength UV light maybe repeated if standard for amphetamine did not appear as a purple spot under step ii.

(v) Respraying the middle portion with ninhydrin–fluorescamine and heating on the hot plate at a temperature of about 220–250°C for a few seconds will cause methamphetamine and amphetamine to undergo different color changes. Heating is continued for 10–30 sec until phenmetrazine (Preludin) standard appears as a bright pink and methylphenidate (Ritalin) standard changes from purple to light yellow.

The intensity of color reactions vary slightly with the different lots of the TLC plates. Detection reagents b–f are then applied in succession in steps as outlined in the procedure described elsewhere[9]. Phenylpropanolamine (PPA) will show as pinkish red color spot or streak after mercuric sulfate spray and amphetamine and/or methamphetamine may appear as dark grey spots. The ITLC plates are then heated in the oven for 5–7 min, where phenothiazine type drugs give two to three varying colored spots depending on the concentrations of various metabolites. Codeine appears as a light orange color spot if the area containing codeine standard was sprayed with mercuric sulfate and then followed by heat in the oven. Spraying with sulfuric acid (0.5%, v/v, in water) and examining under short-wavelength UV light shows unchanged quinine and its major metabolite as brilliant blue fluorescene emitting spots. The plate must be heated in the oven for about 5–7 min prior to the application of the iodoplatinate spray. Morphine gives blue to navy blue colored spot after iodoplatinate spray. The spot due to morphine does not disappear by keeping the plate overnight or for a long period. In fact, spots due to weaker concentration of morphine shows much better by storing the plate overnight. (The plate may be kept covered by paper towel, the use of plastic sheet is not recommended since it gives a false blue spot which disappears on exposure to light.) The spots of morphine with a concentration of 0.2 μg or less can be made visible by holding the chromatogram near the hot plate (a hot plate preheated to a temperature of 220–250°C) for 30–60 sec and then leaving the plate at room temperature for several minutes. Spots (brown or light brown color) due to methadone and its metabolite and grey or blue colored spots or streak due to nor-propoxyphene (metabolite of propoxyphene) must be circled immediately as these spots disappear on keeping the plates (spots due to higher concentration of

methadone do not disappear). Spots due to methadone and its metabolite and grey or blue colored spots due to nor-propoxyphene generally appear at the level of the solvent front if single stage development solvent system is used for opiates detection or they appear at about R_F value of about 0.56–0.6 (8.5–9.0 cm distance from the origin of the spots) if a two-stage development solvent system is used. Higher dosage of propoxyphene ingested by a client will give an additional blue color spot near the level of morphine which, however, can be differentiated from morphine due to different specific shades of color. Propoxyphene (unchanged) and PCP give light brown spots but unchanged cocaine (if any) gives characteristic greyish brown spot. Amphetamine may appear as grey and methamphetamine as reddish color spots depending on the concentration of these drugs and the quality of the TLC plates. (Small concentration of these drugs are not amenable to iodoplatinate.)

RESULTS AND DISCUSSION

Since the inception of drug abuse prevention and treatment programs, the authors have been strong proponents of providing maximum number of drug evaluations (drugs that are invariably abused) per urine specimen at the minimal cost. The only technique which could meet our objectives concomitant with reliability, sensitivity, specificity, productivity and versatility was thin-layer detection and identification of drugs. The modifications reported in this manuscript were constantly made during the past 14 years to meet our goal of testing 12–15 drugs of abuse per specimen at a cost of less than US$2.00 (cost includes technician's salary, thin-layer supplies and ion-exchange resin paper, etc.). Currently a technician analyzes 100 specimens per day (95 specimens plus 10 or 11 spiked controls) thus performing total of 1200–1500 evaluations. A built-in quality assurance program has been incorporated as described earlier in this manuscript. The accuracy and reproducibility of these techniques have been tested by participating in proficiency testing surveys which were conducted by the CDC, and now by a well renowned toxicologist. Thin-layer detection procedures have been reported for any drug or class of drugs which are likely to be abused such as benzodiazepine-type drugs[5], cocaine[3] (as benzoylecgonine), marijuana usage[6], PCP, pentazocine and T's and Blue's[4].

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REFERENCES

1 K. K. Kaistha, *J. Pharm. Sci.*, 61 (1972) 655.
2 K. K. Kaistha, *J. Chromatogr.*, 141 (1977) 145.
3 K. K. Kaistha and R. Tadrus, *J. Chromatogr.*, 135 (1977) 385.
4 K. K. Kaistha and R. Tadrus, *J. Chromatogr.*, 155 (1978) 214.
5 K. K. Kaistha and R. Tadrus, *J. Chromatogr.*, 154 (1978) 211.
6 K. K. Kaistha and R. Tadrus, *J. Chromatogr.*, 237 (1982) 528.
7 K. K. Kaistha and J. H. Jaffe, *J. Pharm. Sci.*, 61 (1972) 679.
8 K. K. Kaistha and J. H. Jaffe, *J. Pharm. Sci.*, 61 (1972) 305.
9 K. K. Kaistha, R. Tadrus and R. Janda, *J. Chromatogr.*, 107 (1975) 359.
10 B. Klein, J. E. Sheehan and E. Grunberg, *Clin. Chem.*, 20 (1974) 272.
11 S. J. Mulé, personal communication, Testing Summary Analysis, Toxicology Drugs of Abuse, Survey III, Center for Disease Control, Atlanta, 1977, p. 10.