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Drug Monitoring and Toxicology

Computer-aided identifications of thin-layer chromatographic patterns in broad-spectrum drug screening

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We have developed a systematic thin-layer chromatographic (TLC) technique for detecting and identifying drugs and drug metabolites on 10-cm-long silica-gel plates with organic binder (fluorescent indicator); a computer program (SPOT CHEK) assists in matching the data from a particular chromatogram with those obtained for known drugs recovered from serum, urine, or other specimens. The plates are developed in a single mobile phase. Visualization and detection reagents used to characterize unknowns include fluorescamine, ferric chloride/perchloric acid/nitric acid, Dragendorff, Marquis, Mandelin, and iodinated Dragendorff solutions, 254 nm ultraviolet light, and vapor from chlorine or hydrochloric acid. Detection limits of 5–200 ng per sample spot were obtained for drugs in the database. The computer program database is based on nine reaction responses plus the plate zone locations for 243 drug substances but requires entry of only one TLC property to generate a matching list. We ran the program with an IBM-compatible 386/486 PC using an MS-DOS operating system (version 6.2).

INDEXING TERMS: drug assays • toxicology

Numerous analytical schemes have been designed to test for drugs in the general unknown context by thin-layer chromatography (TLC) *[1-4]*, gas–liquid chromatography *[5]*, HPLC *[6]*, GC/MS *[7]*, and combinations thereof.[1] Carvalho et al. *[8]* carried out qualitative TLC with color reactions and used a computer program to sort data for 37 drugs. However, their technique required four solvent systems and was limited to 37 drugs. ANSYS, Inc., introduced Toxi-Lab in 1981 *[9]* as a commercially available technique for systematic drug detection and identification and provided a compendium illustrating color reactions of drugs. Toxi-Lab is efficient but has marginal sensitivity for many drugs *[2, 3, 9, 10]*, poor resolution when several drugs are present, and depends on one color reaction (which gives no color with some drugs) and one general staining technique that reacts with nearly all drugs. The accompanying computer program (TOXI-SEARCH) is not currently advertised by ANSYS.

Our goals with the technique presented here included having as many unique sets of reactions as possible (high specificity) within 1–2 h of one chemist's bench time and using routine silica-gel TLC plates and other materials readily available in most toxicology laboratories. We also wanted to meet the criterion that general unknown screening should logically determine what is *not* present as well as what *is* present through the use of reliable exclusion and accurate positive identifications of xenobiotics. The training in TLC required to make accurate identifications has been an important factor in developing the technique we present. A computer program, SPOT CHEK, was developed to minimize the time spent interpreting data by tediously matching observed TLC migration and reaction characteristics with those previously obtained for drug standards. Detection reagents were chosen to detect 100 ng or less per applied spot of sample for a wide variety of drug groups. Results for 9 TLC reactions and the migration position (the 10th defining variable) relative to nicotine for each drug or drug metabolite have been placed in the database to facilitate a search keyed on any one (or more) of these 10 TLC identifying characteristics. After the search is completed, the program presents a list of the matches between test specimen substances and database entries.

Materials and Methods

TLC

Supplies and equipment. All separations and reactions were carried out on scored hard-layer silica-gel plates (prod.

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[1] Nonstandard abbreviations: TLC, thin-layer chromatography; FPN, ferric chloride/perchloric acid/nitric acid; FPIA, fluorescence polarization immunoassay.

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no. 47521; Analtech, Newark, DE). The plates were developed in two-sided chambers with stainless-steel covers (no. 022.5155; CAMAG Scientific, Wilmington, NC). The double-sided TLC chamber allows for a 10-min solvent-preloading time for the plate before development is started. Spray bottles, paper saturation pads, dipping containers, a UV viewing chamber (Spectroline CM-10), and a hand-held UV 254/366-nm lamp (Spectroline ENF 240-C) were supplied by Alltech (State College, PA). Other glassware included pipets, capillary pipets for sample application, conical plastic disposable evaporation cups (Har-Len Medical, Pittsburgh, PA), and screw-cap jars containing various reagents into which the TLC plates would be dipped.

Detection reagents. The 10 TLC detection/visualization reagents used (SPOT ID reagents) were prepared with minor modification of the formulations given elsewhere *[11]*. Composition of the reagents was as follows:

S1, fluorescamine (Sigma Chemical Co., St. Louis, MO), 0.3 g/L; stable 1 month stored in a brown bottle

S2, ferric chloride/perchloric acid/nitric acid (FPN) reagent: 90 mL of 70% perchloric acid, 135 mL of 70% nitric acid, and 15 mL of 50 g/L ferric chloride added to 100 mL of water; stable 6 months in a brown bottle

S3, Dragendorff reagent, prepared as in Clarke *[11]*

S4, Marquis reagent: concentrated sulfuric acid

S5, Mandelin reagent: ammonium metavanadate (A-8050; Sigma Chemical Co.), 800 mg/L in concentrated sulfuric acid; stable at room temperature for at least a year

S6, iodinated Dragendorff reagent: 5 g of potassium iodide, 2 g of iodine, and 0.2 g of bismuth subnitrate (Sigma B9009) mixed in water, followed by 0.5 mL each of glacial acetic acid and concentrated HCl, and brought to 250 mL with deionized water

S7, 254 nm (UV) light projected onto a developed TLC plate (must be fluorescent indicator plate)

S8, chlorine vapor, produced as needed by adding concentrated HCl to commercial bleach (5% hypochlorite)

S9, concentrated HCl fumes, produced as needed in a fume hood

S10, the mobile phase (described below).

Names, application technique, and shelf-life of the reagents are given in Table 1. Before dipping plates 2B and 2C into S4 and S5 reagents, we exposed them for ~5 min to vapors from formaldehyde/methanol solution (37/63 by vol.), then dipped them into the respective acid solutions. Mercuric sulfate (not one of the 10 SPOT ID reagents) was prepared by dissolving 5 g of mercuric sulfate in water plus 20 mL of concentrated sulfuric acid and bringing to 250 mL with water (stable at least a year).

For all chromatographic runs we used a single mobile phase: ethyl acetate/dichloromethane/methanol/concd. ammonium hydroxide (80/90/15/5 by vol.). Migration of drug substances was defined by a "zone," or position relative to a set of known substances present in the normalizing (N) mixture. The compounds included in the N mixture were (Fig. 1, reading spots from bottom to top): benzoylecgonine, morphine, quinine, nicotine, methadone, and cocaine. The area from the origin to the center of the morphine spot was designated Zone 1, from morphine to quinine Zone 2, from quinine to nicotine Zone 3, from nicotine to cocaine Zone 4, and from cocaine to the solvent front Zone 5. Substances that eluted even with a boundary line were referenced to the higher-numbered zone. The TLC chamber must be two-sided or have a small container in which to place mobile phase for preassay vapor-loading of the plate. The calibrating mixtures in M and P (Fig. 1) are neutral drugs (M mixture) and barbiturates (P mixture); S_1, S_2, and S_3 are benzodiazepines and other neutral/acidic drugs; A, B, and C mixtures are basic amine-type drugs. The total number of individual drugs present in the set of mixtures is 48, each at 1 g/L in methanol. Mixture compositions are given below under *Assay Performance (Separations)* and in Fig. 1.

Table 1. TLC reagents used to detect chromatographed drug substances and application–visualization technique.

Reagent	SPOT ID code	How applied	Shelf life
Fluorescamine	S1 fluorescamine	Dip or spray	1 month
FPN	S2 FPN	Light spray, heat	6 months
Dragendorff	S3 D1	Dip or spray	1 year
Marquis[a]	S4 & color	Dip only	1 month
Mandelin[a]	S5 & color	Dip only	1 month
Dragendorff + I_2	S6 D2	Dip or spray	1 year
UV 254 nm light	S7 UV 254 nm	Dark viewing box	——
Chlorine vapor	S8 Cl_2	Cl_2 as needed	[b]
HCl vapor	S9 HCl	HCl as needed	[b]
Migration zone	S10 Zone	Migration position	[c]
Mercuric sulfate[d]	Extra reagent	Dip or spray	1 year

[a] The developed plate was exposed to formaldehyde vapor in a screw-cap bottle for 3–10 min and then was dipped into concentrated H_2SO_4 (S4, Marquis reaction); a second plate similarly exposed to formaldehyde was dipped into Mandelin reagent (S5). Specific colors are noted with the S4 and S5 reactions and compared with the color code stated in the text.

[b] Vapors of Cl_2 and HCl are produced in a fume hood as described under *Assay and Techniques, Plate reactions.*

[c] Migration zone ranges from 1 to 5, according to migration of a TLC spot relative to four calibrators on plate 1A (see *Detection reagents*).

[d] This reagent, which detects barbiturates and several neutral/weakly acidic drugs, is referenced on the data cards but is not included in the computer program.

COMPUTER AND COMPUTER PROGRAM

Data for >240 drug substances have been placed into a PC program, compressed, compiled, and installed onto an MS DOS hard drive, version 6.2. The original program, SPOT CHEK, was written in dBase IV and requires an IBM or IBM-compatible 286 or higher PC, DOS 6.0 or later 100%-compatible version, at least 640 K of memory plus 2 Mb of extended RAM, at least 3 mB of hard disk space (4.5 mB at installation), and an CGA, EGA, or VGA monochrome or color monitor. A peripheral printer is optional but is useful for printing lists of matches after data entry.

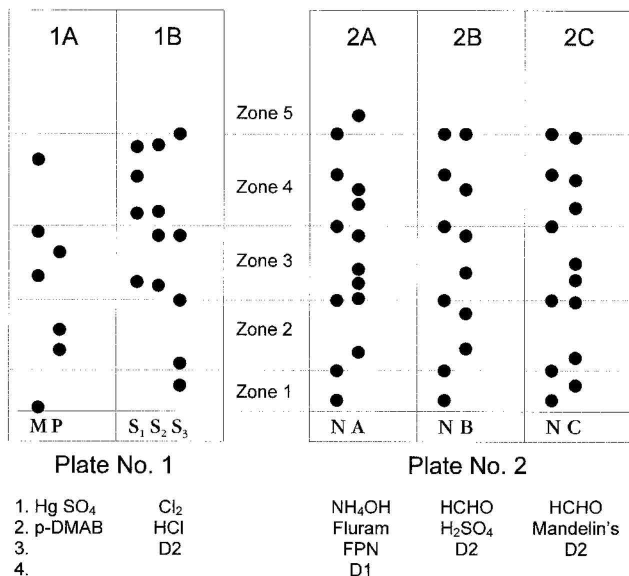
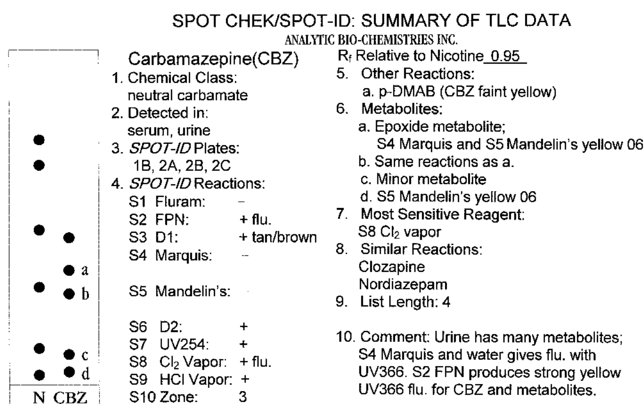


Fig. 1. TLC plate-spotting pattern and reaction sequence for the technique described in the text.

Black circles represent specific drugs and the positions to which they migrate. *Letters* along the origin (M, P, S_1, etc.) designate location and identification of standard mixtures applied in conducting a complete drug screen. *Vertical lines*, outside edges, and *score lines* (on the backs of the plates) permit segmenting the plates for each reaction sequence (shown below each plate segment). *Horizontal lines* pass through the origin and through the drugs that divide the plate into five zones. *M* (*bottom to top*): ibuprofen, meprobamate, methyprylon, glutethimide. *P*: phenobarbital, phenytoin, secobarbital. S_1: lorazepam, temazepam, flurazepam, medazepam. S_2: oxazepam, carbamazepine, nordiazepam, diazepam. S_3: theophylline, dyphylline, acetaminophen, caffeine, methaqualone. *N*: benzoylecgonine, morphine, quinine, nicotine, methadone, cocaine. *A*: phenylpropanolamine, methamphetamine, amphetamine, phenmetrazine, caffeine, imipramine, methylphenidate, phencyclidine. *B*: ephedrine, codeine, nortriptyline, caffeine, diphenhydramine, propoxyphene. *C*: nadolol, triamterene, paroxetine, nordoxepin, dextromethorphan, pentazocine, amitriptyline, lidocaine.

Data cards. TLC reactions were detailed on 250 separate identification cards that give all of the information database for each drug. Fig. 2 shows as an example the data


SPOT CHEK/SPOT-ID: SUMMARY OF TLC DATA

ANALYTIC BIO-CHEMISTRIES INC.

Carbamazepine(CBZ)

1. Chemical Class: neutral carbamate
2. Detected in: serum, urine
3. *SPOT-ID* Plates: 1B, 2A, 2B, 2C
4. *SPOT-ID* Reactions:
   - S1 Fluram: –
   - S2 FPN: + flu.
   - S3 D1: + tan/brown
   - S4 Marquis: –
   - S5 Mandelin's:
   - S6 D2: +
   - S7 UV254: +
   - S8 Cl_2 Vapor: + flu.
   - S9 HCl Vapor: +
   - S10 Zone: 3

R_f Relative to Nicotine 0.95

5. Other Reactions:
   a. p-DMAB (CBZ faint yellow)
6. Metabolites:
   a. Epoxide metabolite; S4 Marquis and S5 Mandelin's yellow 06
   b. Same reactions as a.
   c. Minor metabolite
   d. S5 Mandelin's yellow 06
7. Most Sensitive Reagent: S8 Cl_2 vapor
8. Similar Reactions: Clozapine, Nordiazepam
9. List Length: 4
10. Comment: Urine has many metabolites; S4 Marquis and water gives flu. with UV366. S2 FPN produces strong yellow UV366 flu. for CBZ and metabolites.

Fig. 2. Data card for carbamazepine (1 of 243 prepared) documenting the TLC migration of carbamazepine.

The parent drug (no letter code) and four major metabolites are indicated as *dark circles*. The 10 properties (S1–S10) were placed in the SPOT CHEK database. The data card gives some additional reaction characteristics. Item 9 gives a list length of 4, meaning the 10 reactions are unique for carbamazepine. Note that the metabolites do not have the identical reactions of the parent drug. p-DMAB, *p*-dimethylaminobenzaldehyde; flu., fluorescence.

card for carbamazepine. All substances have a relative R_f, nicotine being assigned R_f = 1.0. The data cards are indexed alphabetically and sorted in order of increasing relative R_f. As shown in Fig. 2, the data cards contain, in addition to the 10 reactions in SPOT CHEK, information that will greatly assist in making accurate drug identifications. Data cards illustrate portions and reactions of metabolites of parent drugs, list drugs that react similarly (can be mistaken for suspect drug), give the most sensitive reagents for visualization, and offer important details of the TLC behavior of the various drugs.

SAMPLE PREPARATION/DRUG RECOVERY FROM URINE AND SERUM

An aliquot of serum (0.5–1 mL) not pH-adjusted was extracted with 6 volumes of a solution of dichloromethane/isopropanol (19/1 by vol.). Whole blood was diluted with 1 volume of water before extraction with the 6 volumes of solvent. Two 5-mL aliquots of urine, one adjusted to pH 9 and the other to pH 12, were extracted with 10 mL of the dichloromethane/isopropanol solvent and combined into one extract fraction. The serum and urine extracts were passed through coarse filter paper and evaporated to dryness in disposable plastic 16-mL conical beakers. Before evaporation, two drops of 1 mL/L HCl in methanol were added to urine extracts to prevent loss of volatile amines. This isolation technique provides a weak acid/neutral fraction and an amphoteric/amine base fraction. The serum extract, which might contain neutral drugs, weakly acidic drugs (barbiturates, acetaminophen), and benzodiazepines, was spotted onto Plates 1A and 1B (Fig. 1), and the basic/amphoteric fraction from urine was spotted onto Plates 2A, 2B, and 2C.

We examined some drugs—from very polar (benzoylecgonine) to nonpolar (phencyclidine)—for approximate recoveries in the liquid–liquid extraction technique described above. Because on the silica-gel TLC plate the drugs migrate according to their polarity, recovery correlates well with migration position; i.e., recovery increases as R_f increases. Benzoylecgonine, found low in Zone 1, has ~45% recovery and phencyclidine ~95% recovery. Morphine recovery is ~70%, and drugs migrating farther than morphine are 75–95% recovered. Benzodiazepines, barbiturates, acetaminophen, caffeine, and other neutral compounds and weak acids are recovered from serum in high yield (>80%) with no pH adjustment. Recovery was determined by measuring the fraction remaining unextracted after the initial extraction.

ASSAY AND TECHNIQUES

Application of samples. The plates used throughout were factory-scored hard-layer silica-gel plates, 20 cm wide × 10 cm high (running distance), with an organic binder and fluorescent indicator. Biological fluid extracts, powders, and other solid dosage forms (e.g., tablets, pills, residues) were applied directly to the plates in methanol or dichloromethane. Residues from both the neutral and the pH 9

plus 12 fractions were taken up in a few drops of dichloromethane/isopropanol solvent and applied in 10-μL capillaries or by an automated applicator. The neutral extract was spotted about equally on Plates 1A and 1B and the pH 9 plus 12 fraction from urine was spotted about equally on Plates 2A, 2B, and 2C, ~20% of this residue being reserved for optional tests. The plates being spotted were placed over a 2.54-cm-wide heating tape so that application spots dried quickly and fit within a standard 3-mm-diameter size for mixtures and extraction fractions (see Fig. 1 for spotting pattern). The scored plates were sectioned *after* development, except that Plate 2C (Fig. 1) was separated from the other sections before development. This permitted the placing of all 5 sections of plate into a single small development chamber (2C was placed facing the other plate sections) and developed in the mobile phase described above. Serum and urine extracts from one or two subjects were accommodated on a 12.5 × 10 cm (*w* × *l*) plate. Larger plate sections of 1A, 1B, 2A (etc.) and tanks holding 20 × 10 cm plates were used when we were analyzing specimen extracts from more than two subjects.

Plate development. A paper pad was placed on one side of a two-sided TLC chamber for 10 × 10 cm plates, and 6 mL of developing solvent was run (pipetted) down the pad, followed by 3 drops of concentrated NH_4OH (pad side). The plate was placed on the dry side of the chamber for 10 min before developing to full plate height by the addition of 6 to 8 mL of solvent. Chromatography per se took 10.5 min. The plates were then dried and observed in the viewing chamber at 254 and 366 nm, where we marked the UV-quenching spots lightly with a pencil and made note of any fluorescent spots. Relative R_f values were not routinely measured, but zone positions were accurately noted by reading the positions of detected substances vs those of the N calibrator drugs, either under the UV 254 light (reaction S7) or after a visualization reaction.

Plate reactions. Reagents were applied (after segmenting the plates as in Fig. 1) by classical techniques, either dipping or spraying as indicated in Table 1. Barbiturates, meprobamate, glutethimide, and other nonbarbiturate sedatives were detected or ruled out by use of mercuric sulfate dip (neutral/weak acid extract) on plate 1A. After positioning plate 1B over a 600-mL beaker on a warm hot plate (in a fume hood), we added several milliliters of commercial bleach followed immediately by the same volume of concentrated HCl. This produced sufficient Cl_2 gas to react with drugs on the developed plate. After exposure to Cl_2 vapor for 10–15 s, some drugs give a visible color (acetaminophen), whereas others fluoresce strongly (some benzodiazepines, carbamazepine). We next exposed plate 1B to HCl vapors (in a fume hood). After each reaction (Cl_2 and HCl) we examined plate 1B for fluorescence with UV 254 and 366 nm light. Detection is vastly improved by placing the plate over a hand-held UV lamp (face up) inside the dark chamber rather than having the light source above the plate.

Plates 2A, 2B, and 2C were processed according to the scheme in Fig. 1 and all reactions were noted. The sequence of reactions for plate 2 was as follows: 2A, NH_4OH vapor, fluorescamine, FPN, D1; 2B, formaldehyde for 5–10 min, then concentrated H_2SO_4 dip; 2C, formaldehyde for 5–10 min, then Mandelin reagent dip. After the acid dips for 20–50 s, plates 2B and 2C were dipped first into water and then into reagent D2 for 2–5 min, which fixes the location of most drug substances. The two acid dips produce colors in the spots, so a color number is assigned to all spots on the plates that give colors with the acid dips. The color code was: 1, pink; 2, red; 3, copper/rust/red-brown; 4, orange/peach/yellow-orange; 5, tan/salmon/gold; 6, yellow; 7, green; 8, olive green/gray-green; 9, blue-green/aqua/lime; 10, blue (light and dark); 11, violet/purple/lavender; and 12, gray/blue-gray. Reaction results and the zone positions of unknown spots on the plate were recorded. These nine reactions plus the zone position, collectively called SPOT ID reactions, are numbered S1 through S10 for convenient tabulating and entry into the SPOT CHEK program.

ASSAY PERFORMANCE

Separations. The silica-gel plates and mobile phase used resolved individual drugs within classes sufficiently so that 48 drugs seen very frequently could be applied as calibrators and fully separated. Mixtures of these calibrators [1 g/L (1 μg/μL) each] were applied to TLC plates as long as 2 weeks before plate development, ~2 μg per spot. No decomposition of the calibrators occurred if the prepared plate was covered with a glass plate and kept in a closed box. The components of each calibration mixture are listed in the legend to Fig. 1. This large number of calibrators allows for visual determination that an unknown drug is between two calibrators rather than measuring the R_f or relative R_f of the unknown. For example, for a drug that migrates between methadone and cocaine (in the N mixture), this area of the TLC plate presents few matches. Migration relative to a known calibrator is more consistent than relative R_f, which varies as much as 15% from plate to plate. Some drugs are more susceptible to slight solvent changes than others. For example, phenobarbital is moderately dependent on the ammonia concentration within the chamber, and benzoylecgonine migration depends on the moisture content of the TLC plate. Whereas relative R_f numbers can change from plate to plate, migration positions relative to the N mixture set remain constant if the developing conditions are according to the protocol given above.

Detection limits. Detection limits, in terms of quantity (ng)/spot, are dependent on spotting technique and the reaction used to visualize the drugs. Table 2 indicates the approximate detection limits expected for several drugs and visualization reagents, assuming that the procedure is

Table 2. TLC[a] visual detection limits of several drug types determined with SPOT ID reagents and techniques.

Drug	Detection reagent, technique	Detection limit, ng/spot
Amphetamine	S1 366 nm, fluorescence	10
Amitriptyline	S3 D1, visual observation	100
Benzoylecgonine	S3 D1, visual observation	50
Benzoylecgonine	S6 D2, visual observation	25
Caffeine	S6 D2, visual observation	200
Caffeine	S7 UV 254 nm, in viewing box	25
Carbamazepine	S8 Cl_2 vapor and 366 nm light	5
Desipramine	S2 FPN, lightly heat, blue color	20
Diphenhydramine	S4 Marquis, yellow color	100
Lorazepam	S8 Cl_2 vapor and 366 nm light	20
Mesoridazine	S5 Mandelin, pink color	20
Methampetmine	S1 366 nm, fluorescence quench	30
Morphine	S4 Marquis, purple color	40
Nortriptyline	S4 Marquis, red-brown color	20
Phencyclidine	S3 D1, visual observation	200
Phencyclidine	S6 D2, visual observation	100

[a] Analtech no. 47521 scored silica-gel plates with fluorescent indicator were developed to 10 cm with the solvent system described in the text.

performed by someone with reasonable analytical skill and experience. Detection limits in terms of concentrations in urine and serum (ng/mL specimen) are dependent on recovery, spotting technique, and reagent for visualization. Tables 3 and 4 present results for several drugs added to 0.5-mL serum and 5-mL urine samples and are typical of results for all 240 drugs studied. These results should be considered approximate because experience, plate quality, and TLC skills are factors in the overall detection. Nevertheless, the technique presented here offers lower detection limits for all classes of drugs relative to previously published detection limits *[2, 3, 9, 10, 12]*, either per spot or per volume of specimen.

Validation. Data on many drugs and drug metabolites have been accumulated over several years in the process of broad-based drug screening of >15 000 individual urine and serum/blood sets. Validation of the TLC techniques was accomplished by using at least one of several alternative techniques—RIA, gas chromatography, HPLC, UV spectrophotometry, fluorescence polarization immunoassay (FPIA), and GC/MS—to confirm the TLC identifications. All positive results for cocaine/benzoylecgonine, opiate, barbiturate, phencyclidine, amphetamine, and benzodiazepine were tested by RIA; tricyclics, barbiturates, and various neutral drugs positive by TLC were tested by FPIA; drugs with unique UV spectra such as naproxen and methaqualone were scanned by UV spectrophotometry; gas chromatography, GC/MS, or HPLC was used in numerous instances where TLC findings were not clearcut.

The Toxi-Lab compendium, consisting of color photographs of spots after the Mandelin reaction, helped us confirm how drugs and metabolites react with this reagent. Urines from persons taking a single known medication were processed on several occasions to obtain metabolite patterns (e.g., for tramadol, ibuprofen, and codeine). Patient histories in which drug intake was reasonably documented provided a check on the overall reliability of the SPOT CHEK technique. Information for the data cards (Fig. 2) was based on positive, confirmed identifications with authentic drugs.

Table 3. TLC visual detection limits for several drugs extracted from 0.5 mL of unbuffered serum.

Drug	Detection limit, mg/L			
	UV 254 nm	Cl_2	HCl	$HgSO_4$
Acetaminophen	0.5	2.5	NR	NR
Caffeine	0.1	NR	NR	NR
Carbamazepine	0.2	0.02	0.04	NR
Carisoprodol	NR	NR	NR	1.0
Nordiazepam	0.1	0.05	0.1	NR
Theophylline	0.2	NR	NR	NR
Secobarbital	NR	NR	NR	0.2

NR, no obvious reaction with the reagent indicated.

Table 4. TLC visual detection limits for several basic and neutral drugs recovered by liquid–liquid extraction from 5 mL of pH 9 buffered urine.

Drug substance	Detection limit, mg/L			
	UV 254 nm	Fluorescamine	FPN	D1
Amphetamine	Weak[a]	0.2	NR	Weak
Benzoylecgonine	0.2	NR	NR	0.15
Codeine	0.3	NR	NR	0.2
Desipramine	0.1	0.2	0.1	0.5
Diphenhydramine	Weak	NR	NR	0.5
Imipramine	0.1	NR	0.1	0.5
Mesoridazine	0.05	NR	0.1	0.4
Methamphetamine	Weak	0.1	NR	0.4
Morphine	0.3	NR	2	0.4
Nortriptyline	0.2	0.2	NR	0.4
Phencyclidine	Weak	NR	NR	0.15[b]

NR, no obvious reaction with the reagent indicated.
[a] Weak reaction observed.
[b] Dragendorff (D1) oversprayed with iodinated Dragendorff (D2).

Specificity. Identification problems most often involved choices between closely related drugs or drugs with few or no SPOT ID reactions other than migration position. For example, orphenadrine and diphenhydramine have very similar reagent reactions but can be differentiated by slight migration differences. Brompheniramine, pheniramine, and chlorpheniramine are difficult to distinguish by these TLC techniques and when present in a mixture could not be resolved with the solvent system presented here.

Persons who are color-blind can use SPOT CHEK, but they need to use the Marquis and Mandelin color codes guardedly or have others' eyes note the colors.

DATA ENTRY AND EFFECTIVE USE OF SPOT CHEK COMPUTER PROGRAM

To operate the program, the user notes systematically the reactions and zone for each spot. Weak reactions are noted as such and colors are described in words, then assigned the corresponding number from the color key. The program, installed in MS DOS onto a PC, is then opened. The result of each SPOT ID reaction (the 9 reactions) is asked for with a yes/no (Y/N) statement or a prompted color number (reactions 4 and 5), as follows.

S1, fluorescamine reaction—Quench, Fluoresce, None? (Q/F/N)
S2, FPN reaction? (Y/N)
S3, D1 Dragendorff reaction ? (Y/N)
S4, Marquis reaction? Enter number corresponding to color, or NN for no color
S5, Mandelin reaction? Enter number corresponding to color, or NN for no color
S6, D2—iodinated Dragendorff reaction? (Y/N)
S7, UV 254 nm reaction—quench fluorescence? (Y/N)
S8, chlorine vapor reaction? (Y/N)
S9, HCl vapor reaction? (Y/N)
S10, zone portion on plate? Enter 1, 2, 3, 4, or 5
Would you like a printout? (Y/N)
Are all entries correct ? (Y/N)
If N (no), go back to start (S1).

Initially, only those reactions that were carried out and gave very clearcut spots should be entered. Leaving the response blank means that the reaction will not be considered. After the last entry, the program produces on the screen a list of drugs matching the designated properties. Only if the list contains more than four entries is one advised to enter more reactions. Specificities and probability matches vary with the compound and to some extent with the skill and experience of the analyst. Once a small list has been generated, the data cards are to be examined carefully for best matches and for those substances similar to the suspect drug.

Results

Table 5 gives the results from seven individual drug screens processed according to the procedure given and to the TLC reactions entered into the SPOT CHEK computer program. Not all reactions were used for the individual cases; in some cases, only a single plate was used to acquire a listing or identification. After obtaining a list, whether one drug or more, we compared each drug with a data card of the listed drugs such as the one given in Fig. 2. The exact position (R_f relative to nicotine) and the metabolite pattern assisted in distinguishing close matches. Case 1 was from a pH 7 serum extract spotted onto plates 1 and 2 (Fig. 1). If only reactions S6, S7, S8, S9, and Zone are entered into SPOT CHEK, the search list is too long for quick identification. By including plates 2A

Table 5. Summary of SPOT CHEK computer program listings from the input of SPOT ID reactions from seven typical patients.

Patient no.	Plates used	Charateristic in program										No. of matches[a]
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	
1	1B						+	+	+		3	44
	1B						+	+	+	+	3	22
	1B, 2A	−	+	+			+	+	+	+	3	11
	1B, 2A, 2B	−	+	+	−		+	+	+	+	3	4[b]
2	2A	−	−	+				+			3	31
	2A, 2B	−	−	+	9		+	+			3	2
	2A, 2B, 2C	−	−	+	9	9	+	+			3	1[c]
3	2A	−	−	+				+			2	12
	2A, 2B	−	−	−	11		+	+			2	2[d]
4	2A	−	+	+				−			3	1[e]
5	2A	Q	−	+							1	3
	2A, 2B	Q	−	+	5		+	+			1	1[f]
6	2A	Q	−	+							4	6
	2A, 2B, 2C	Q	−	+	−	4	+	−			4	1[g]
7	2A	−	−	+				+			5	6
	2A, 2B	−	−	+	6		+	+			5	1[h]

[a] Relative to the reactions entered. (−) indicates a negative reaction and (+) indicates a positive reaction; a blank means the reaction was not entered; Q indicates fluorescence quenching; under S4 and S5, the color is given by the number (see code in text).
[b] Carbamazepine, lorazepam, nordiazepam, oxazepam.
[c] Dextromethorphan.
[d] Guaifenesin, methocarbamol.
[e] Erythromycin.
[f] Azacyclonol.
[g] Lidocaine metabolite (MEG-X).
[h] Amiodarone.

and 2B, the program lists four possible drugs, which are readily differentiated by close inspection of the TLC plate for relative R_f values of the three benzodiazepines and carbamazepine, all having been present in the calibrators applied. In case 2, SPOT CHEK selected dextromethorphan as the only choice after plates 2A, 2B, and 2C were developed, reacted, and read. The acid-neutral side of the reaction system (plate 1A and 1B) was not needed to select dextromethorphan: Basic drugs can usually be identified with plates 2A, 2B, and 2C, whereas benzodiazepines and various neutral compounds can be detected and identified on Plate 1. The third set of plates (case 3) initially used plate 2A, which resulted in 12 possible identifications. Adding plate 2B shortened the selection list to 2 substances but did not reduce to 1 "hit." Guaifenesin and methocarbamol can be differentiated with *p*-dimethylaminobenzaldehyde, because methocarbamol is a carbamate and guaifenesin is not.

Some drugs can be selected on the basis of only one TLC plate, e.g., case 4 in Table 5. The absence of UV absorbance and the charring with FPN reagent are highly characteristic of erythromycin. The last three searches in Table 5 resulted in single-drug listings: case 5, azacyclonol; case 6, lidocaine metabolite (monoethylglycinexylidine or MEG-X), and case 7, amiodarone. Not all reactions were required for the specific identification of these basic drugs.

Discussion

By accumulating TLC information systematically in a PC database, we have developed a convenient program, meaningful to chemists, for assisting in the detection and identification of large numbers of drugs, as would be sought in general unknown screening. The approach can be applied to urine extracts, serum/blood extracts, solid dosage forms (e.g., tablets, pills), and mystery powders. The logic of the SPOT CHEK technique is towards discovery of drug classes (e.g., benzodiazepines, barbiturates), functional groups (primary, secondary, and tertiary amines), and conjugation (as in aromatic structures) by fluorescamine, FPN, and Dragendorff reagents and UV absorbance. Color reactions lend high specificity to the technique. Speed (bench time) and sensitivity (detection of low nanogram quantities) are not sacrificed by the TLC technique described. Moreover, new drugs can be readily added to the database: Most drugs will react in some way with one of the reagents used. Drug identifications in simple one- or two-drug urine specimens, such as those obtained in proficiency tests, are readily accomplished by the TLC technique presented here. However, experience with the technique is required when several drugs—and their metabolites—are present in a urine extract. SPOT CHEK can be used for selective drug screens by using the most sensitive or selective reagents for the drugs that are to be included in screening.

The technique described here can be carried out without the assistance of the computer program. Advantages of the TLC technique alone are: (*a*) in a single developing system, plates provide good separations for drug substances and have a high peak (spot) capacity; (*b*) plates are convenient to use because ruggedness of the layer allows dips and sprays to be applied without disintegrating the layer; (*c*) the last reaction of each sequence can be read weeks after it has been carried out; (*d*) reagents used will detect drugs in quantities <100 ng/spot, in some cases as little as 5 ng/spot; (*e*) scored plates are efficient and economical; (*f*) reagents are stable and do not need to be made up daily, (*g*) new drugs can be readily added to the total screen, and (*h*) the drug recovery technique is flexible; i.e., laboratories can continue in-house procedures or use the one described here. However, the tedious and somewhat subjective interpretation of TLC results can now be made faster and more objective by utilization of a versatile but simple computer program SPOT CHEK.

In the two papers published on the use of a computer for storage and retrieval of TLC data *[8, 13]*, both studies rely on R_f determinations in several mobile-phase solvents, thereby increasing analysis time. The SPOT CHEK program, however, has the following advantages: (*a*) from 1 to 10 TLC characteristics (identification variables) can be entered, depending on how many reactions were done (i.e., partial use of the system is feasible); (*b*) the program works in practical situations, as demonstrated by several years of experience with >15 000 full drug screens from clinical and forensic referrals; (*c*) the program will often list results by drug class; (*d*) the identification technique is reaction-based and visual and does not require tedious measurements of migration positions or the use of more than one mobile phase. The program is also very helpful in training analysts in TLC and in understanding drug chemistry. Finally, and most importantly, the computer-assisted interpretation of results obviates much of the tedium involved in broad-spectrum drug screening.

In conclusion, the extent and degree of confirmation of drug substances identified or selected by the TLC technique described here depend on how the results are to be used. The decision of how and when to confirm drug finding will depend on laboratory protocols and on the chemist's perspective, experience, and insight. Some of the drugs in the SPOT CHEK database and card file are uniquely defined by their TLC reactions and metabolite patterns. The approach of Ojanpera and Vuori *[4]*, who presented TLC identifications by scanning individual spots in situ in the UV range, is especially compatible with the SPOT ID reactions and SPOT CHEK program presented here because the UV spectrum can be obtained nondestructively. With a "hit" list from a UV scan, selective SPOT ID reactions can follow. TLC offers an excellent first step in broad-spectrum drug screens and in general unknown protocols; once applied to the plate, the sample is not lost (unless volatile). The techniques to corroborate TLC findings can be intelligently selected because (after TLC) the analyst will know something

about the functional groups, aromaticity, polarity, and often the likely identification of the unknown(s) before the confirmatory step. Non-TLC confirmatory techniques appropriate in clinical and forensic toxicology include immunoassay (RIA, FPIA, Emit), UV spectrophotometry, gas and liquid chromatography, and GC/MS.

We dedicate this paper to the memory of E. Paul Elson, the PC specialist and author of the original SPOT CHEK software program written in dBase IV. Revisions of the program were made by us.

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