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The General Toxicology Unknown I. The Systematic Approach

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ABSTRACT: The general toxicology unknown often presents challenges and interests to toxicologists. A systematic analytical approach to search for drugs or poisons is presented here. The preliminary screening analyses were as follows: alcohol by gas chromatography (GC), ethchlorvynol colorimetric analysis, enzyme multiplied immunoassay technique (EMIT), basic drug screening by GC, and neutral and weakly acidic drug screening by GC. Other additional analyses were performed depending on the special circumstance of each individual case and the results of these preliminary analyses. Positive findings were confirmed by computerized gas chromatography/mass spectrometry when practical. Quantitation was performed by GC whenever possible.

KEYWORDS: toxicology, chromatographic analysis, screening procedures, general toxicology unknown, systematic approach, mass spectrometric analysis

The general toxicology unknown often presents challenges and interests to toxicologists [*1-4*]. Many analytical methods have been developed for screening, confirmation, and quantitation of drugs and poisons in human blood and tissues [*1-34*]. Which of these methods should be included in an analytical approach for the general toxicology unknown depends largely on an individual laboratory's expertise, personnel, instrumentation, and needs [*1,4*].

In Cook County, IL, there have been an average of 4200 toxicology cases annually. The systematic approach used for the general toxicology unknown is presented here.

Equipment and Methods

Equipment

A Beckman Model 25 spectrophotometer with a thermal control, sipper system, and Model 701 timer-printer was used for all enzyme-multiplied immunoassay technique (EMIT) assays. A Beckman ACTA M VI spectrophotometer was used for the colorimetric analyses.

Four computerized Hewlett-Packard 5840A gas chromatographs (GC), equipped with either a flame ionization detector (FID), a nitrogen-phosphorus detector (NPD), or a ^{63} Ni electron capture detector (ECD), were used for the GC analyses.

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A Finnigan 3200 gas chromatograph/mass spectrometer (GC/MS) with an Incos 2300 data system was used for the GC/MS analyses. An Eberbach horizontal shaker was used for the solvent extraction. A Brinkmann concentrator was used for the evaporation of solvent.

Methods

All standards used in the following experiments were authentic standards obtained from either various manufacturers or from U.S. Pharmacopeial Convention, Inc., Rockville, MD.

Alcohol Analysis—Five millilitres of postmortem specimens (blood, urine, bile, or tissue homogenate) were steam-distilled [*35*] with 5 mL of 1-propanol (160 mg/dL) internal standard. Twenty-five millilitres of steam distillate were collected. Two microlitres of the steam distillate were injected into a GC. Five millilitres of 160 mg/dL ethanol standard were also processed accordingly and used as a calibration standard for ethanol GC analysis. The GC column was a 1.2-m (4-ft) by 2-mm inside diameter glass column packed with 0.2% Carbowax 1500 on Carbopack C, 80-100 mesh (Hewlett-Packard, Avondale, PA). Conditions for GC were as follows: column temperature, 80°C; injector temperature, 130°C; FID temperature, 150°C; and nitrogen carrier gas flow rate, 30 mL/min.

Ethchlorvynol Screening—Part of the steam distillate from alcohol analysis was used for the diphenylamine colorimetric method described by Finkle and Bath [*36*]. Absorbance at 510 nm was measured for each of the samples and two ethchlorvynol standards (30 and 100 mg/L).

EMIT Assays—The EMIT reagents were obtained from Syva in Palo Alto, CA. Manufacturer's assay procedures were followed. Urine was assayed without any prior treatment. Bile, blood, or tissue homogenates (1 or 2 mL) were extracted and assayed. The extraction procedures were similar to those described in the following sections for basic and weakly acidic drugs, except that acid hydrolysis and pH 8.7 extraction were performed for opiate assay, and the residue was reconstituted with 200 μL of EMIT buffer solution.

Basic Drug Screening—In a 15-mL screw cap glass culture tube, 2.0 mL of blood, bile, urine, or tissue homogenate spiked with 20 μL of diphenhydramine (100 mg/L) internal standard were made basic with 200 μL of concentrated ammonium hydroxide and 1 mL of pH 10 carbonate/bicarbonate buffer (1.0*M*), and extracted with 10 mL of hexane:ethyl acetate (1:1). After centrifugation, the solvent was pipetted into a 15-mL screw cap glass culture tube. Two millilitres of 1.0*N* sulfuric acid were then added and the mixture was shaken for 5 min. After centrifugation, the solvent layer was aspiratd and the acid layer was transferred to a 15-mL screw cap glass culture tube. The acid layer was made basic with 300 μL of concentrated ammonium hydroxide and 1 mL of pH 10 carbonate/bicarbonate buffer (1.0*M*) and was extracted with 10 mL of hexane:ethyl acetate (1:1) for 5 min. After centrifugation, the solvent was transferred to a 30-mL Brinkmann concentrator cup and evaporated to dryness at 50°C on a Brinkmann concentrator. The residue was reconstituted with 20 μL of ethyl acetate and 1 μL was injected into the GC. A 1.2-m (4-ft) by 2-mm inside diameter glass column packed with 3% OV-101 on Chromosorb WHP, 80-100 mesh (Hewlett-Packard, Avondale, PA), connected to an FID was used as the initial GC screening column. Conditions for GC were as follows: column temperature, 190°C isothermal for 2 min, then 20°C/min to 240°C, and then isothermal for 5 min or longer; injector temperature, 275°C; FID temperature, 275°C; and nitrogen carrier gas flow rate, 30 mL/min. A 1.2-m (4-ft) by 2-mm inside diameter glass column packed with 5% OV-225 on Chromosorb WHP, 80-100 mesh (Hewlett-Packard, Avondale, PA), connected to an NPD was used for presumptive confirmation.

Neutral and Weakly Acidic Drug Screening—In a 15-mL screw cap glass culture tube, 1.0 mL of blood, bile, urine, or tissue homogenate was spiked with 20 μL of phensuximide (1000-mg/L) internal standard, made neutral with 1 mL of pH 7.0 carbonate/bicarbonate buffer (1.0*M*), and extracted with 5 mL of ethyl acetate for 3 min. After centrifugation, the

organic layer was pipetted into a 15-mL screw cap glass culture tube and brought to dryness on a water bath. Two millilitres of 0.1*N* hydrochloric acid and 0.5 mL of hexane were then added and the mixture was shaken for 3 min. After centrifugation, the solvent was aspirated and the aqueous layer was transferred to a 15-mL screw cap glass culture tube. Five millilitres of chloroform were added to the tube and the mixture was shaken for 3 min. After centrifugation, the chloroform layer was transferred to a 30-mL Brinkmann concentrator cup and evaporated to dryness at 50°C on a Brinkmann concentrator. The residue was reconstituted with 50 μL of ethyl acetate and 1 μL was injected into the GC. The GC conditions were the same as described for basic drug screening, except the OV-101 column temperature was 160°C isothermal for 1 min, then 30°C/min to 250°C, and then isothermal for 2 min or longer. A quantitative standard of meprobamate (50 mg/L), glutethimide (10 mg/L), and methaqualone (10 mg/L) was extracted and analyzed accordingly with each set of biological specimens.

GC/MS Search—The appropriate solvent extraction procedure as described above for GC was used with another aliquot of the biological specimen. The residue was reconstituted with 20 μL of ethyl acetate. One microlitre was injected into a gas chromatograph/mass spectrometer (GC/MS). An 0.6-m (2-ft) by 2-mm inside diameter glass column packed with 5% OV-1 on Chromosorb-WHP, 80-100 mesh (Hewlett-Packard, Avondale, PA), was used. The conditions depended on the retention times of the unknown peaks detected from the previous GC screening. The helium carrier gas flow rate was 20 mL/min. The quadrupole mass spectrometer was operating in the electron impact (EI) mode with an electron energy of 70 eV. All GC/MS data were stored and processed with an Incos 2300 data system containing a computer library of more than 25 000 EI mass spectra.

Results and Discussion

Our systematic approach for the general toxicology unknown is outlined and discussed below. The first five categories of analyses are performed initially. The sixth through the eighth category follow, depending on the results of the first five categories of analyses and the special circumstance of each individual case.

Blood Alcohol Analysis by GC

The retention times obtained under our GC conditions for methanol, ethanol, acetone, isopropanol, and 1-propanol (internal standard) were 0.18, 0.33, 0.42, 0.63, and 0.91 min, respectively. This analysis was qualitative as well as quantitative. Ethanol concentration of at least one more tissue or fluid was performed when blood ethanol concentration exceeded 100 mg/dL.

Ethchlorvynol Screening by Colorimetry

The diphenylamine colorimetric method [*36*] was performed on an aliquot of the steam distillate from alcohol analysis.

EMIT

Those drugs routinely screened for included barbiturates, opiates, amphetamines, phencyclidine, methadone, propoxyphene, cocaine metabolites, and benzodiazepine metabolites. When urine was not available, bile, blood, or tissue homogenate were extracted and assayed. The extracted specimens generally provided increased sensitivity and a cleaner sample for analysis [*30*].

Basic Drug Screening by GC

With each set of specimens analyzed, a qualitative mixture of some common basic drugs was injected. These included the following compounds, with their respective retention times shown in parentheses: diphenhydramine (1.15 min, internal standard), phencyclidine (1.31 min), tripelennamine (1.71 min), chlorpheniramine (1.86 min), dexbrompheniramine (2.55 min), methadone (2.91 min), propoxyphene (3.23 min), imipramine (3.39 min), pentazocine (3.73 min), codeine (4.19 min), diazepam (4.50 min), chlorpromazine (4.83 min) and flurazepam (6.70 min). Sensitivity was approximately 0.2 to 0.5 mg/L for the Group I basic drugs.

Relative retention times, or the relative eluting order, for most commonly used GC columns can be found in the literature [*4,5,7–9,11,13–15*] and in our own toxicology files. Under the conditions described for OV-101 column, meperidine and caffeine eluted before the internal standard diphenhydramine; amoxapine [*33*] eluted after chlorpromazine and before trifluoperazine; maprotiline coeluted with codeine; doxepin, desipramine, and protriptyline eluted after imipramine and before pentazocine; and amitriptyline, cocaine, and propoxyphene coeluted. In every case, when the presence of a basic drug was suspected from the gas chromatogram, a qualitative drug standard, if not already included in the original basic drug mixture, was injected on the same day to compare the retention time of the suspected peak with that of the standard. A 5% OV-225 column was used routinely to confirm presumptively those peaks detected by using the 3% OV-101 screening column. For example, a peak was observed at the retention time of amitriptyline from the OV-101 gas chromatogram. This only suggested the possible presence of amitriptyline, propoxyphene, or cocaine. The specimen was injected into the OV-225 column; at a 200°C column temperature, the retention times for propoxyphene, amitriptyline, nortriptyline, and cocaine were 1.07, 1.14, 1.55, and 1.96 min, respectively. A qualitative standard of propoxyphene, amitriptyline, nortriptyline, and cocaine was injected into the OV-225 column to compare the retention time of the specimen with that of the standard. Possible identity of the peak could be established after this two-column presumptive confirmation method.

Caution must be exercised when using these results without any other confirmatory measures. This is especially true when multiple peaks are seen in the gas chromatogram.

Neutral and Weakly Acidic Drug Screening by GC

This was designed to be a qualitative and quantitative analysis for some commonly encountered neutral drugs, such as meprobamate, glutethimide, and methaqualone. The retention times obtained from the GC conditions were 1.27, 2.02, 2.18, and 3.38 min for phensuximide (internal standard), meprobamate, glutethimide, and methaqualone, respectively. Caffeine was coeluted with meprobamate. Separation of meprobamate (4.26 min) and caffeine (2.88 min) was achieved on the OV-225 column at 200°C. This example further illustrates the importance of the two-column presumptive confirmation method. Sensitivity for these compounds was in the range of 1 to 5 mg/L. In addition to other neutral drugs (such as methyprylon), barbiturates and phenytoin also appeared in this GC screening. Therefore, this occasionally served as a double check for the EMIT barbiturate assay. The relative retention times of neutral and weakly acidic drugs for most commonly used GC columns can also be found in the literature [*4,5,7,8,12,13,15*] and in our toxicology files. A qualitative standard was always injected on both an OV-101 and an OV-225 column to compare the retention time of the specimen with that of the standard.

Other Analyses

The extent of a comprehensive search in this category varies greatly from case to case. The analyses for specific substances may be dictated to a certain extent by the case history or a pathologist's request or both. It could include a carbon monoxide analysis (usually performed

on a stat basis), or more screening by GC or by GC/MS with or without prior hydrolysis or derivatization procedures. It could also include a GC/MS search to follow up some unknown peak(s) revealed from previous GC screening, or a heavy metal or cyanide analysis. The type of test could include any other practical analyses that were needed for each particular case. The procedures used are those previously published [*4,5,16,17,20*], or modifications thereof.

Confirmation

All EMIT results were further presumptively confirmed by GC on both an OV-101 column and an OV-225 column. All substances detected by GC screening were also first presumptively confirmed on a second column. All qualitative confirmations were performed on a GC/MS when practical. Acceptable criteria for GC/MS confirmation should include the mass spectrum, the retention time, and the extraction characteristics of the peak in the specimen. All the above criteria must be consistent with those of the standard [*16,20*]. Standards were extracted and analyzed by GC/MS with each set of biological specimen. Electron impact mass fragmentography was used for the confirmation and quantitation of free morphine and codeine in blood [*34*].

Quantitation

Quantitation was performed with internal standard methods on GC whenever possible. The FID, NPD, and ECD were used whenever appropriate. The choice of internal standard depended on the retention time and chromatographic and extraction characteristics of the drugs being quantitated [*10*]. Quantitation was based on the peak area ratio of each specific drug to the internal standard. All biological specimens were properly diluted to fall within the linear range for each specific drug. With each set of biological specimens analyzed daily, a standard of appropriate concentration was processed accordingly, and its GC response was used for the automatic quantitation for that day. The average of two determinations was reported. Quantitation was performed on blood and at least one more fluid or tissue.

The above systematic approach has served our needs successfully in dealing with medical examiner's cases, as well as toxicology proficiency testing samples from the College of American Pathologists, the Center for Disease Control, and the Center for Human Toxicology.

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