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Aviation Accident Forensic Assessment: Comprehensive Single-Extraction Urine Screening Procedure

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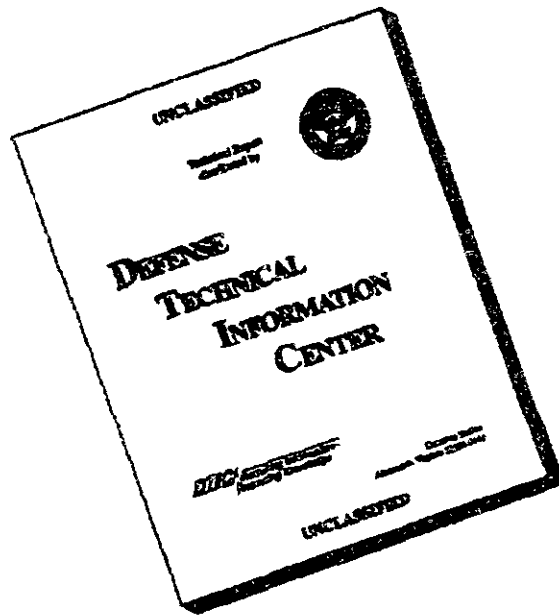
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16. Abstract One of the missions of the Civil Aeromedical Institute (CAMI), Office of Aviation Medicine (OAM), Federal Aviation Administration (FAA), is to help assess the role of potential medical or drug related pilot impairment in aviation accidents. This requires the ability to identify a wide range of drugs and the medical conditions for which these drugs are prescribed. A single extraction screening procedure was developed to identify as many drugs as possible in urine, with minimal effort and cost. Triamterene, a diuretic, is easily identified using HPLC and TLC, whereas it can not be easily identified using standard GC Mass Spectroscopy. On the other hand, atenolol is easily identified using HPLC and Mass Spectroscopy, but is not detected using TLC at therapeutic levels. Methadone is difficult to detect using HPLC with a photo diode array and fluorescence detector, but can easily be detected using TLC and confirmed by mass spectroscopy. Urine specimens are hydrolyzed using β -glucuronidase, and the specimen is then extracted using commercially purchased TOXI-LAB A® extraction tubes. After removing the TOXI-LAB® organic phase, the remaining aqueous layer is washed with chloroform. The organic phase and the chloroform wash are combined, evaporated to dryness, and then reconstituted in 50 μ L of a methanol solution. A 10 μ L quantity of the methanol solution is then chromatographed, using HPLC with a photo diode array and fluorescence detector. The methanol solution is next analyzed using either TOXI-LAB® or GC Mass Spectroscopy, depending on the compound detected using HPLC. When HPLC is negative, the extract is analyzed using the TOXI-LAB® procedure. This method has proven successful in the identification of benzodiazepines (alprazolam, temazepam, diazepam, nordiazepam, and oxazepam), cardiovascular medications, antihistamines, and other drugs that could have been missed using only one of the methods listed above. The number of positive benzodiazepines identified has increased as a result of using this new method, and the time required to complete cases has been reduced substantially. Using a single aliquot, extraction, and analysis of a urine specimen by several different methods makes it possible to screen for a wide variety of drugs. Screening for these drugs would typically require several different sample aliquots and procedures. The new procedure helps prevent false negative results, that might lead investigators in aviation accidents to the wrong conclusions. The fact that drugs can be uniquely identified by HPLC, and then confirmed using GC mass spectroscopy, reduces the time necessary to complete cases by reducing the number of aliquots needed to specifically identify the drugs present in the body.			
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AVIATION ACCIDENT FORENSIC ASSESSMENT: COMPREHENSIVE SINGLE-EXTRACTION URINE SCREENING PROCEDURE

INTRODUCTION

The Civil Aeromedical Institute (CAMI), Office of Aviation Medicine (OAM), Federal Aviation Administration (FAA), is required under federal law 100-591 [H.R.4686] to help assess the role of potential medical or drug related pilot impairment in aviation accidents. This includes the identification of abused drugs such as cocaine, amphetamines, and benzodiazepines; or prescription drugs such as cardiovascular and neurological medications. Finally, over-the-counter drugs such as pseudoephedrine, chlorpheniramine, and diphenhydramine must be identified. This requires the ability to extract and identify a wide range of drugs and to identify the medical conditions for which these drugs are prescribed.

It is essential for the full-service FAA Forensic Toxicology laboratory to identify a wide variety of drugs, with the least amount of specimen and analysis time. In addition, screening procedures should limit the number of presumptive false positive tests. Positive screening tests require additional aliquoting and complex analytical tests that require additional work and cost. In all cases there are only limited specimens available for testing, and presumptive false positive screening tests may make it impossible to confirm true positive results. The cost in time and money required for testing false positive results is enormous. The CAMI laboratory utilizes fluorescence polarization immunoassay (FPIA), to screen urine for abused drugs (marihuana, cocaine, amphetamine, methamphetamine, opiates, barbiturates, PCP (phencyclidine), and benzodiazepines). Theophylline, phenytoin, salicylates, and acetaminophen are also screened by FPIA. Other prescription and over-the-counter drugs are screened using a combination of high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and Mass Spectroscopy.

The pilot's drug history is usually not available to assist in selecting the proper analytical procedure for testing. Therefore, a single extraction and screening procedure was developed to identify as many drugs and other foreign substances as possible in urine with minimal effort and cost.

Extraction procedures were evaluated to identify the optimum extraction for the isolation of a wide range of drugs from urine. No single analytical procedure is capable of screening for all drugs. Triamterene, a diuretic, is easily identified using HPLC and TLC, whereas it can not easily be identified using standard GC Mass Spectroscopy. On the other hand, atenolol is easily identified using HPLC and Mass Spectroscopy, but is not identified using TOXI-LAB at therapeutic levels. TOXI-LAB® may identify drugs that would be difficult to detect using HPLC with a photo diode array and fluorescence detector. Many drugs, however, may easily be detected using TOXI-LAB and confirmed by mass spectroscopy.

METHOD

Three extraction procedures were evaluated for possible use in the extraction of drugs for the initial screen. All urine specimens were hydrolyzed using β -glucuronidase before extraction. Initially specimens were extracted using commercially purchased TOXI-LAB A® extraction tubes. After removing the TOXI-LAB® organic phase, the remaining aqueous layer was washed with chloroform to extract atenolol which does not extract well with the standard TOXI-LAB A® procedure. The organic phase and chloroform were combined, evaporated to dryness, and then reconstituted in 50 μ L of a methanol solution; 10 μ L is then injected in the HPLC (Figure 1). Originally a 1:3 chloroform/methanol solution was used to reconstitute

Figure 1. Methanol reconstituted specimen.

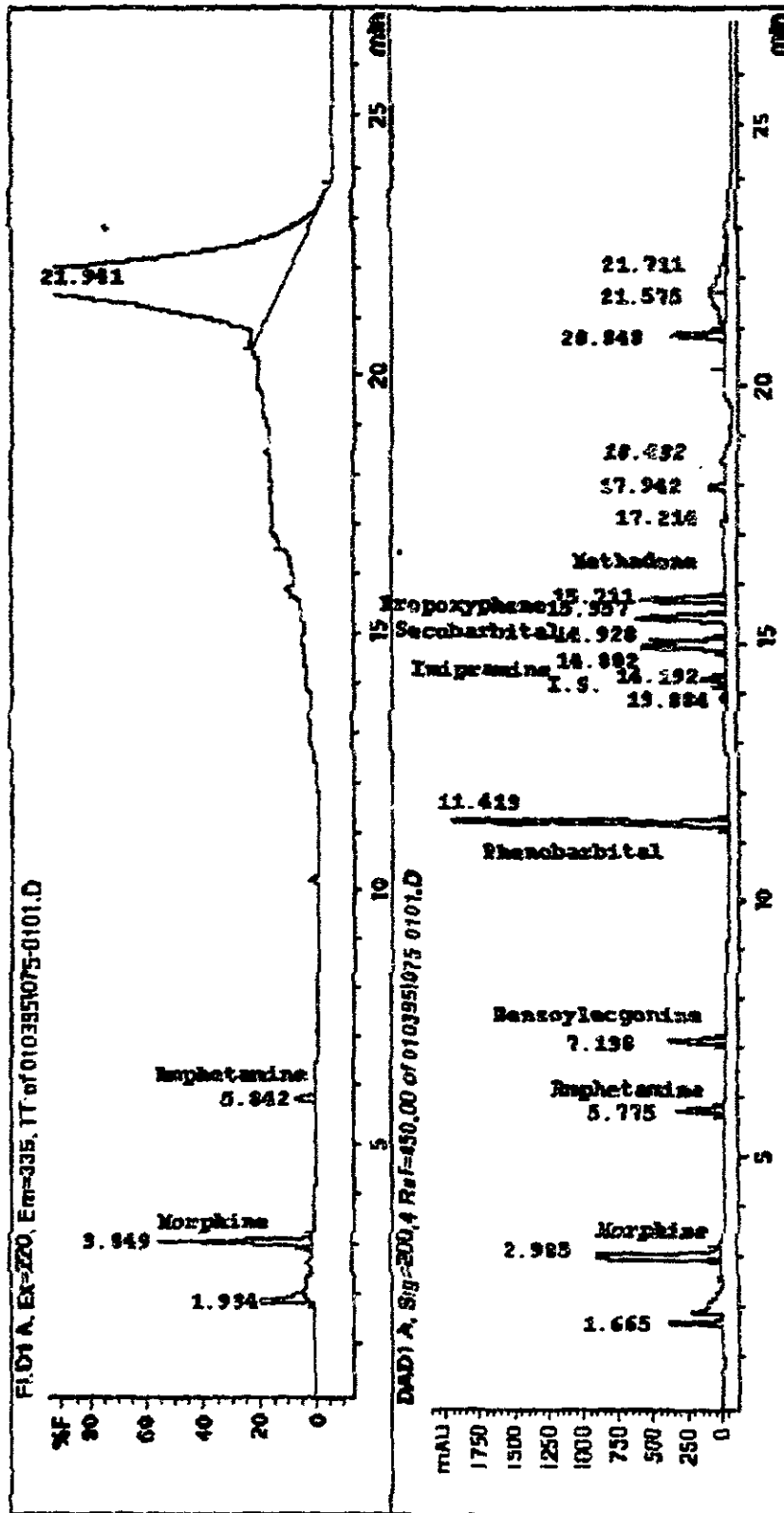
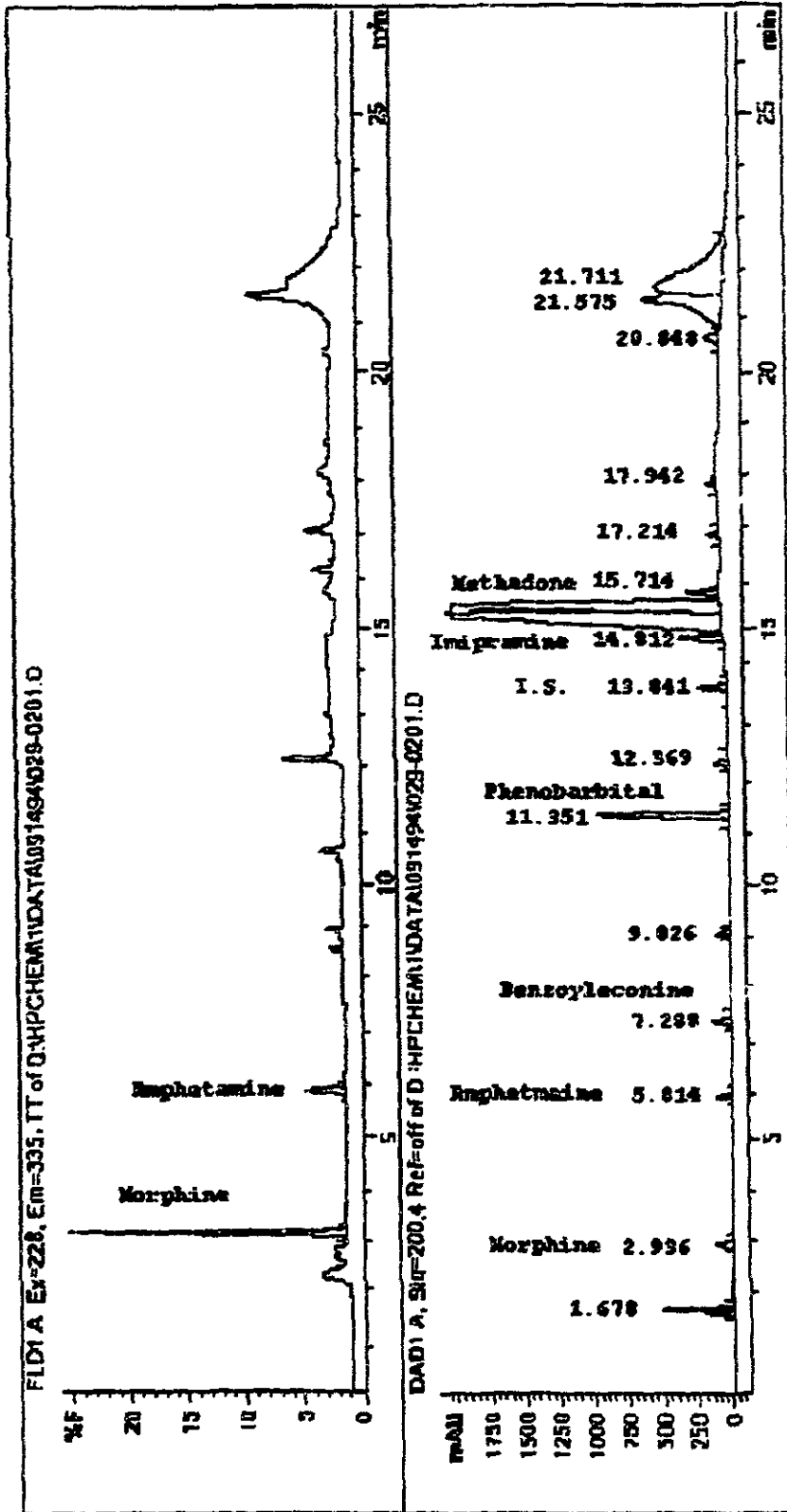


Figure 2. Extract reconstituted in chloroform/methanol 1:3 showing chloroform peak at ≈ 15 mins



the sample for injection; however, it was determined that the chloroform was destroying the HPLC stationary phase, and causing a large peak at ~ 15.0 mins (Figure 2).

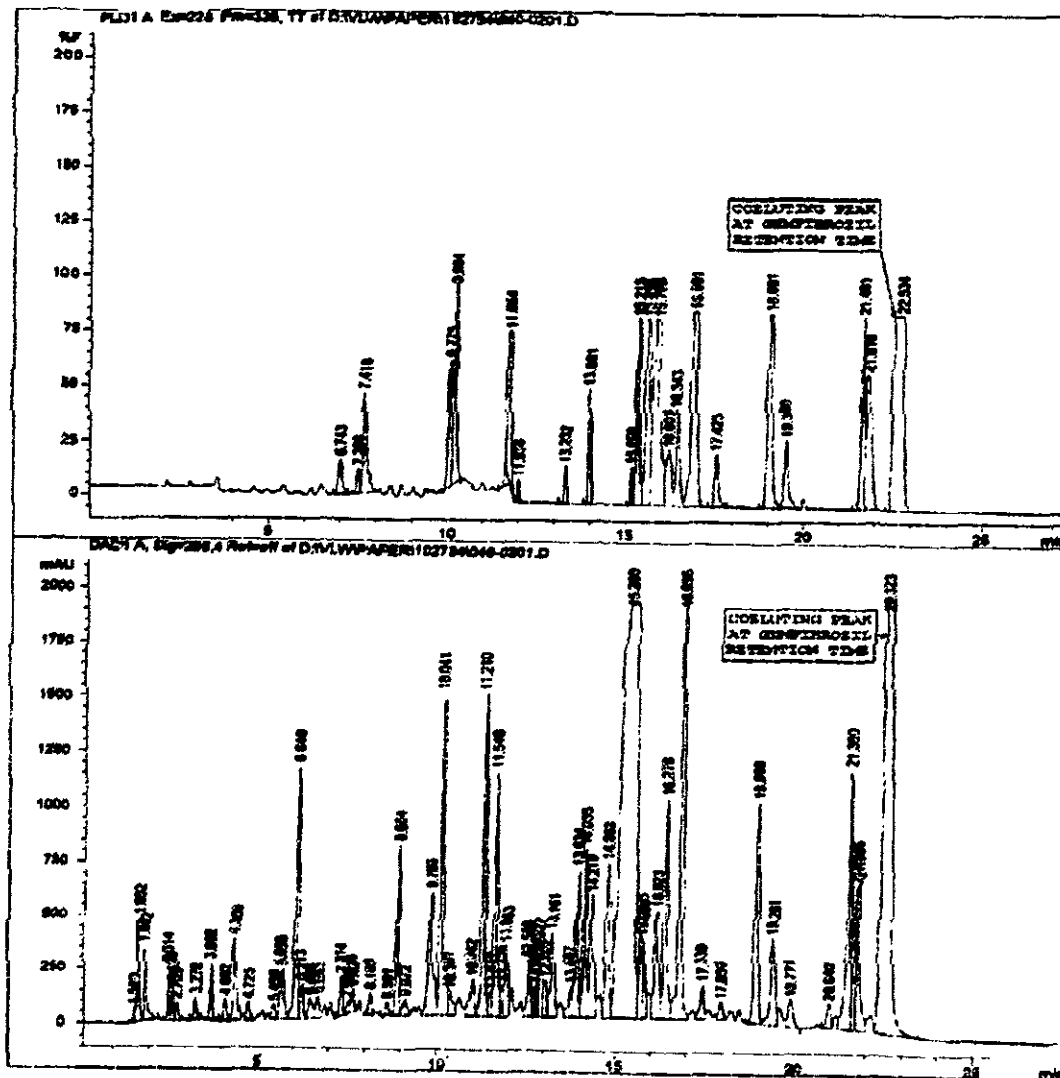
The TOXI-LAB A® extraction tubes had a high recovery of the compounds of interest and high reproducibility. However, the TOXI-LAB A® extraction had a high background (Figure 3) which made it difficult to evaluate the chromatogram.

Two solid phase extraction procedures were evaluated for possible replacement of the TOXI-LAB A® extraction tubes. A Bond Elut Certify™ LRC procedure was evaluated (1), but several drugs of interest could not be extracted with this procedure. The procedure that extracted the widest range of drugs was the ABN Bond Elut Certify™ LRC procedure (2), used

for the sequential extraction of acidic/neutral and basic drugs. This procedure utilizes Varian Analytichem Bond Elut Certify LRC, solid phase extraction columns.

Prepare specimen by pipetting 5 mLs of urine into a test tube. Add internal standard (I.S.) and adjust urine pH to 5.0. Pipet 5000 units *P. vulgaris* β glucouronidase, (Sigma), and incubate at 60-65° C for 2 to 3 hours. Centrifuge specimen at 2000 rpm, and pour off supernatant. Pipet 2 to 3 mLs 0.1M phosphate buffer, pH 6.0 into specimen tube and vortex sample. Condition the Bond Elut Certify LRC extraction column with 2 mLs of methanol, then 2 mLs of 0.1M phosphate buffer, pH 6. Note: DO NOT ALLOW CARTRIDGE TO RUN DRY! Add prepared samples to cartridges using no more than 5

Figure 3. Example of background from TOXI-LAB®



in. Hg vacuum. Wash the column with 1 mL of 80/20% 0.1M phosphate buffer, pH6/methanol solution. Under full vacuum, dry the column for 5 min. Rinse the column with 1 mL 1.0M acetic acid. Dry the column under full vacuum for 10 min. Rinse the column with 1 mL hexane. Elute the acidic/neutral drugs with 4 mLs methylene chloride into labeled conical tubes and cap. The acidic/neutral extract is only used if acidic drugs are found by immunoassay. Rinse the column with 6 mLs of methanol. Elute the basic drugs with 2 mLs of 2% ammonium hydroxide in ethyl acetate, by gravity, into labeled conical tubes.

Evaporate the extracts in an N-Evap, under slow nitrogen, to dryness. Reconstitute with 50mL methanol. Pipet the methanol solution into a labeled autosampler vial and crimp seal. Inject 10µL into HPLC.

The instrumentation used was a Hewlett Packard 1090 Series II HPLC equipped with photo diode array detector and fluorescence detector linked to a 3-D DOS Chemstation. The Merck column, by E.M. Separations, was a Lichrospher 60 RP-select B 3µm, 250 x 4mm. The HPLC procedure utilized was reported by Logan (4). The times, excitation, and emis-

Table 1. The Excitation and Emission wavelengths

TIME	Drug Detected	Excitation (nm)	Emission (nm)
0	Morphine	228	335
3.3	Atenolol	228	308
4.2	Pseudoephedrine	230	315
5.0	Nadolol	198	303
7.0	Triamterene	231	427
8.0	Metoprolol	228	305
9.6	Quinine/Quinidine	246	440
10.5	Oxprenolol	229	315
10.8	Labetalol	190	417
11.5	Propranolol/ Alprenolol/ Dextromethorphan	229	340
12.5	Diltiazem	232	340
14.0	Oxazepam	230	310
14.8	Verapamil	229	314
15.7	Fluoxetine	228	303
16.0	Risperine/ Naproxen	229	350

sion wavelengths used to detect cardiovascular drugs, and other drugs that have a strong fluorescence, can be found in Table 1.

The remaining 40µL of methanol solution is analyzed using either TOXI-LAB®, or GC Mass Spectroscopy, depending on the compound detected using HPLC. When the HPLC screen is negative, the remaining 40µL of extract is analyzed using the TOXI-LAB® procedure. TOXI-LAB® is used to identify drugs that can easily be detected and differentiated by TLC, such as quinine, quinidine, acetaminophen, and nicotine. TOXI-LAB® is also used for drugs that cannot be run using GC/MS, such as triamterene. All other positive HPLC samples are confirmed by GC/Mass Spectroscopy. After identification of the drugs in urine, blood is accessioned, and the drugs are identified and quantitated in blood, if present.

RESULTS

A comparison of the relative amount of drug recovered for each extraction procedure can be seen in Table 2. Based on these results, it was determined that

the sequential extraction of acidic/neutral and basic drugs procedure (ABN) would be adopted for use on actual case work.

This new procedure was initiated in April of 1994, and has proven successful in the identification of benzodiazepines (alprazolam, temazepam, diazepam, nordiazepam, and oxazepam). Cardiovascular medications, antihistamines, and other drugs have also been detected. These drugs could have been missed if only one of the methods listed previously had been used.

Several pilots were found to have beta blockers, which would have been missed using a standard TOXI-LAB® screen. One beta blocker identified in a pilot, using the new method, was nadolol, a cardiovascular medication used to control hypertension. The drug was found using HPLC (Figure 4) and confirmed using mass spectroscopy (Figure 5). The blood of this pilot was found to contain 0.096 µg/mL of nadolol. It was later determined through interviews that the pilot was prescribed 40mg nadolol (Corgard®) once per day. It is unlikely this drug would have been identified, at the reported concentration, using only the standard TOXI-LAB® procedure.

**Table 2. Ratio of Drug to External Standard (Normalized to 100)
For Three Extraction Procedures**

Drug	TOXI-LAB®/Chloroform	ABN	SPE
Morphine	75	98	100
Amphetamine	21	28	100
Benzoyllecgonine	100	42	None Detected
Propoxyphene	50	100	76
Imipramine	61	100	82
Methadone	46	100	55
Phenobarbital	100	14	None Detected
Secobarbital	100	12	None Detected
Atenolol	95	100	51
Oxazepam	43	63	100

Figure 4. HPLC chromatogram of extracted nadolol sample.

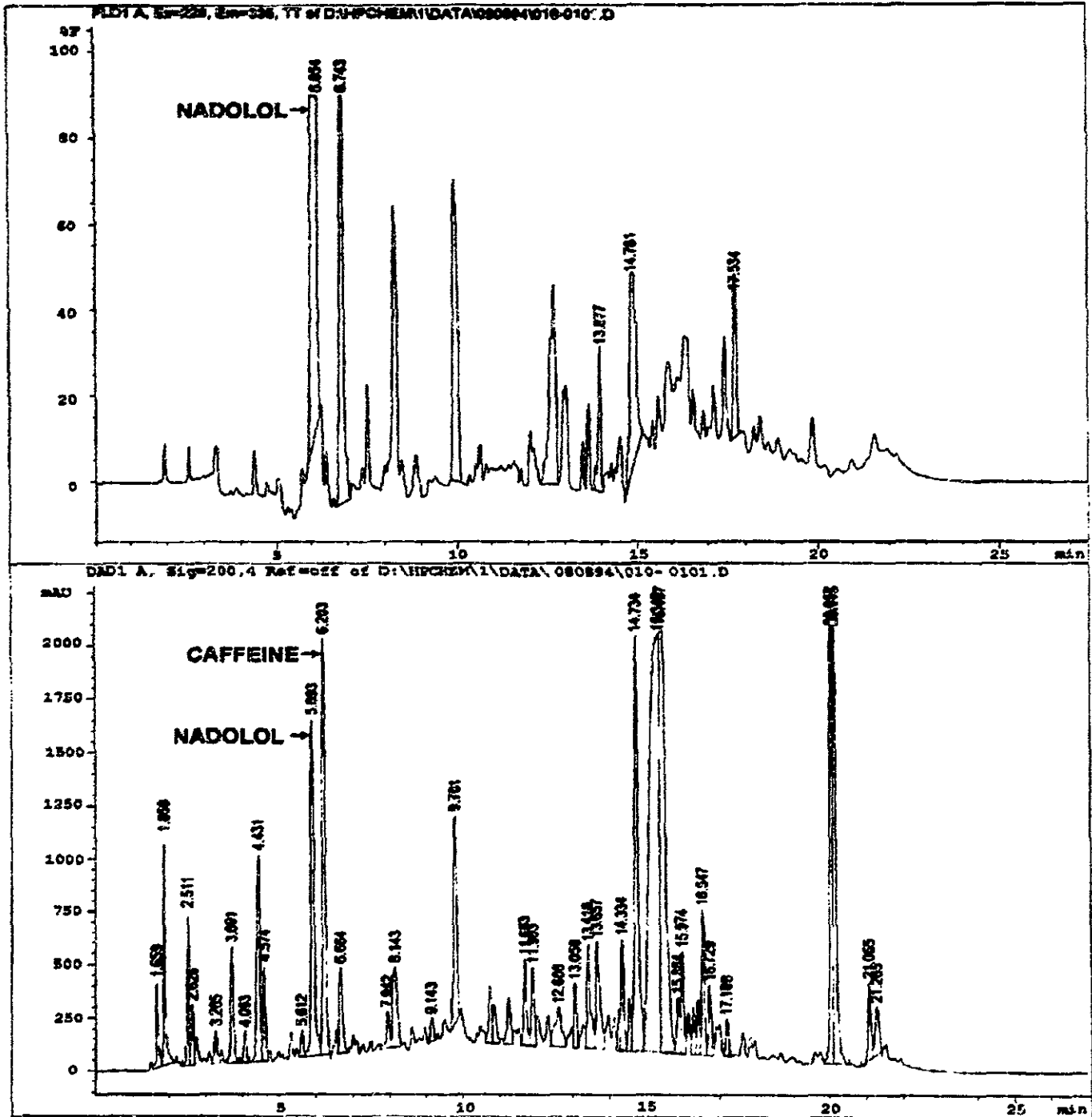
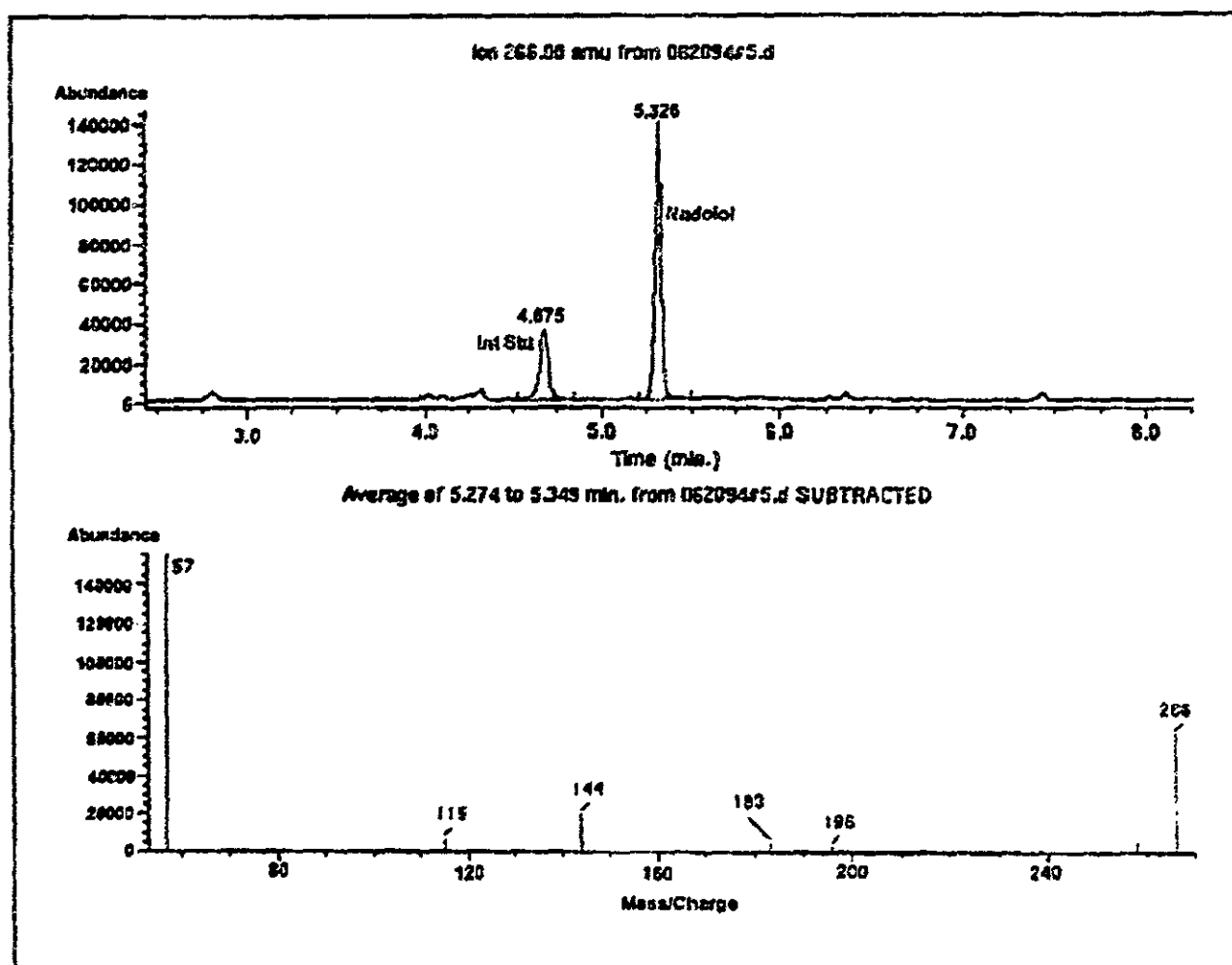


Figure 5. Nadolol detected using GC/MS

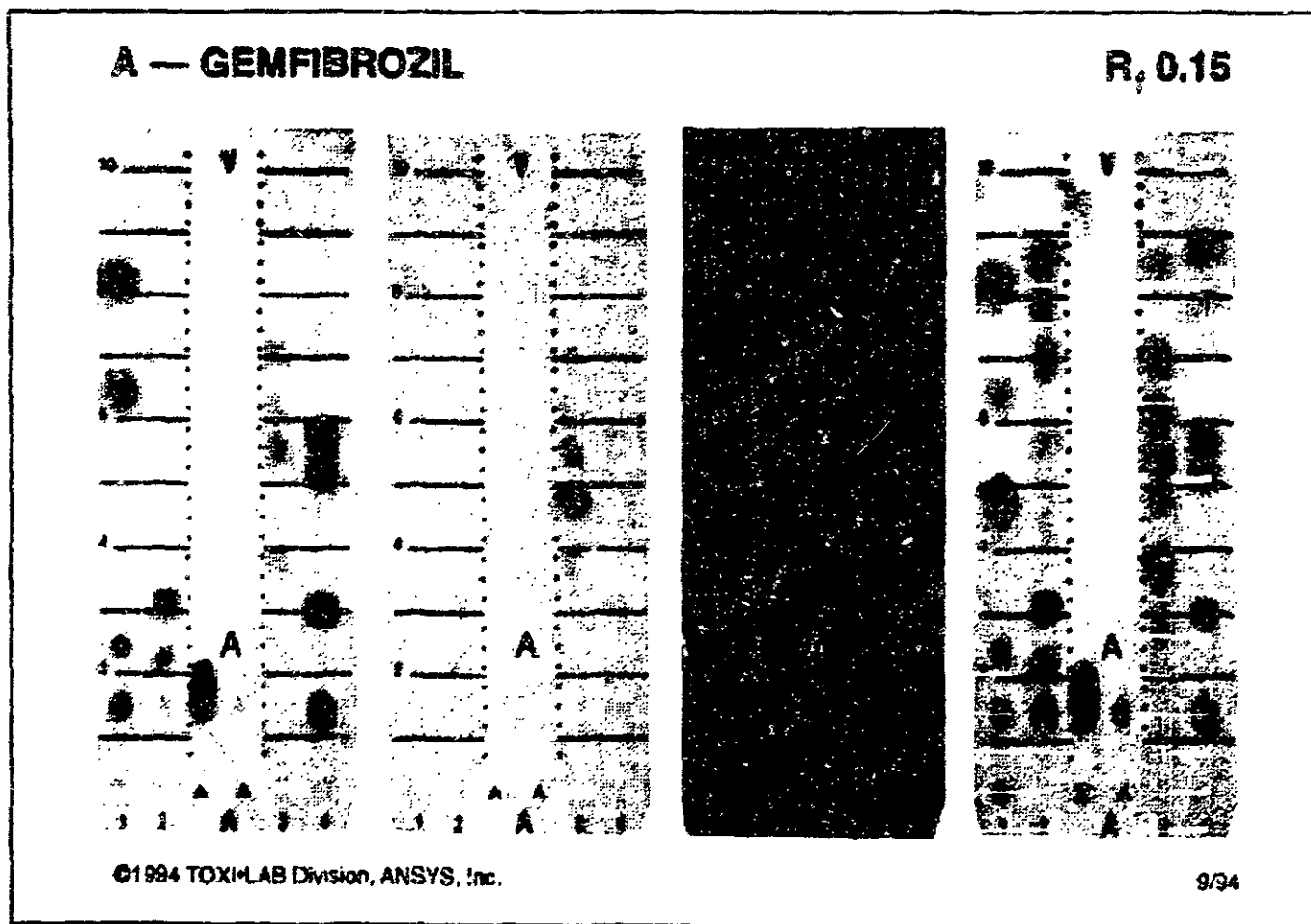


Triamterene, a diuretic, was found in several pilots by using HPLC with fluorescence and diode array detectors. The drug was confirmed using the quinine/quinidine TOXI-LAB® differentiation procedure. This drug would have been missed using only a standard GC/mass spectroscopy screening procedure (3).

In several cases, the HPLC screen was negative, and a drug was detected in the remaining 40uL of specimen using TOXI-LAB®. In one case, gemfibrozil was missed by using HPLC due to a coeluting peak, which interfered with the identification (Figure 3). The drug, however, was identified using the standard TOXI-LAB® procedure (Figure 6). This drug would have been missed using only an HPLC screening procedure.

There has been a threefold increase in the number of positive benzodiazepines identified using this new method. In several actual cases, benzodiazepines, which would have been missed using the standard TOXI-LAB® screening procedure, were detected by using HPLC. Benzodiazepines are also detected in the immunological screens used by the laboratory (RIA, FPIA). Using HPLC and mass spectroscopy together in the initial screen has made it possible to identify the specific benzodiazepine without the need for a second aliquoting process, extraction, and analysis.

Figure 6. TOXI-LAB A⁹ of gemfibrozil.



CONCLUSION

It is possible, in a screening procedure to use a single aliquot and a single extraction, and to analyze the urine extract by several different methods. This allows the identification of a wide variety of drugs that would typically require several different aliquots, multiple extractions, and several analyses. This procedure helps prevent presumptive false negative results, which might lead aviation accident investigators to the wrong conclusions. This also prevents presumptive false positive results, which would require a new aliquot, additional extraction, and further analysis. This single sequence of uniquely identifying drugs by using HPLC, and then validating by using GC mass spectroscopy, or TLC, reduces the time necessary to complete cases. It must be emphasized that in all cases screening positives are later confirmed with a second aliquot using GC/MS, GC/FTIR, or some other acceptable confirmation procedure.

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