CASE REPORT

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Documentation of a Doxylamine Overdose Death: Quantitation by Standard Addition and Use of Three Instrumental Techniques

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ABSTRACT: To answer the question, "Is this death due to a drug overdose?" requires at least that the drug be unequivocally identified and a blood concentration reliably determined. The approach taken in this case as standard addition technique and use of three different chromatographic techniques—high performance liquid chromatography (HPLC), high performance thin—layer chromatography (HP-TLC) and gas chromatography/mass spectrometry (GC/MS). Each of the chromatographies was carried out on the same extract by splitting the residue three ways. HPLC provided a quantitative result which was 1.2 mg/L in blood and HP-TLC and GC/MS confirmed this result with additional quantitative data, information about two metabolites (nordoxylamine and dinordoxylamine) and conclusive identification. Blood nordoxylamine was 0.52 mg/L and doxylamine plus metabolites in urine was 25 mg/L.

KEYWORDS: toxicology doxylamine overdose, standard addition technique, instrumental TLC, calibration matrix, postmortem blood determination

Reports on doxylamine overdose are few, especially those in which doxylamine is the sole drug of consequence. In 1983, Wu Chen and co-workers [*1*] reported a combination doxylamine-pyrilamine death with appropriate analytical detail. However, it is not clear from their paper if calibration standards in blood matrix were used to determine the drugs.

Professional forensic toxicologist groups such as the American Academy of Forensic Sciences and Society of Forensic Toxicologist have recently published guidelines [*2*] for good practice in doing postmortem quantitative determinations and one recommendation is to use the same matrix or fluid for calibration standards as the test specimen. Recent papers reporting postmortem toxicology findings [*3–5*] indicate that some forensic laboratories are following this guideline. Appropriate matrices are unquestionably hard to come by, especially since each postmortem blood is aged and decomposed to a different degree. If time and expense in completing a case is limited, steps such as obtaining a "blank" blood and fortifying to specified concentrations can be costly and time consuming. A convenient way to neutralize the matrix effect on drug recovery is to use standard

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addition technique, an approach frequently used in atomic absorption spectrophotometry. The Guidelines (Ref 2, Appendix page 9) indicated standard addition as an appropriate approach when matrix blank is unavailable.

This paper reports a standard addition technique to blood for the determination of doxylamine by HPLC with follow-up by HP-TLC and GC/MS for qualitative and quantitative verification. Effective use of specimen, materials and time is achieved by preparing one extract for all three chromatographic techniques.

Materials and Methods

Preliminary Tests

The case history presented to the laboratory was brief: A 45-year-old male was suspected to have taken an overdose of "sleeping pills," about 15 mL of whole blood in a redtop tube and a graytop tube and 20 mL of urine was received. The urine was subjected to TLC, RIA, and color test screens, and doxylamine and it metabolites were detected. Analysis of the basic fractions of the urine and blood by ultraviolet spectrophotometry revealed doxylamine plus metabolite concentrations of 25 mg/L and 2.9 mg/L respectively. While these concentrations are only approximate, it provided sufficient information from which to prepare standard additions of doxylamine to the blood, which would yield accurate quantitative results.

The source of doxylamine analytical reference material was Wm. S. Merrell doxylamine succinate and Sigma Catalogue No. A-8404 amitriptyline hydrochloride; the doxylamine contained a 1% impurity, but both drugs were used without purification steps and were considered 100% when weighed out.

Standard Addition

Anticipating a blood doxylamine concentration of 1 to 2 mg/L, 4 × 0.5 mL aliquots of the blood to be tested were added to separate 13 × 100 mm disposable borosilicate glass tubes and 3 of the aliquots were seeded with doxylamine concentrations of 2.0, 4.0 and 6.0 mg/L; amitriptyline internal standard at 4.0 mg/L was added to each tube with vortexing to complete preliminary steps of the procedure. Amitriptyline was chosen because it was suitable for the three chromatographic procedures used. Table 1 details the test set up and volumes of additions to each tube. Figure 1 flow charts the sequence of isolation steps for the instrumental chromatographic techniques used to obtain qualitative and quantitative results.

Each addition to the tubes was followed by vortexing. After the addition of organic solvent (hexane-isoamyl alcohol 97/3), the tubes were vortexed three times for about 20

TABLE 1—*Doxylamine determination in blood by standard addition.*

Tube no.	Doxylamine added[a]	0.1N NaOH	Hexane-isoamylalcohol
1	No addition	0.25 mL	5 mL
2	10 μL	0.25 mL	5 mL
3	20 μL	0.25 mL	5 mL
4	30 μL	0.25 mL	5 mL

[a]Doxylamine standard, 0.10 mg/mL, added to tubes 2, 3 and 4 gave spiked concentrations of 2.0, 4.0 and 6.0 mg/L doxylamine in the tested blood. Amitriptyline, 20 μL × 0.10 mg/mL was added to all tubes.

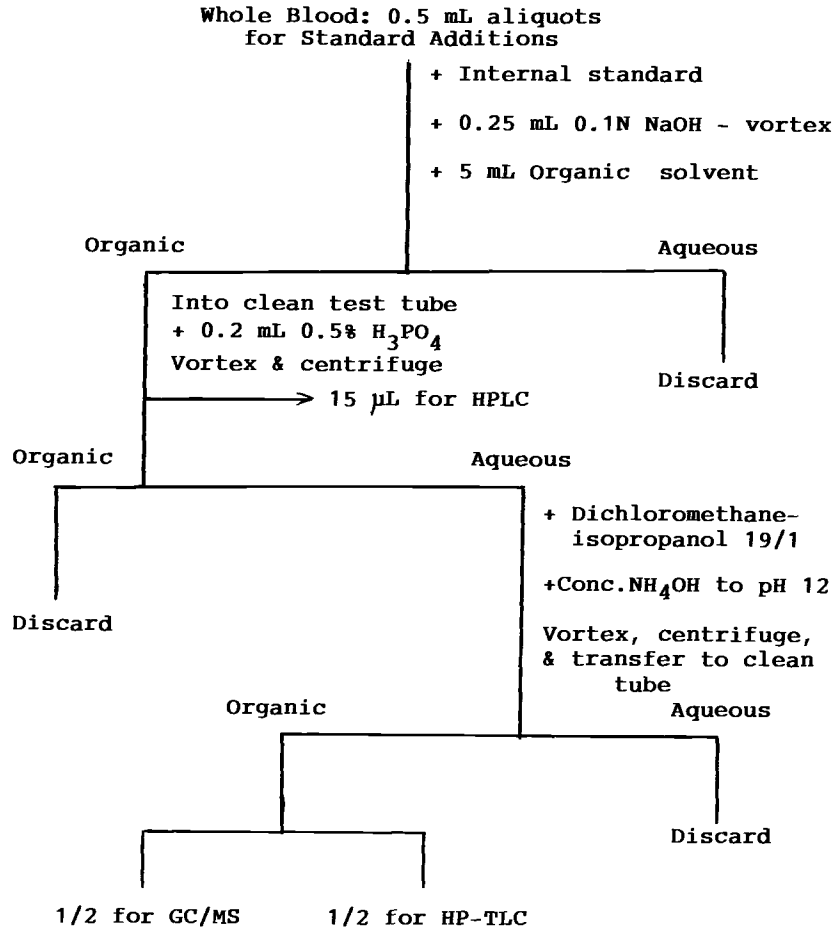

FIG. 1—*Flow chart diagram of blood specimen extraction for three instrumental techniques in series.*

s (60 s total) and centrifuged. The organic layer was transferred to a second 13 × 100 mm borosilicate glass test tube and 0.2 mL of dilute aqueous phosphoric acid (0.5 mL of 85% H_3PO_4 to 100 mL with deionized water) was added to each tube followed by vortexing and centrifuging. A 15 µL portion of the aqueous phase was sandwiched with 40 µL of mobile phase and subjected to HPLC as indicated below. The unused aqueous was saved for HP-TLC and GC/MS processing (see Fig. 1).

HPLC Column and Conditions

A Beckman Ultrasphere ODS C-18 reverse phase column (4.6 × 150 mm) with an attached 20 mm pellicular guard column (Alltech Nos. 28978 B24 and 28950, packing and column respectively) was used throughout studies reported here. The mobile phase was prepared by combining 420 mL of HPLC grade acetonitrile, 4 mL of triethylamine and water to 1000 mL. The pH was adjusted to 5.3 with 85% *ortho*-phosphoric acid after addition of most of the water. A flow rate of 1.0 mL/min. was maintained. The instrumentation included a Beckman Model 331 isocratic pump with a Model 160 fixed 254

nm detector, a 50 μL Rheodyne injector and a peripheral Spectra-Physics Data-Jet Model 4600 reporting integrator.

HP-TLC Instrumentation and Procedure

The TLC plates used were Analtech silica gel high performance Uniplates with organic binder (10 × 20 cm scored to 2.5 cm, No. 59527). CAMAG Twin-Trough development chambers for 10 × 20 cm plates were used to enable "pre-loading" of plates with solvent vapors prior to chromatography. Application to the plates was with a CAMAG Linomat IV automated band spotter. Densitometry was carried out on a CAMAG TLC Scanner II with a peripheral Spectra-Physics model 4290 reporting integrator.

To the tubes remaining after HPLC (two layers) was added 0.2 mL of 0.1 NH_2SO_4 followed by vortexing 3 × 20 s. The organic phase was aspirated without disturbing the aqueous layer (a residual of hexane may be left on the surface). The tubes were next made alkaline with NH_4OH and extracted with 2.5 mL of dichloromethane-isopropanol 19/1, vortexed and centrifuged. The organic phase was isolated and evaporated in a water bath.

The extracts were applied to TLC plates in bands of 3 mm beginning 12 mm from the plate edge with track distances of 5 mm. The application rate was 5 s/μL, each extract made up with 30 μL and applied in 4/30 and 7/30 μL portions. The unspotted portions of extract were saved for GC/MS. The plate was placed in a CAMAG Twin-Trough chamber with mobile phase for 10 minutes prior to development with ethyl acetate/dichloromethane/methanol/concentrated NH_4OH (80/90/15/5 by vol) to full plate height. After drying, densitometry was carried out with a CAMAG TLC Scanner II. Table 2 gives complete conditions for scanning the plate to obtain doxylamine and metabolite concentrations.

GC/MS Studies

The residue from the TLC run was taken up in ethyl acetate for qualitative and quantitative studies by GC/MS. GC/MS conditions are given in Table 3.

TABLE 2—*Steps preceding the scan of the developed TLC plate.*

Program the Scanner	
Start X	14 mm (center of Track No. 1)
Start Y	3 mm below spot origin
Scan length	65 mm (10 mm above amitriptyline)
Track space	5 mm apart band edge to band edge
Zero all tracks	Yes
Span	75 (expands peaks from Scanner)
Offset	10% above baseline
Scan speed	1.0 mm/sec. (speed plate moves)
Wavelength	261 nm in absorbance-reflectance mode
Sensitivity manual adjust	No
Micro bean	0.3 mm width, 2 mm length, 30 nm bandpass
Program the Integrator	
Peak width (PW)	3
Peak threshold (PT)	7800
Timebase (TB)	1
Attenuation (AT)	128
Chart speed (CS)	4 (1.0 cm/min)
Peak height mode (PH)	1 (integrates by peak height)

TABLE 3—*GC/MS operating conditions for doxylamine determination.*

Instrument:	Finnigan ITS40 with a COMPAQ 386/20e computer, version 1.10 software.
Injector:	Split 100/1, 275°C.
Column:	15 m by 0.25 mm ID DB-5 (1.0 μm film) bonded phase fused silica capillary, J&W Scientific.
Column conditions (oven):	150°C for 1 min, 20°C/min to 310°C for 0.2 min hold.
Transfer:	310°C.
Mass spectrometer:	Manifold temperature, 220°C, electron impact mode, 50–280 amμ at 1 sec/scan. Quantitation by the 58 amμ peak ratios.

Results

Figure 2 plots the linear regression line from the addition of 2.0, 4.0, and 6.0 mg/L to the test blood. The concentration of the blood with no additions is found by the X intercept; in this case a value of 1.2 mg/L was obtained (see Table 4) by HPLC with standard addition, 1.1 mg/L with no calibration, 1.2 mg/L by both HP-TLC and GC/MS. The metabolites were not located in the HPLC run but were obvious on TLC by comparing blood extracts to the urine extract. The urine doxylamine metabolites were readily identified by TLC because of previous experience with doxylamine-containing urines.

Figure 3 shows the HPLC and HP-TLC chromatograms side by side. Blood and urine concentrations are given in Table 5.

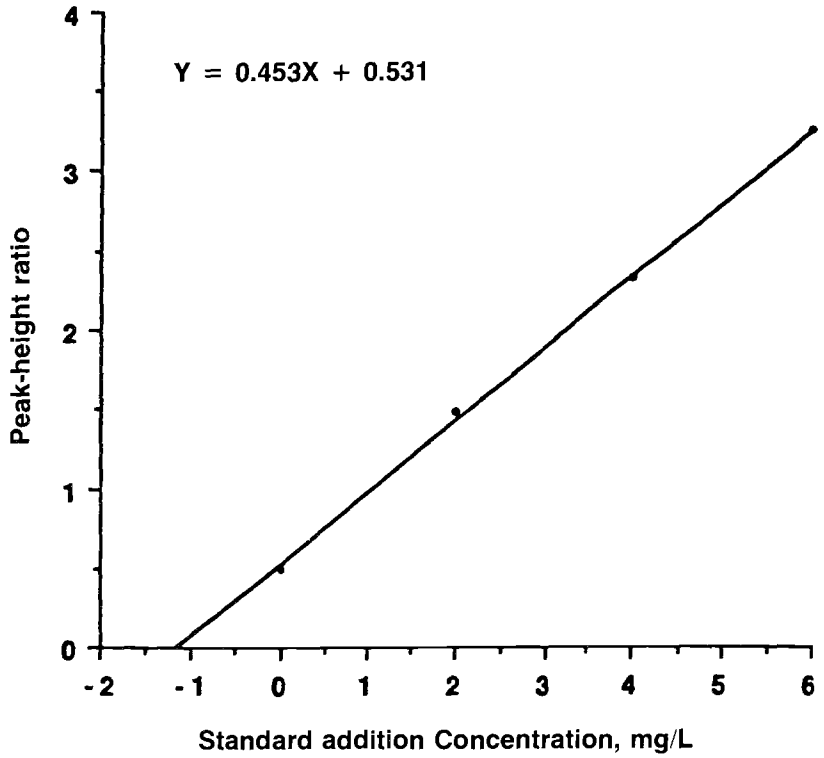

FIG. 2—*Standard addition plot of three additions of doxylamine to the test blood. The X intercept (to the left of 0) indicates the concentration of doxylamine without addition. The linear regression equation is:* $Y = 0.453X + 0.531$.

TABLE 4—*Results of whole blood determinations and comparisons of analytical techniques.*

Instrumental technique	Doxylamine	Nordoxylamine
HPLC	1.2 mg/L	Not done
HP-TLC 1	1.2 mg/L	0.52 mg/L
HP-TLC 2	1.2 mg/L	Not done
GC/MS	1.2 mg/L	Not done

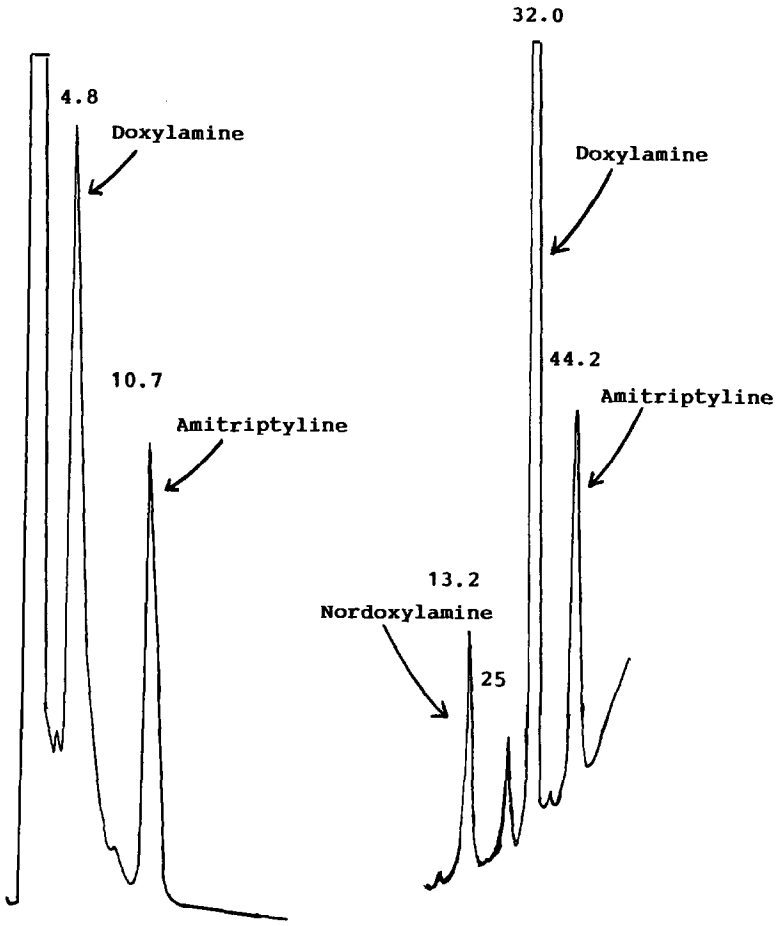

FIG. 3—*HPLC (left set of peaks) and HP-TLC chromatograms from doxylamine determination. Peaks and retention data are given for both chromatograms.*

TABLE 5—*Distribution of doxylamine and metabolites.*

	Whole Blood	Urine
Doxylamine	1.2 mg/L	15.7 mg/L
Nordoxylamine	0.52 mg/L	6.6 mg/L
Dinordoxylamine	—	2.7 mg/L

The concentration (C_x) in blood may be calculated from a single standard addition (C_x) by the relationship:

$$C_x = C_{add}\left(\frac{R_x}{R_{x+} - R_x}\right)$$

where R_x is the peak height ratio of the test specimen, and R_{x+} is the ratio of the standard addition specimen.

Discussion

The postmortem blood concentration found in this case, 1.2 mg/L, greatly exceeds the therapeutic range of approximately 0.05 to 0.15 mg/L [6]. This fact, the high urine concentrations of drug and metabolites, and the case history caused a ruling of death due to doxylamine overdose by the coroner. The doxylamine concentration in the overdose death case presented by Wu Chen et al. was 0.7 mg/L [1].

Our approach to general unknown toxicology or a suspected drug overdose is typical of many toxicology laboratories in that drugs are first identified in a specimen like urine or gastric contents and quantitations are set up in blood and other specimens that may be provided. In postmortem cases, we have found it expedient to obtain some initial estimate of the blood concentration(s) of drugs by a simple technique such as ultraviolet spectrophotometry or by a run through of the specimen without calibrators but with internal standard, and then set up appropriate calibrators.

A ratio of an unextracted equal parts mixture of analyte and internal standard will give a close estimate of the concentration in those cases where recovery is high and the chromatography is uncomplicated by interfering peaks. The estimate is usually within 20% of what a completely calibrated determination would yield. Once the estimate is known, a standard addition series can be selected, which may follow the initial run. Standard additions of 2X, 4X, and 6X the estimate will give a plot or equation that will yield accurate and precise determinations. In the case of limited specimen, standard additions of 3X and 6X are suitable. Standard addition satisfies two primary concerns of the analytical toxicologist—matrix effect and bracketing of the test specimen concentration with calibrators and in addition provides replicate determinations: two to four determinations are accomplished depending on the number of standard additions.

A second aspect of the case report presented here is a workup that allows for more than one instrumental technique to be carried out on the same extract. This is efficient and confirms and validates the quantitation by other chromatographic techniques. The extraction technique is rapid in that no lengthy mixing and partitioning steps are involved. The small volume of phosphoric acid extraction from hexane-isoamyl alcohol is efficient—from this point on, all volumes of solvents are scaled down, minimizing solvent and glassware contamination.

The matrix problem is realistically handled by the standard addition technique. No "blank" blood is just like the test blood particularly in postmortem cases. One of the pitfalls of standard addition technique is addition of too little or too much analyte. To prevent this, the unspiked blood can be processed first and additions made to the other aliquots on the basis of an estimate of the analyte concentrations. In our case we estimated the doxylamine concentration from ultraviolet spectrophotometry. The HPLC quantitation provided an accurate value for doxylamine, as demonstrated by Fig. 2.

The HP-TLC chromatograph (by scan and by visualization reactions), presented all metabolites and their relative quantities since on the TLC plate nothing is lost between origin and solvent front. Metabolites in the urine were readily visualized and identified as doxylamine substances by classical TLC spray reagents. GC/MS provided a check on

the concentration and unequivocal identification of the drug. The use of instrumental thin-layer chromatography for qualitative and quantitative determinations is not widespread in toxicology laboratories in the United States, possibly due to the past reputation of TLC as being a qualitative technique. Fig. 2 shows TLC chromatogram having low noise and sharp, symmetrical peaks.

The role of instrumental thin-layer chromatography here is to complement HPLC and confirm metabolite concentrations. Quantitation by HP-TLC in the ultraviolet absorbance-reflectance mode does not yield a linear plot of concentration to peak height ratio, preventing a plot such as that in Fig. 2. However, quantitative results can be obtained by plotting the points in a quadratic fit (done by the SP 4290) or by comparing peak height ratios of an unextracted mixture of analyte and internal standard.

The practical importance of completing postmortem cases within a few days of their receipt should not be overlooked by forensic toxicologists. Because of outside pressures, delayed results can sometimes lead medical examiners and coroners into giving results on cause of death before toxicology results are completed. We hope this report will assist toxicologists in expeditiously completing their cases with forensically acceptable validation.

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