

Detection of Benzodiazepines in Hair Using ELISA and LC-ESI-MS-MS

Eleanor I. Miller*, Fiona M. Wylie, and John S. Oliver

Forensic Medicine and Science Department, University of Glasgow, University Place, Glasgow, G12 8QQ, Scotland

Abstract

This study was designed to validate an enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for the detection of nine benzodiazepines in hair. Sixteen hair case samples were tested from drug-related deaths where a positive benzodiazepine blood result was obtained. The case samples were decontaminated with 0.1% sodium dodecyl sulfate, distilled water, and dichloromethane. For ELISA analysis, the samples were extracted by incubation in monobasic phosphate buffer for 1 h and then neutralized with dibasic phosphate buffer. They were diluted 1:5 with phosphate buffer saline (PBS) prior to analysis. For LC-MS-MS, the samples were incubated overnight in methanol/25% ammonium hydroxide (20:1). The benzodiazepines were extracted by solid phase. Thirteen samples were confirmed positive by LC-MS-MS. The benzodiazepines detected included diazepam, nordiazepam, temazepam, oxazepam, nitrazepam, and lorazepam. Using a cut-off concentration of 0.1 ng/mg oxazepam, the Immunalysis® Benzodiazepine Microplate ELISA demonstrated a sensitivity and specificity of 100% and 81%, respectively, compared with LC-MS-MS results.

Introduction

Benzodiazepines are the most frequently prescribed drugs in the world as they are cheap, effective, and safe. In addition to this, they are addictive and, thus, widely abused (1). They tend to appear more often in overdose cases than any other type of drug. One study showed that 45% of drug-related deaths tested positive for diazepam, and 33% of these cases tested positive for temazepam (2). The Scottish Health Statistics show that diazepam is currently the most commonly prescribed benzodiazepine in Scotland (3).

Benzodiazepines include a large number of drugs, which are used as sedatives, hypnotics, muscle relaxants, and anticonvulsants. They exert an additive effect when taken simultaneously with alcohol and other drugs, including opiates, sedatives, antidepressants, and neuroleptics (4).

Despite benzodiazepine abuse being the most significant in

the world, it is the detection of other drugs in hair, such as heroin, amphetamines, cocaine, and cannabis, that have been well researched and documented. Benzodiazepine detection has, however, been reported in hair collected from living and deceased users using gas chromatography-mass spectrometry (GC-MS) (5-10) and liquid chromatography-tandem mass spectrometry (LC-MS-MS) (11-13). Another study identified five benzodiazepines using high-performance liquid chromatography (HPLC) and a restriction access extraction column (14).

Enzyme-linked immunosorbent assay (ELISA) is a fast, automated, and sensitive technique that can be used to screen large numbers of unknown samples. Immunoassay systems used for hair testing must display cross-reactivity with the parent drug and metabolites that are found in hair, must not be affected by interference from the extracted hair matrix, and must be capable of detecting drug concentrations found in hair (typically in the pg/mg range). There appear to be a limited number of publications that have studied ELISA screening for benzodiazepines in hair. In one study, two postmortem hair samples tested positive for benzodiazepines by ELISA, and these were confirmed as positive for flunitrazepam and its metabolite using GC-negative ion chemical ionization-MS at a cut-off of 0.1 ng/mg 7-aminoflunitrazepam (15). Benzodiazepines in hair have been detected using fluorescence polarization immunoassay and radioimmunoassay (16,17).

The aim of this study was to validate an ELISA and LC-MS-MS method for detecting benzodiazepines in hair. For this purpose, the results from the diagnostic testing of 16 post-mortem case hair samples using both methods were compared.

Experimental

Samples

The 16 hair samples tested were postmortem case samples submitted to our laboratory. One scalp hair sample was collected in each case. The samples selected for testing were from cases in which the blood result was positive for benzodiazepines using a validated in-house LC-MS-MS method. Thirteen negative hair samples were obtained from volunteers and post-

* Author to whom correspondence should be addressed. E-mail: ei_miller2003@yahoo.co.uk.

mortem case samples. All samples were wrapped in aluminium foil and stored at room temperature prior to analysis.

Apparatus

Microplate ELISA. A Miniprep 75 automatic pipettor, purchased from Tecan (San Jose, CA), was used to dilute samples and to pipette all calibrators, controls, and samples into the microplate wells. The plates were washed using a Columbus Plus washer system from Tecan (Grödlg, Austria) and read using a Sunrise Remote EIA autoreader from Tecan. Disposable borosilicate glass culture tubes (75 × 12 mm, VWR International, Poole, U.K.) were used for the samples and dilutes.

LC-MS-MS. This consisted of a Surveyor HPLC system with an LCQ Deca XP Plus ion trap MS. HPLC was performed on a Gemini C18 column (150 mm × 2.0 mm, 5- μ m particle size), fitted with a guard column with identical packing material (4 mm × 2.0 mm, Phenomenex, Torrance, CA).

Chemicals and reagents

Methanol, acetonitrile, ammonium hydroxide, cyclohexane, ethyl acetate, formic acid, dichloromethane, and propan-2-ol were purchased from BDH (Poole, U.K.) and were of analytical grade. Ammonium formate and sodium dodecyl sulfate were purchased from Sigma-Aldrich (Dorset, U.K.). 7-Aminoflunitrazepam, flunitrazepam, oxazepam, lorazepam, chlor-diazepoxide, temazepam, diazepam, nordiazepam, nitrazepam, 7-aminoflunitrazepam-d₇, flunitrazepam-d₇, oxazepam-d₅, lorazepam-d₄, temazepam-d₅, diazepam-d₅, and nordiazepam-d₅ were obtained from Promochem (Teddington, U.K.). Solid-phase mixed-mode cation exchange-hydrophobic phase extraction columns (ZSDAU 020, 200 mg) were purchased from United Chemical Technologies (Bristol, PA). Benzodiazepine Direct ELISA kits were purchased from Immunalysis (Pomona, CA). The kits contained a 96-well antibody-coated microplate, benzodiazepine conjugate labelled with horseradish peroxidase, substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB), and stop solution containing 1N hydrochloric acid. Monobasic phosphate buffer [0.025M, pH 2.7 with 0.1% bovine serum albumin (BSA)], dibasic phosphate buffer (0.5M, pH 9.0 with 0.1% BSA), and phosphate buffer saline (PBS) (0.1M, pH 7.0 with 0.1% BSA) were also purchased from Immunalysis.

Methods

Washing of hair samples

The root-0.5 cm section was removed from each sample where roots were present. The 0.5 cm-tip section was weighed out into a vial for analysis and cut into 2-3-mm segments using a pair of clean scissors. Each sample was then split into two separate vials for the ELISA and LC-MS-MS extraction procedures.

The samples were washed with 1 mL of 0.1% sodium dodecyl sulfate with a 10-min sonication, 2 × 1 mL deionized water with a 10-min sonication, and 2 × 1 mL dichloromethane with a 10-min sonication. The washings were not analyzed because the likelihood of benzodiazepine contamination from an external source was very low. Blank hair was also washed using this

procedure prior to spiking to produce the calibrators for the ELISA and LC-MS-MS procedures.

ELISA method

An aqueous extraction method previously used for ELISA screening for cocaine and amphetamine in hair was used (18,19). Monobasic phosphate buffer (0.5 mL) was added to each sample. The samples were incubated in an oven for 1 h at 60°C. After cooling, 50 μ L of dibasic phosphate buffer was added to each sample to neutralize the acidic medium. The samples were vortex mixed, then diluted 1:5 on-line with PBS. The diluted extracts were also vortex mixed.

Each ELISA run contained a set of calibrators consisting of a blank and spiked hair samples at a concentration of 0.1, 0.2, and 0.5 ng/mg oxazepam. A positive control (0.3 ng/mg oxazepam) and a negative control were used to check kit performance. These were distributed to wells at the beginning and end of the plate. The calibrators and controls were prepared by spiking approximately 10 mg of drug-free decontaminated hair with oxazepam at the desired concentration, then extracting the spiked samples in the same way as the case samples.

Fifty microliters of diluted hair extract was added to the microplate wells in duplicate and left to infiltrate the antibodies for 1 h. After this time passed, 100 μ L of benzodiazepine enzyme conjugate reagent was added to each well. The plate was then left in the dark at room temperature for an incubation period of 1 h.

Following incubation, the microplate wells were washed with deionized water (6 × 350 μ L) in order to remove any unbound sample or residual conjugate reagent that may be left in the wells. TMB substrate reagent (100 μ L) was added to the wells, and the plate was left to incubate in the dark at room temperature for an additional 30 min. After 30 min, the reaction was stopped by adding 100 μ L of stop reagent to each well. The contents of the wells turned yellow following addition of the stop reagent, and this was to enable the chromophore to be detected at 450 nm. A reference wavelength of 620 nm was used for background correction.

Validation of ELISA method

Dose-response curve. A dose-response curve was generated for approximately 10 mg of drug-free decontaminated hair spiked at concentrations of 0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5

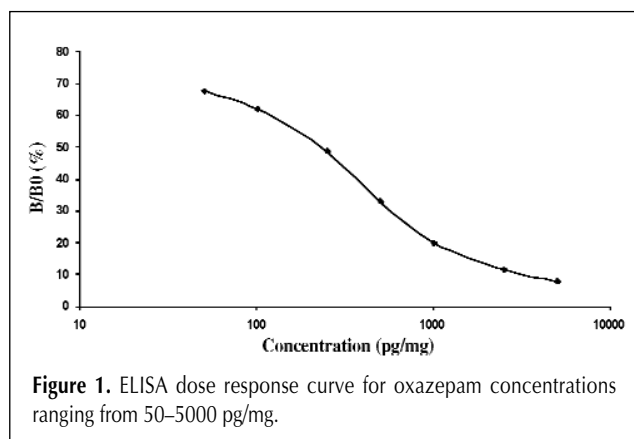


Figure 1. ELISA dose response curve for oxazepam concentrations ranging from 50-5000 pg/mg.

ng/mg oxazepam (Figure 1). The B/B_0 (%) values were calculated where B is the absorbance value of the bound calibrator, and B_0 is the absorbance value of the blank calibrator.

Limit of detection (LOD) and assay precision. The LOD was calculated from the mean absorbance value for 13 negative samples using equation 1:

$$\text{LOD} = A_0 - 3\sigma \quad \text{Eq. 1}$$

where A_0 is the mean absorbance value, and σ is the standard deviation of the absorbance values (20,21).

The intraday precision of the B/B_0 (%) values was calculated by spiking 10 drug-free decontaminated hair samples at 0.10 ng/mg oxazepam. These were tested on the same day and on the same plate. This process was carried out on five separate days, and an interday precision of the B/B_0 (%) values was calculated.

Sensitivity and specificity. The sensitivity and specificity of the assay were calculated by applying equations 2 and 3, respectively, which require the total number of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN) to be counted.

$$\text{Sensitivity} = (\text{TP} \times 100)/(\text{TP} + \text{FN}) \quad \text{Eq. 2}$$

$$\text{Specificity} = (\text{TN} \times 100)/(\text{TN} + \text{FP}) \quad \text{Eq. 3}$$

A TP sample produced both positive screening and confirmation results. A TN sample produced both negative screening and confirmation results. An FP sample produced a positive screening and negative confirmation result. An FN sample produced a negative screening and positive confirmation result.

Cross-reactivity. The microplate ELISA is directed towards oxazepam and cross-reacts 100% with it. To calculate cross-reactivity, PBS was spiked at 10–200 ng/mL oxazepam to establish a calibration graph. The cross-reactivity of other benzodiazepines was calculated relative to the calibration graph constructed from oxazepam-spiked calibrators. Benzodiazepine standards were also prepared in PBS, and the cross-reactivity at 100 ng/mL oxazepam was calculated (Table I).

Table I. Cross-Reactivity of Benzodiazepines Tested by the Immunalysis Benzodiazepine Microplate ELISA

Compound	Approx. ng/mL Equivalent to 100 ng/mL Oxazepam	% Cross-Reactivity Quoted by Immunalysis	% Cross-Reactivity Found
7-Aminoflunitrazepam	1300	Not quoted	8
Chlordiazepoxide	750	17	13
Diazepam	125	91	80
Flunitrazepam	310	31	32
Lorazepam	860	13	12
Nitrazepam	320	33	31
Nordiazepam	70	150	143
Temazepam	130	83	77

The cross-reactivity of the assay with some unrelated drugs was also tested at a concentration of 10,000 ng/mL.

LC–MS–MS Method

An alkaline pre-extraction was used prior to benzodiazepine extraction by solid phase (22). Ammoniated methanol was found to produce slightly higher recoveries than just methanol for diazepam, oxazepam, and flunitrazepam in another study (14). Methanol/25% ammonium hydroxide solution (1.5 mL, 20:1) was added to each calibrator and sample, left to sonicate for 1 h, and stored at room temperature overnight. The solvent was removed from the vials and transferred to a test tube. The hair was washed twice more with 0.75 mL solvent, and the washings were removed and transferred to the corresponding test tube. The contents of the test tubes were evaporated to dryness under nitrogen and reconstituted in 1 mL of phosphate buffer (0.1M, pH 6.0).

Solid-phase extraction (SPE) method

World Wide Monitoring Clean Screen® columns (ZSDAU 020) were conditioned sequentially with 3 mL methanol, 3 mL distilled water, and 1 mL of phosphate buffer (0.1M, pH 6.0). The vortex mixed samples were loaded onto the columns and allowed to drip through without the presence of a vacuum. The columns were washed sequentially with 2 mL distilled water, 2 mL 20% acetonitrile in phosphate buffer (0.1M, pH 6.0), 2 mL cyclohexane, and 2 mL distilled water. The columns were dried for 5 min under full vacuum after the second part of wash step, for 1 min after the third part of the wash step, and for 5 min after the final part of the wash step. The analytes were eluted using 1.5 mL 2% ammoniated ethyl acetate (followed by a 2-min drying step on full vacuum) and 1.5 mL dichloromethane/isopropanol/ammonium hydroxide (78:20:2). The samples were reconstituted with 100 μ L of the mobile phase initial conditions.

LC–MS–MS conditions

The column was maintained at 25°C. The mobile phase consisted of 3mM ammonium formate/0.001% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.3 mL/min. The initial gradient conditions were 65% A, decreased to 20% A after 13 min. The mobile phase composition was 10% A from 13.5 to 16.5 min. Initial conditions were restored from 16.5 to 20 min, and the system was allowed time to equilibrate. The total run time was 20 min. A divert valve taking LC flow to waste was used for the last 7 min of the run to preserve the MS source.

All mass spectral data were collected in electrospray positive ion mode. The capillary temperature, sheath and auxiliary gas flow rates, and collision energies were optimized for each analyte. The probe voltage used was 4.5 kV. Internal standard data was collected in selected ion monitoring mode, and analytes were identified on the basis of their retention time and full MS–MS spectra. The analyte product ion ratios were monitored to gain further qualitative identification data. The optimum tuning parameters as well as precursor and product quantitation ions are shown in Table II.

LC-MS-MS validation

Linearity. Linearity was established over the concentration range of 2–100 ng/30 mg of blank hair to produce a regression line for each analyte.

Recovery. Total extraction recovery was calculated for each analyte at 10 ng/30 mg and 50 ng/30 mg by comparing the average analyte peak-area ratio of five extracted standards with the average analyte peak-area ratio of two unextracted standards of the same concentration.

An additional experiment was conducted on one authentic hair case sample to determine the efficiency of the extraction. The sample was cut up and divided into four portions. Each portion was left to incubate in the alkaline extraction medium for 1, 2, 4, and 16 h and subsequently extracted using the SPE method already described.

Stability

Alkaline pre-extraction. The stability of the analytes during the alkaline overnight pre-extraction was assessed by comparing the results of four extracted samples with those from three unextracted samples at the same concentration.

Evaporation. In one study, the total extraction recovery of benzodiazepine amino metabolites was found to be at least 25% lower than the extraction recovery of their respective parent drugs (1).

In this study, evaporation of the eluant in the SPE method was investigated as a potential means of lowering analyte recovery. Four spiked samples were prepared in eluant and blown down with nitrogen at 40°C, and the results were compared with three spiked samples that were not prepared in eluant or heated during blowing down with nitrogen.

Intraday precision. Five extracted standards were prepared at 10 ng/30 mg and 50 ng/30 mg. Standards were also prepared at 2, 5, 10, 25, 50, and 100 ng/30 mg to produce linearity data. A blank was prepared containing no internal standard as well as a blank containing only internal standard. The blown down extracts were reconstituted in 100 µL of mobile phase, and 20 µL was injected. The mean product ion/peak-area ratio value was substituted into the regression equation for a particular drug, and the mean concentration was calculated along with the % relative standard deviation (RSD).

Interday precision. The interday precision between extracts was calculated at 10 ng and 50 ng/30 mg. The intraday experiment described in the previous section was carried out on five different days. The mean product ion/peak-area ratio value for the five days was substituted into the regression equation for a particular drug, and the mean concentration was calculated along with the % RSD.

LOD and limit of quantitation (LOQ). The LOD for each drug was calculated statistically as the intercept of the calibration graph plus three times the standard error of the line, and the LOQ was calculated statistically as the intercept of the calibration graph plus five times the standard error of the line (23).

ELISA validation results

Dose-response curve. The dose-response curve is shown in Figure 1. The y-axis is linear scale, and the x-axis is log scale. The optical density values showed a hyperbolic decrease with increasing oxazepam concentration. This is because the higher the drug concentration in the sample, the lower the amount of enzyme conjugate that binds to the antibody sites, producing a lower optical density value.

LOD and assay precision. The LOD of the ELISA assay was calculated to be 2 ng/mL (equivalent to 0.1 ng/mg using 10 mg blank

hair) using equation 1. The intraday precision of the B/B₀ (%) values for drug-free decontaminated hair spiked at 0.10 ng/mg oxazepam (n = 10) was 3.1%. The interassay precision (n = 50) was determined as 5.9% at this concentration.

Cross-reactivity. The cross-reactivity values for the benzodiazepines tested in the LC-MS-MS method are shown in Table I. The values obtained are comparable to the values provided by the manufacturers in the package insert.

The absorbance values for the unrelated drugs were all greater than the assay sensitivity level of 2 ng/mL oxazepam and, hence, did not cross-react with the assay.

LC-MS-MS validation results

The gradient and intercept values are shown in Table III,

Table II. Optimum Tuning Parameters, Precursor, and Product Ions for Each Analyte

Analyte	Sheath Gas (AU)	Auxiliary Gas (AU)	Capillary Temperature (°C)	Collision Energy (%)	Precursor ion (MH+) m/z	Product ions m/z
7-Aminoflunitrazepam	20	20	280	40	284	264*, 256
Chlordiazepoxide	20	20	300	29	300	283*, 241
Diazepam	20	20	300	42	285	257*, 222
Flunitrazepam	20	20	280	43	314	286*, 268
Lorazepam	30	20	290	30	321	303*, 275
Nitrazepam	30	20	300	42	282	254, 236*
Nordiazepam	20	15	300	41	271	243*, 140
Oxazepam	20	20	300	29	287	269*, 241
Temazepam	20	20	300	29	301	283*, 255

* Quantitation ion

Table III. Gradient, Intercept, and R² Values for LC-MS-MS Calibration Graphs

Analyte	Equation of Line	R ² Value
7-Aminoflunitrazepam	y = 0.0017x - 0.0028	0.9981
Chlordiazepoxide	y = 0.0129x - 0.0156	0.9994
Diazepam	y = 0.0016x - 0.0032	0.9984
Flunitrazepam	y = 0.0019x - 0.0011	0.9992
Lorazepam	y = 0.0092x + 0.0106	0.9974
Nitrazepam	y = 0.0036x + 0.0082	0.9977
Nordiazepam	y = 0.0012x - 0.0009	0.9995
Oxazepam	y = 0.0093x - 0.0122	0.9996
Temazepam	y = 0.0113x - 0.0323	0.9985

along with the correlation coefficient (R^2) values for the regression lines. All regression lines had an R^2 value > 0.99 . Examples of the chromatograms obtained for a blank hair sample, lowest temazepam standard and a temazepam positive sample are shown in Figure 2.

Recovery

The total extraction recovery for each analyte is given in Table IV for 10 and 50 ng/30 mg and ranged from 53 to 98%. Generally, the analytes had total extraction recoveries $> 70\%$ at both concentrations. 7-Aminoflunitrazepam and chlordiazepoxide recoveries were lower than the other analytes.

The experiment conducted on the authentic hair case sample to determine the efficiency of the alkaline extraction showed that the greatest concentration of diazepam and nordiazepam were detected following a 16 h incubation (4.26 and 1.67 ng/mg, respectively). The concentrations of diazepam and nordiazepam detected after a 1, 2, and 4 h incubation were similar and were within a small range (2.40–2.70 ng/mg and 0.90–1.16 ng/mg, respectively).

Stability

Alkaline pre-extraction. The recovery for all analytes following the overnight alkaline extraction ranged from 87 to 102% (Table V). These results show that this milder alkaline extraction using methanol/25% ammonium hydroxide (20:1) is suitable for benzodiazepines. The lower 7-aminofluni-

trazepam and chlordiazepoxide recoveries were not a result of alkaline hydrolysis. Lorazepam was hydrolyzed to a greater extent than temazepam or oxazepam, which have a similar chemical structure to lorazepam. It is possible that the presence of an electron withdrawing chlorine atom on both aromatic rings in the lorazepam structure increases the lability of the ring C=N to attack by the base used in the extraction, promoting hydrolysis. Temazepam and oxazepam only have an electron withdrawing chlorine atom and are, therefore, less susceptible to hydrolysis.

Evaporation. With the exception of 7-aminoflunitrazepam and chlordiazepoxide, the analyte recoveries were all $> 98\%$. 7-Aminoflunitrazepam recovery was 72%, and chlordiazepoxide recovery was 89%, which could partly explain the lower total extraction recovery values found for these analytes. The recovery values for the evaporation step are given in Table VI.

Intra- and interday precision. The intra- and interday precision at 10 ng/30 mg and 50 ng/30 mg were acceptable (all $< 20\%$). The % RSD values were generally lower at the higher concentration. These values are shown in Tables VI and VII. The % RSD values were relatively high for some analytes at both the low and high spike concentrations. It may be the case that precision is slightly lower because of the “soft” ionization mode used in the MS–MS system.

LOD and LOQ. The LOD and LOQ for all the benzodiazepines tested ranged from 0.03 to 0.62 ng/30 mg and 0.05 to 1.02 ng/30 mg. The values are shown in Table VIII.

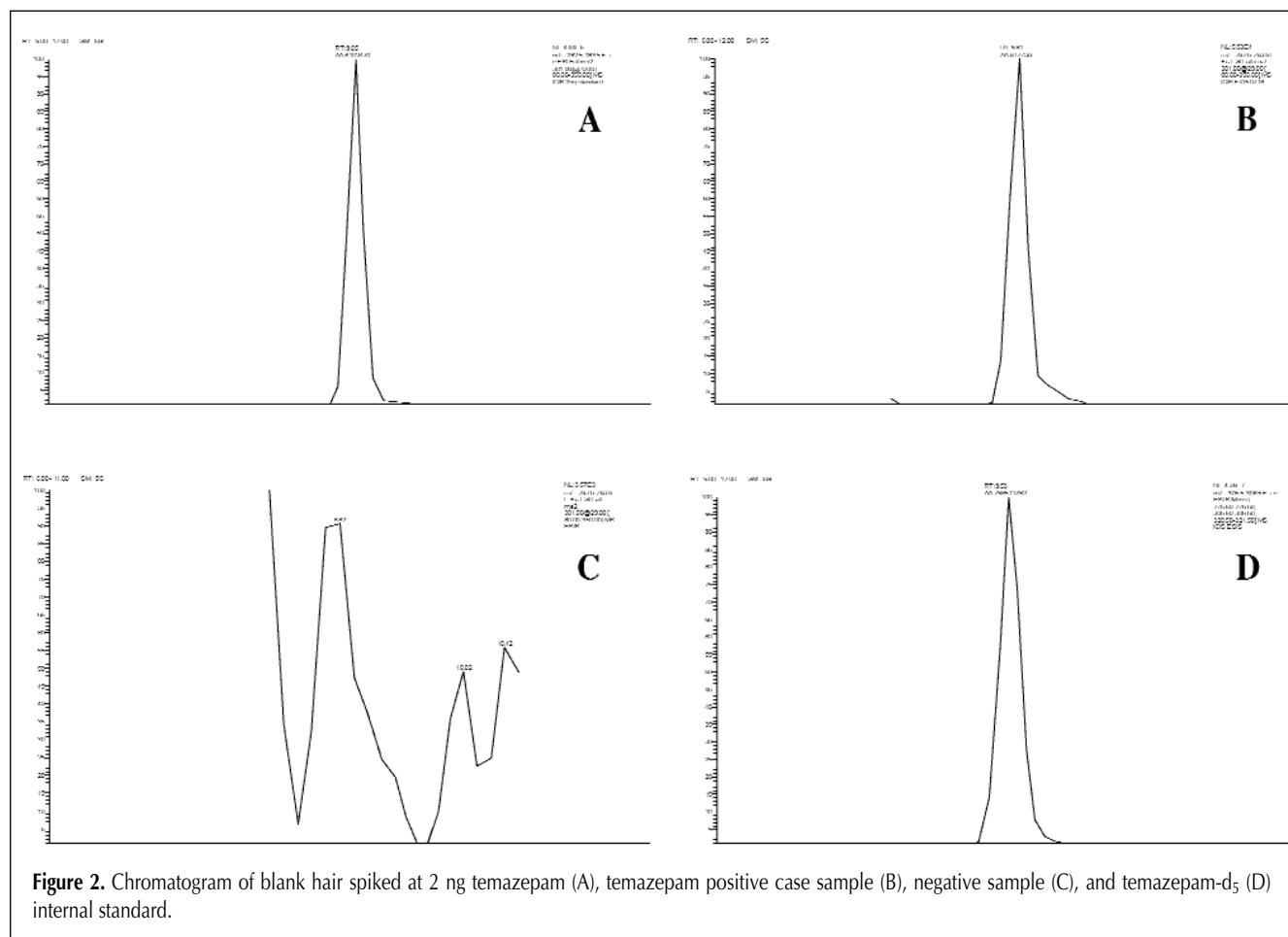


Figure 2. Chromatogram of blank hair spiked at 2 ng temazepam (A), temazepam positive case sample (B), negative sample (C), and temazepam- d_5 (D) internal standard.

Case sample results

The validated ELISA and LC–MS–MS methods for the detection of benzodiazepines in hair have been successfully applied to 13 postmortem positive case samples. Table IX shows the ELISA and LC–MS–MS results for benzodiazepines in the 13 TP samples. Overall, the ELISA and LC–MS–MS results correlated well. Thirteen of the 16 hair case samples were screened and confirmed as TP using a cut-off value of 0.1 ng/mg oxazepam. This cut-off value was used as it produced no FN results. Cut-off values of 0.2 and 0.5 ng/mg oxazepam produced one FN and two FN results, respectively. Using a 0.1 ng/mg cut-off, the sensitivity and specificity of the Immunalysis Benzodiazepine Microplate ELISA were 100% and 81%, respectively, versus LC–MS–MS.

Benzodiazepines detected by LC–MS–MS in the samples included diazepam (10 samples), nordiazepam (10 samples), oxazepam (3 samples), temazepam (10 samples), lorazepam (1 sample), and nitrazepam (1 sample). The diazepam levels detected in 3 of the 10 samples were below the LOQ; therefore, only 7 samples were reported as positive. Using LC–MS–MS, benzodiazepine hair concentrations ranged from 0.01 to 2.86 ng/mg diazepam, 0.27 to 1.79 ng/mg nordiazepam, 0.35 to 0.89 ng/mg oxazepam, and 0.22 to 0.73 ng/mg temazepam. One sample tested positive for lorazepam at 0.38 ng/mg, and another sample tested positive for nitrazepam at 0.24 ng/mg.

Table IV. Recovery in Spiked Hair

Analyte	Mean % Recovery (% RSD) for <i>n</i> = 5	
	10 ng/30 mg	50 ng/30 mg
7-Aminoflunitrazepam	53 (13)	55 (10)
Chlordiazepoxide	59 (19)	63 (5)
Diazepam	77 (13)	69 (2)
Flunitrazepam	90 (11)	89 (6)
Lorazepam	76 (11)	94 (10)
Nitrazepam	98 (10)	91 (9)
Nordiazepam	88 (12)	82 (5)
Oxazepam	71 (10)	83 (8)
Temazepam	73 (16)	88 (8)

Table V. Stability of Benzodiazepines During Alkaline Pre-Extraction and Loss During Evaporation Step

Analyte	Mean % Recovery % RSD (<i>n</i> = 4)	
	Alkaline Extraction	Evaporation Step
7-Aminoflunitrazepam	97 (2)	72 (6)
Chlordiazepoxide	95 (2)	89 (8)
Diazepam	92 (4)	101 (5)
Flunitrazepam	102 (4)	110 (1)
Lorazepam	87 (9)	103 (5)
Nitrazepam	100 (6)	104 (4)
Nordiazepam	93 (12)	100 (12)
Oxazepam	99 (6)	98 (4)
Temazepam	93 (3)	100 (7)

Discussion

A comparison of the hair and blood results, shown in Table X, found that diazepam was detected in 10 TP hair case samples where a diazepam-positive result was found in blood. One sample contained a diazepam level in hair < LOD. Nordiazepam was not detected in one sample in which the blood result was positive. Interestingly, temazepam was detected in four samples in which the blood result was negative. There was, however, one case where the hair tested negative for temazepam and the blood results were positive. The level of temazepam in blood was very low in this case (0.02 mg/L). This level could be for a naïve

Table VI. Intraday Precision Between Extracts

Analyte	Intraday Precision (% RSD) for <i>n</i> = 5	
	10 ng/30 mg	50 ng/30 mg
7-Aminoflunitrazepam	9.2 (6)	48.6 (2)
Chlordiazepoxide	8.0 (17)	46.0 (6)
Diazepam	8.7 (6)	47.1 (1)
Flunitrazepam	9.3 (7)	50.3 (2)
Lorazepam	7.7 (17)	47.6 (10)
Nitrazepam	9.8 (12)	50.6 (5)
Nordiazepam	9.4 (3)	50.2 (4)
Oxazepam	6.6 (13)	54.1 (7)
Temazepam	5.9 (14)	48.2 (6)

Table VII. Interday Precision Between Extracts

Analyte	Interday Precision (% RSD) for <i>n</i> = 5	
	10 ng/30 mg	50 ng/30 mg
7-Aminoflunitrazepam	9.3 (7)	49.8 (5)
Chlordiazepoxide	6.8 (14)	48.7 (6)
Diazepam	6.6 (19)	44.4 (7)
Flunitrazepam	8.4 (11)	48.7 (2)
Lorazepam	7.0 (13)	45.2 (9)
Nitrazepam	8.8 (10)	46.0 (8)
Nordiazepam	8.9 (5)	47.9 (8)
Oxazepam	5.7 (18)	45.2 (18)
Temazepam	5.7 (11)	45.6 (4)

Table VIII. LOD and LOQ

Analyte	LOD (ng/30 mg)	LOQ (ng/30 mg)
7-Aminoflunitrazepam	0.14	0.23
Chlordiazepoxide	0.07	0.13
Diazepam	0.13	0.22
Flunitrazepam	0.30	0.50
Lorazepam	0.62	1.02
Nitrazepam	0.03	0.05
Nordiazepam	0.24	0.41
Oxazepam	0.11	0.19
Temazepam	0.09	0.16

user, but there is no information in the police report to support this. Oxazepam was detected in one sample for which the blood result was positive. Interestingly, oxazepam was detected in two samples for which the blood result was negative. In contrast, five hair case samples tested negative for oxazepam where the blood result was positive. The blood results were very low in three of these cases (samples 3, 5, and 13). Lorazepam and nitrazepam were detected in one hair sample for which the blood result was negative.

In general, benzodiazepine levels were in the ranges reported in the literature. With the exception of sample 8, nordiazepam was always detected at a higher concentration than diazepam.

This finding is consistent with other studies (5,9).

Oxazepam was only detected in three samples. A possible reason for this low detection rate may be that oxazepam has a relatively short half life compared with nordiazepam, for example, and is a polar compound, which may not readily incorporate into hair (14).

Temazepam levels have not been widely reported in hair. In this study, temazepam levels were present within a relatively short range of 0.22–0.36 ng/mg, with the exception of sample 8, which had a higher temazepam level of 0.73 ng/mg. A possible reason for the lower temazepam levels found in our study is that temazepam could be present as a metabolite of diazepam, rather

than the parent compound. Lorazepam levels in hair have also not been widely documented. The lorazepam-positive sample was found to contain 0.38 ng/mg, which is not within the reported ranges in the literature (5,8). The nitrazepam level (0.24 ng/mg) detected in one case sample was present at a higher level than reported in another study (1).

Overall, it is difficult to directly compare the benzodiazepine levels detected in our study with those reported by other authors because of the range of extraction and analytical methods used.

It is also difficult to interpret the hair results because the amount and type of drug taken over the months prior to death is not known in the majority of cases. The individual from which hair case sample 8 was collected was being prescribed 4 × 5-mg diazepam tablets a day and 5 mg nitrazepam at night. It is not known how long the individual had been under this treatment. The highest diazepam level of all the hair case samples tested was found in this particular case sample, but no nitrazepam was detected. The individual from whom sample 12 was collected had been prescribed diazepam 6 months prior to death.

A problem with the interpretation of results is that diazepam is metabolized to nordiazepam and temazepam, which can both be metabolized further to oxazepam. As well as potentially being present in hair samples because of diazepam metabolism, nordiazepam, temazepam, and oxazepam can also be ingested as parent drugs or they could be present as an association with both metabolism and parent drug use.

In this study, the benzodiazepine levels detected in the postmortem samples indicate chronic use.

Conclusions

The validated ELISA and LC–MS–MS methods detected six therapeutic benzodi-

Table IX. Immunalysis Benzodiazepine Microplate ELISA and LC–MS–MS Positive Hair Results*

Sample number	Immunalysis® Microplate ELISA Benzodiazepine Equivalents		Weight (mg)	LC–MS–MS (ng/mg)					
	Weight (mg)	Concentration (ng/mg)		DZ	NDZ	TZ	OX	NTZ	LOR
1	8.96	> 0.5	13.23	–	–	0.35	–	–	–
2	10.18	> 0.5	9.88	< LOD	0.28	0.22	–	–	–
3	8.19	> 0.5	9.29	0.65	1.20	0.24	–	0.24	–
4	4.30	> 0.5	7.13	< LOD	0.36	0.31	0.89	–	0.38
5	10.03	> 0.5	9.90	< LOQ	0.27	–	–	–	–
6	6.36	> 0.5	5.94	0.32	0.47	0.36	–	–	–
7	10.33	> 0.5	9.86	0.46	–	–	–	–	–
8	10.45	> 0.5	6.47	2.86	1.79	0.73	0.35	–	–
9	20.74	> 0.5	40.56	0.39	1.07	0.30	–	–	–
10	10.23	> 0.5	9.95	< LOQ	0.31	0.22	–	–	–
11	9.94	> 0.5	10.02	0.03	0.43	–	–	–	–
12	9.89	> 0.5	9.80	0.06	0.28	0.23	0.59	–	–
13	5.51	0.48	7.96	< LOQ	–	0.27	–	–	–

* Abbreviations: DZ, diazepam; NDZ, nordiazepam; TZ, temazepam; OX, oxazepam; NTZ, nitrazepam; and LOR, lorazepam.

Table X. Benzodiazepine Results in Hair (ng/mg) and Blood (mg/L)

Sample number	DZ*		NDZ		TZ		OX		NTZ		LOR	
	Hair	Blood	Hair	Blood	Hair	Blood	Hair	Blood	Hair	Blood	Hair	Blood
1	–	–	–	–	0.35	2.81	–	0.16	–	–	–	–
2	< LOD	0.03	0.28	0.01	0.22	–	–	–	–	–	–	–
3	0.65	0.72	1.20	1.42	0.24	0.13	–	0.08	0.24	–	–	–
4	–	0.1	0.36	0.04	0.31	0.01	0.89	–	–	–	0.38	–
5	< LOQ	0.15	0.27	0.20	–	0.02	–	0.02	–	–	–	–
6	0.32	0.38	0.47	0.44	0.36	0.09	–	0.21	–	–	–	–
7	0.46	0.11	–	–	–	–	–	–	–	–	–	–
8	2.86	0.31	1.79	0.54	0.73	0.05	0.35	0.12	–	–	–	–
9	0.39	0.07	1.07	0.06	0.30	–	–	–	–	–	–	–
10	< LOQ	0.04	0.31	0.08	0.22	–	–	–	–	–	–	–
11	0.03	0.02	0.43	0.04	–	–	–	–	–	–	–	–
12	0.06	0.06	0.28	0.11	0.23	–	0.59	–	–	–	–	–
13	< LOQ	0.51	–	0.25	0.27	0.12	–	0.03	–	–	–	–

* Abbreviations: DZ, diazepam; NDZ, nordiazepam; TZ, temazepam; OX, oxazepam; NTZ, nitrazepam; and LOR, lorazepam.

azepines in hair. Thirteen samples screened as TP using the ELISA versus the LC–MS–MS method. The Immunalysis Benzodiazepine Microplate ELISA showed 100% sensitivity and 81% specificity using a cut-off value of 0.1 ng/mg oxazepam.

The LC–MS–MS method used provided the sensitivity necessary for quantitation of benzodiazepines in postmortem hair samples. It appears that mild alkaline extraction conditions are suitable for benzodiazepines and does not result in any significant hydrolysis. An incubation time of 16 h in the alkaline medium produced the greatest diazepam and nordiazepam recoveries compared with the shorter incubation times for one authentic hair case sample. The evaporation step in the SPE method resulted in a relatively high loss of 7-aminoflunitrazepam and chlordiazepoxide, which is partly responsible for the lower total extraction recoveries for these analytes.

The comparison of the hair and blood results has shown that some benzodiazepines were detected in hair but not in blood and vice versa. Hair analysis for benzodiazepines should be used to complement blood analysis.

References

1. R. Kronstrand, I. Nystrom, M. Josefsson, and S. Hodgins. Segmental ion spray LC–MS–MS analysis of benzodiazepines in hair of psychiatric patients. *J. Anal. Toxicol.* **26**: 479–484 (2002).
2. A. Seymour, M. Black, and J.S. Oliver. Drug related deaths in the Strathclyde region of Scotland, 1995–1998. *Forensic Sci. Int.* **122**: 52–59 (2001).
3. www.isdscotland.org/isd/files/final_F05.xls.
4. *Benzodiazepines and GHB, Detection and Pharmacology*. S.J. Salamone, Ed. Humana Press, Totowa, NJ, 2001.
5. M. Yegles, F. Mersch, and R. Wennig. Detection of benzodiazepines and other psychotropic drugs in human hair by GC/MS. *Forensic Sci. Int.* **84**: 211–218 (1997).
6. Progress in hair analysis for illegal drugs. In *Workshop Proceedings of the International Society of Hair Testing*, June 18 to 20, 2000, R. Klaus Muller and D. Thieme, Eds. Kreischa, Sport and Buch Strauss, Cologne, Germany, 2000, pp 22–34.
7. A. Negrusz, A.M. Bowen, C.M. Moore, S.M. Dowd, and M.J. Strong. Deposition of 7-aminoclonazepam and clonazepam in hair following a single dose of Klonopin™. *J. Anal. Toxicol.* **26**: 471–478 (2002).
8. V. Cirimele, P. Kintz, and P. Mangin. Detection and quantification of lorazepam in human hair by GC-MS/MS in a case of traffic accident. *Int. J. Legal Med.* **108**: 265–267 (1996).
9. V. Cirimele, P. Kintz, and B. Ludes. Screening for forensically relevant benzodiazepines in human hair by gas chromatography–negative ion chemical ionization–mass spectrometry. *J. Chromatogr. B* **700**: 119–129 (1997).
10. P. Kintz, V. Cirimele, F. Vayssette, and P. Mangin. Hair analysis for nordiazepam and oxazepam by gas chromatography–negative ion chemical ionization mass spectrometry. *J. Chromatogr. B* **677**: 241–244 (1996).
11. R. Kronstrand, I. Nystrom, J. Strandberg, and H. Druid. Screening for drugs of abuse in hair with ion spray LC–MS–MS. *Forensic Sci. Int.* **145**: 183–190 (2004).
12. M. Villain, M. Concheiro, V. Cirimele, and P. Kintz. Screening method for benzodiazepines and hypnotics in hair at pg/mg level by liquid chromatography–mass spectrometry/mass spectrometry. *J. Chromatogr. B* **825**: 72–78 (2005).
13. M. Cheze, M. Villain, and G. Pepin. Determination of bro-mazepam, clonazepam and metabolites after a single intake in urine and hair by LC–MS/MS. Application to forensic cases of drug facilitated crimes. *Forensic Sci. Int.* **145**: 123–130 (2004).
14. A. El Mahjoub and C. Staub. Determination of benzodiazepines in human hair by on-line high-performance liquid chromatography using a restricted access extraction column. *Forensic Sci. Int.* **123**: 17–25 (2001).
15. A. Negrusz, C. Moore, D. Deitermann, D. Lewis, K. Kaleciak, R. Kronstrand, B. Feely, and R.S. Niedbala. Highly sensitive micro-plate enzyme immunoassay screening and NCI-GC–MS confirmation of flunitrazepam and its major metabolite 7-aminoflunitrazepam in hair. *J. Anal. Toxicol.* **23**: 429–435 (1999).
16. P. Kintz, B. Ludes, and P. Mangin. Detection of drugs in human hair using Abbott ADx with confirmation by GC/MS. *J. Forensic Sci.* **37**: 328–331 (1992).
17. J.J. Sramek, W.A. Baumgartner, T.N. Aherns, V.A. Hill, and N.R. Cutler. Detection of benzodiazepines in human hair by radioimmunoassay. *Ann. Pharmacother.* **26**: 469–471 (1992).
18. C. Moore, M. Feldman, E. Harrison, D. Irvan, D. Kuntz, W. Ross, N. Giorgi, A. Agrawal, S. Rana, M. Vincent, and J. Soares. Analysis of cocaine and metabolites in hair, oral fluid and urine. *Annal. Toxicol. Anal. (SFTA)* **XVII No. 4**: 221–228 (2005).
19. C. Moore, M. Feldman, N. Giorgi, W. Ross, E. Harrison, D. Irvan, D. Kuntz, A. Agrawal, S. Rana, M. Vincent, and J. Soares. Analysis of amphetamines in hair, oral fluid and urine. *Annal. Toxicol. Anal. (SFTA)* **XVII No. 4**: 229–236 (2005).
20. S.A. Sweeney, R.C. Kelly, J.A. Bourland, T. Johnson, W.C. Brown, H. Lee, and E. Lewis. Amphetamines in hair by enzyme-linked immunosorbent assay. *J. Anal. Toxicol.* **22**: 418–424 (1998).
21. M. Cassani and V. Spiehler. Analytical requirements, perspectives and limits of immunological methods for drugs in hair. *Forensic Sci. Int.* **63**: 175–184 (1993).
22. K.S. Scott and Y. Nakahara. A study into the rate of incorporation of eight benzodiazepines into rat hair. *Forensic Sci. Int.* **133**: 47–56 (2003).
23. J.N. Miller and J.C. Miller. *Statistics and Chemometrics for Analytical Chemistry*, 4th ed. Pearson Education, Harlow, U.K., 2000.

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