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Use of the Randox Evidence Investigator immunoassay system for near-body drug screening during post-mortem examination in 261 forensic cases.

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Highlights

- The Evidence Investigator screens for a range of drugs in postmortem specimens
- Qualitative results can be obtained in the time it takes to complete an autopsy
- Assay results for each specimen was in good agreement with confirmatory analysis

Abstract

Background

This paper describes the performance of four Randox drug arrays, designed for whole blood, for the near-body analysis of drugs in a range of post-mortem body specimens.

Methods

Liver, psoas muscle, femoral blood, vitreous humor and urine from 261 post-mortem cases were screened in the mortuary and results were obtained within the time taken to complete a post-mortem. Specimens were screened for the presence of amphetamine, barbiturates,

benzodiazepines, benzoylecgonine, buprenorphine, cannabinoids, dextropropoxyphene, fentanyl, ketamine, lysergide, methadone, metamfetamine, methaqualone, 3,4-methylenedioxymetamfetamine, opioids, paracetamol, phencyclidine, salicylate, salicylic acid, zaleplon, zopiclone and zolpidem using the DOA I, DOA I+, DOA II and Custom arrays.

Results

Liver and muscle specimens were obtained from each of the 261 post-mortem cases; femoral blood, vitreous humor and urine were available in 98%, 92% and 72% of the cases, respectively. As such, the equivalent of 12,978 individual drug-specific, or drug-group, immunoassay tests were undertaken. Overall >98% of the 12,978 screening tests undertaken agreed with laboratory confirmatory tests performed on femoral blood.

Conclusions

There is growing interest in the development of non-invasive procedures for determining the cause of death using MRI and CT scanning however these procedures are, in most cases, unable to determine whether death may have been associated with drug use. The Randox arrays can provide qualitative and semi-quantitative results in a mortuary environment enabling pathologists to decide whether to remove specimens from the body and submit them for laboratory analysis. Analysis can be undertaken on a range of autopsy specimens which is particularly useful when conventional specimens such as blood are unavailable.

Keywords: Post-mortem, Toxicology, Near-body Drug Screening, Forensic Pathology, Laboratory Tests.

Introduction

Interest is increasing in the use of non-invasive techniques to ascertain the cause of death as a replacement for the traditional post-mortem examination. Magnetic resonance imaging (MRI) and computerised tomography (CT) scanning have been suggested in lieu of invasive post-mortems [1 – 3]. Whilst CT and MRI scanning may be suitable for the diagnosis of certain types of death there are weaknesses [3] and pathologists must still rely on the removal of

tissue specimens for laboratory analysis before drug or poisoning related deaths may be identified. The removal of post-mortem tissue can be complicated by legislation or religious custom hence a rapid near-body screen that can provide an indication of the presence of drugs in a body could be beneficial. Emergency departments, drug treatment clinics and workplace drug testing programs already use near-body drug screening to determine an individual's recent drug use [4-9] but few reports describe the use of near-body methodologies for post-mortem drug screening.

Immunoassays are simple to use however the majority of drug screening immunoassays designed for clinical settings are not suitable for post-mortem cases as their cut-off limits are too high and drugs of forensic importance may be missed. Furthermore, clinical near-patient tests are limited to detecting a small selection of common drugs of abuse and are also based on technology that requires relatively non-viscous specimens e.g. urine or oral fluid in order to operate.

The Randox Evidence Investigator system relies on biochip technology rather than conventional lateral flow technology and is subsequently amenable for use with a wide range of tissue specimens. Each biochip measures 9 x 9 mm and can have up to 13 drug specific antibodies immobilised in predefined regions on the biochip. Chemiluminescence is employed to obtain semi-quantitative drug concentrations. Light signals generated from each antibody site on the biochip are simultaneously detected using digital imaging technology and compared to that from a calibration curve. Nine biochips are mounted within individual wells on a single cassette enabling analysts to screen either nine different cases or fewer cases but a wider range of tissue specimens simultaneously. We previously reported on the performance of the Randox DOA I and DOA II panels in 106 forensic post-mortems [10]. In this paper we describe the use of the DOA I, DOA I+, DOA II and a "Custom Array" panel to detect a range of drugs (Table 1) in a variety of post-mortem toxicology specimens obtained from 261 post-mortem cases.

Table 1 Drugs detected using the DOA I, DOA I+, DOA II and Custom arrays

DOA I (calibration range, LOD)	DOA I+ (calibration range, LOD)	DOA II (calibration range, LOD)	Custom (calibration range, LOD)
Amfetamine (0-108 ng/mL, 1.60 ng/mL)	Amfetamine (0-108 ng/mL, 1.60 ng/mL)	Buprenorphine (0-80 ng/ml, normalised value of 71)	Paracetamol (0-695 µg/mL, 1.045 µg/mL)
Barbiturates (0-356 ng/mL, 1.59 ng/mL)	Barbiturates (0-356 ng/mL, 1.59 ng/mL)	Dextropropoxy phene (0-2000 ng/ml, normalised value of 71)	Salicylate (0 - 665 µg/ml, 0.495 µg/mL)
Benzodiazepines I ^a (0-244 ng/mL, 0.37 ng/mL)	Benzodiazepines I ^a (0-244 ng/mL, 0.37 ng/mL)	Fentanyl (0-40 ng/ml, normalised value of 76)	Salicylic acid (0 - 665 µg/ml, 0.495 µg/mL)
Benzodiazepines II ^a (0-280 ng/mL, 0.29 ng/mL)	Benzodiazepines II ^a (0-280 ng/mL, 0.29 ng/mL)	Ketamine (0-2000 ng/ml, normalised value of 10)	Zaleplon (0 - 202 ng/mL, 0.1 ng/mL)
Benzoylcegonine (0-244 ng/mL, 0.36 ng/mL)	Benzoylcegonine (0-244 ng/mL, 0.36 ng/mL)	Lysergide (0-8000 ng/ml, normalised value of 76)	Zolpidem (0 – 137 ng/ml, 0.075 ng/mL)
Cannabinoids (0-40 ng/mL, 0.98 ng/mL)	Buprenorphine (0.08 ng/mL)	Methaqualone (0-500 ng/ml, normalised value of 10)	Zopiclone (0 - 516 ng/mL, 0.375 ng/mL)
Metamfetamine (0-368 ng/mL, 6.75 ng/mL)	Cannabinoids (0-40 ng/mL, 0.98 ng/mL)	3,4- Methylenedioxy metamfetami ne (0-4000 ng/ml, normalised value of 77)	
Methadone (0-132 ng/mL, 0.16	Metamfetamine	Opioids ^c (0-2000 ng/ml,	

ng/mL)	(0-368 ng/mL, 6.75 ng/mL)	normalised value of 53)	
Opiates ^b (0-132 ng/mL, 0.09 ng/mL)	Methadone (0-132 ng/mL, 0.16 ng/mL)	Oxycodone I ^d (0-2000 ng/ml, normalised value of 43)	
Phencyclidine (0-48 ng/mL, 0.10 ng/mL)	3,4-Methylenedioxyamphetamine (1.93 ng/mL)	Oxycodone II ^d (0-2000 ng/ml, normalised value of 18)	
	Opiates ^b (0-132 ng/mL, 0.09 ng/mL)		
	Phencyclidine (0-48 ng/mL, 0.10 ng/mL)		
	Tricyclic Antidepressants (1.06 ng/mL)		

^aBenzodiazepine I has high cross reactivity for oxazepam, midazolam, flunitrazepam, diazepam, temazepam, ethylflurazepam and clobazam whereas benzodiazepine II is specific for lorazepam.

^bOpiate antibody cross reacts with morphine (100%), 6-acetylmorphine (1214%), codeine (106%), morphine-3-glucuronide (16%) and hydromorphone (27%).

^cOpioid antibody cross reacts with hydrocodone (2282%), ethyl morphine (867%), hydromorphone (163%), codeine (291%), oxycodone (100%), dihydrocodeine (82%), thebaine (21%) and morphine (9%).

^dOxycodone I has higher cross reactivity for noroxycodone and hydrocodone compared to Oxycodone II.

Materials and Methods

Femoral blood, urine, vitreous humor, liver and psoas major muscle specimens were obtained from 261 consecutive forensic post-mortem cases carried out at the Sir James Black Mortuary, Dundee. Ethics permission was not necessary as the information obtained was included in the toxicology case report, following the guidelines by the Procurator Fiscal.

Specimen *Preparation*

Blood was collected from the ligated femoral vein and urine was syringed from the bladder using a 10 mL syringe. Vitreous humor was collected with a 5 mL syringe and a 19 gauge needle via puncture through the sclera. Body fluids were collected into tubes containing sodium fluoride and potassium oxalate. The liver specimen was obtained from the right lobe and a 1 cm³ section of the psoas major muscle was also collected. On completion of screening all tissue specimens, other than blood, were returned to the body.

Liver and psoas major muscle (1 cm³; approximate weight 1 g) were cut into small pieces and homogenised with 1 mL of the manufacturer's diluent. Specimens were blended to form a smooth homogenate using a Janke Kunkel Ultra Turrax t25 homogeniser (IKA Laboratory). Homogenates were centrifuged for 10 minutes at 3000 rpm and 25 µL aliquots of the supernatant were removed. 25 µL aliquots of blood, vitreous humor, urine, and liver and muscle supernatant were diluted 1:3 v/v with assay diluent as per the kit manufacturer's instruction. Specimens were thoroughly mixed using a vortex mixer prior to introduction of the specimen onto the biochip.

Immunoassay Protocol

A two day training course was provided by Randox to operate the Evidence Investigator system, the protocol was as follows. Assay diluent (120 µL) was added to each biochip followed by either 60 µL of a calibrator, control, or specimen. A calibration curve cassette and quality controls (low and high), provided by Randox, for each array were run alongside each set of case specimens. A conjugate (120 µL) consisting of horseradish peroxidase labelled analytes was added to compete for specific antibodies with any drug present in the specimen. Biochips were then incubated and agitated at 25°C for 30 minutes at 330 rpm in a 'Thermoshaker'. The DOA II array protocol differs from the DOA I, DOA I+ and Custom array only in that the DOA II array has an incubation temperature of 30°C and an increased agitation of 370 rpm. Subsequent to incubation each biochip was relieved of unbound analytes by 6 quick washes followed by 6 two-minute soaks using the manufacturer's wash buffer. Fluid remaining in the cassettes was drained onto absorbent paper. Luminol:peroxide signal reagent

(250 μ L) was added to each biochip and the cassette was protected from light for 2 minutes prior to insertion into the Evidence Investigator imaging system for measurement of the chemiluminescence reaction.

Confirmation

Confirmatory analysis of the femoral blood specimens of all 261 cases was performed at the Centre of Forensic and Legal Medicine toxicology laboratory (Dundee) using LC-MS/MS, LC-DAD and GC-MS. LC-MS/MS assay was performed using an AB Sciex 3200 Qtrap (Warrington, UK) with Agilent (Wokingham, UK) 1200 series HPLC system. A Phenomenex Gemini column (150 mm x 2 mm) was used for analysis. A mobile phase of 3% acetonitrile for 3 minutes increased to 65% acetonitrile over 20 minutes was used, total run time of 23 minutes. Mobile phase A comprised 1 mM ammonium formate and 0.1% formic acid. Mobile phase B comprised 70% acetonitrile, 1 mM ammonium formate and 0.1% formic acid.

Detection by LC-DAD was based on a previously published method [11] using a Dionex (Camberley, UK) HPLC system fitted with a Phenomenex Synergi 4m Fusion 150 mm x 4.6 mm column. Data acquisition was achieved using a diode array detector recording between 200 nm and 595 nm.

GC-MS was performed using an Agilent (Stockport, UK) 7890A gas chromatograph interfaced with an Agilent 5975C mass spectrometer using an adapted version of a previously published method [12]. The GC conditions involved splitless injection onto a HP-5MS column (30 m x 0.25 mm ID, 0.25 μ m film thickness); injection port temperature, 175°C; carrier gas, helium; flow rate, 1.7 ml/min; column temperature, 60°C for 1.5 min and increased by 10°C /min to 220°C and held for an additional 9 min. The limit of detection (LOD) employed for barbiturates was 0.1 mg/L, tricyclic antidepressants, amine drugs and methadone 0.01 mg/L, and a LOD of 0.004 mg/L for opioids and benzodiazepines.

Results

Blood, urine, vitreous humor, liver and muscle from 261 post-mortem cases were screened and results were obtained within 60 minutes of the pathologist providing the necessary specimens. Although the Evidence Investigator is reported to be semi-quantitative, the calibrators and controls were designed for whole blood. Therefore the assay results obtained for the biological specimens were only qualitative. Validation studies would have to be undertaken to determine the matrix effects to allow semi-quantitative data to be reported. Liver and muscle specimens were obtained from all post-mortem cases whereas blood, vitreous humor and urine were available in 98%, 92% and 72% of the cases respectively. The post-mortem cases were screened using the DOA I, DOA I+, DOA II, or the custom array, or a combination of two arrays. In total, 1,207 biochips across the four arrays were run that screen for several drugs, or drug classes, simultaneously (Table 1), equating to 12,978 individual drug/drug group screens being processed.

Table 2 Summary of confirmed screening tests in different tissues compared against confirmed blood analysis

	Liver		Urine		Muscle		Blood		Vitreous	
	Cases Confirmed	% Agreed	Cases Confirmed	% Agreed	Cases Confirmed	% Agreed	Cases Confirmed	% Agreed	Cases Confirmed	% Agreed
Amfetamine	3	100	2	100	3	100	3	100	3	100
Barbiturates	1	100	1	100	1	100	1	100	1	100
Benzodiazepines	70	99	56	95	70	96	68	94	63	78
Benzoylcegonine	1	100	1	100	1	100	1	100	1	100
Methadone	20	100	17	100	20	95	19	95	18	89
Opiates ^a	83	99	61	100	81	94	83	96	80	95

Opioids ^a	15	100	10	100	15	100	15	87	13	100
Oxycodone ^a	2	100	2	100	2	100	2	100	2	100
Paracetamol	12	100	7	100	12	100	12	100	11	82
Salicylate	11	100	10	100	11	100	11	91	11	91
Tricyclic Antidepressants	20	100	15	100	20	95	18	94	18	89

Comparison of the number of cases in which confirmed screening test results (cases confirmed) are shown against the number of results confirmed in femoral blood (% agreed) for liver, urine, muscle, blood and vitreous humor. ^aOpiates, and opioids and oxycodone not grouped as different antibodies were used in the DOA I+ and DOA II arrays, respectively.

Of the 261 post-mortem blood specimens that underwent confirmatory analyses, 151 were positive for one or more analytes. The analytes detected by confirmatory analysis in the blood are listed in Table 2. Opiates and benzodiazepines were the most common groups of drugs detected whilst barbiturates and benzoylcegonine were only infrequently encountered. The liver screens were in $\geq 99\%$ agreement with confirmatory analysis, urine $\geq 95\%$, muscle $\geq 94\%$, blood $\geq 87\%$ and vitreous humor $\geq 78\%$.

Table 3 Kappa analysis between the Radox microchip screening test results in different tissues and the gas chromatography / mass spectrometry confirmatory analysis undertaken on whole blood specimens.

	Liver	Muscle	Blood	Vitreous Humor	Urine
Amphetamine	.488	.316	.414	.854	.318
Barbiturates	.664	1.00	1.00	1.00	1.00

Benzodiazepines	.945	.978	.932	.809	.956
Benzoyllecgonine	1.00	.956	1.00	1.00	1.00
Methadone	1.00	.944	.970	.935	.967
Opiates ^a	.888	.896	.958	.922	.820
Opioids ^a	.871	.936	.870	.931	1.00
Oxycodone ^a	.783	1.00	1.00	1.00	.425
Paracetamol	.805	.684	.867	.842	.903
Salicylate	.801	.801	.784	.702	.676
Tricyclic Antidepressants	.885	.938	.901	.865	.859

Statistical measurements using Kappa scores enables sets of data to be compared to observe agreement between the data sets. Kappa scores between 0.81 -0.99 represent almost perfect agreement, 0.61-0.8 substantial agreement, 0.41-0.6 moderate agreement and 0.1 -0.2 slight agreement. ^aOpiates, and opioids and oxycodone not grouped as different antibodies were used in the DOA I+ and DOA II arrays, respectively.

To take into account the possibility of chance agreement, Cohen's Kappa testing [13] was undertaken. Kappa testing may be used to demonstrate the degree of agreement corrected for the element of chance agreement between the different screening tests and confirmatory analysis. Table 3 summarises the results of Kappa analysis between the Randox microchip screening test results in different biological specimens and confirmatory analysis undertaken on whole blood specimens.

Table 4 Number of cases where non-concordant immunoassay results were obtained

	Liver	Muscle	Blood	Vitreous	Urine
Amfetamine	6	12	8	1	8

Barbiturates	1	-	-	-	-
Benzodiazepines	4	1	2	1	-
Metamfetamine	9	8	5	2	11
Methadone	-	1	-	-	1
3,4-Methylenedioxyamfetamine	10	9	1	1	7
Opiates ^a	10	3	1	3	13
Opioids ^a	2	1	-	1	-
Oxycodone ^a	1	-	-	-	4
Paracetamol	3	5	2	-	1
Salicylic acid	3	3	2	3	4
Tricyclic antidepressants	4	1	2	2	4
Total discrepancies (false positives) excluding THC ^b	53	44	24	14	53
Percentage of tests that disagreed with confirmatory analysis, false positives (excluding THC ^b)	2.0%	1.7%	0.9%	0.6%	2.8%
Percentage of tests that disagreed with confirmatory analysis, false positives and false negatives (excluding THC ^b)	2.1%	2.0%	1.4%	1.6%	2.9%

^aOpiates, and opioids and oxycodone not grouped as different antibodies were used in the DOA I+ and DOA II arrays, respectively. ^bTHC Tetrahydrocannabinol - analyte that gives a positive indicator for cannabinoids.

Non-concordant immunoassay results were detected <3% of the post-mortem specimens (Table 4). Liver and urine had the greatest occurrence of additional positives compared to their respective confirmed blood specimen, followed by muscle, blood and vitreous humor. False positive immunoassay results were detected in <1% of all blood specimens tested.

In addition to the drugs listed in Table 2, cannabinoids were also detected using the DOA I and DOA I+ array. One or more tissue specimens obtained from 33 post-mortem cases gave positive cannabinoid screens, however confirmatory analysis for cannabinoids was not undertaken and therefore these results could not be included in Table 4.

Although the test systems evaluated were reported to be capable of detecting buprenorphine, dextropropoxyphene, fentanyl, ketamine, lysergide, methaqualone, PCP, salicylic acid, zaleplon, zopiclone or zolpidem, no cases were encountered during the study period where these drugs were deemed present by the confirmatory methods. In addition, no false positives were obtained for the aforementioned drugs.

Discussion

Tissue selection for toxicological analysis during a post-mortem examination is dictated by availability and whilst analysis of peripheral blood specimens is the preferred option for interpretative purposes, alternative specimens are important in cases where blood may not be available. In putrefied bodies blood or urine may not be available and muscle may be less affected than liver or other tissues. For interpretation peripheral blood is the desired specimen however, if no blood is obtainable liver is an excellent tissue for providing evidence of drug use as it is the major organ in the body where drugs are metabolised. Vitreous humor is particularly useful as a tissue for drug analysis in bodies subjected to major trauma although much has still to be learned concerning the relationship of drugs in this tissue to that in other

tissues. In this study we therefore investigated the use of a microchip screening technique to observe how it would perform using a variety of different post-mortem tissues.

The routine procedure employed at the Centre of Forensic and Legal Medicine (Dundee) was to perform confirmatory analysis on blood only. In cases where blood is unavailable an alternative tissue is analysed. Since only blood was used for confirmatory analysis, an assumption was made that where a positive screening result was observed in urine, vitreous humor, liver and muscle, the blood could be used to corroborate the screening result. Excellent correlation was observed between blood confirmation and liver and urine screening results (Table 2). This is not surprising because liver is the principal organ responsible for drug metabolism and urine has a long drug detection window. Muscle and blood also presented excellent overall agreement with the confirmatory results. In the cases where positive correlation between screening and confirmatory analysis in blood and muscle was not observed it was noted that the blood drug concentrations were usually close to cut-off and patient case histories indicated infrequent or low drug consumption. Vitreous humor screening results were the most varied, agreement with confirmatory analyses ranged from 78-100%. However the Kappa analysis (Table 3) shows that there was almost perfect agreement between screening and confirmatory analysis for the majority of drugs, with amfetamine being the exception; scores between 0.81-0.99 represent almost perfect agreement, 0.61-0.8 substantial agreement, 0.41-0.6 moderate agreement and 0.1-0.2 slight agreement [13].

In general the additional drug positives (Table 4) could be supported by the individual's case history with the exception of amine based drugs, and paracetamol and aspirin (indicated by salicylate, salicylic acid) as their use was not always recorded. The majority of non-concordant results were attributed to cross reaction between putrefactive amines and the amfetamine antibody. False positives for amfetamine, metamfetamine and 3,4-methylenedioxymetamfetamine occurred in blood specimens from decedents who were left undiscovered for a number of weeks or were located in a warm environment. Urine, liver and

muscle were also adversely affected by putrefactants, as shown by GC/MS, however few false positive amphetamine, metamphetamine and 3,4-methylenedioxymetamphetamine results were obtained in vitreous humor specimens. In general fewer presumed false positives were detected in vitreous humor compared to blood, muscle, liver and urine (0.6% compared to 0.9%, 1.7%, 2.0% and 2.8% respectively). This is most likely due to the isolated compartment in which vitreous humor is located and the selectivity of the tissue membrane. The selectivity of the vitreous membrane may also inhibit or delay the incorporation of some drugs into eye fluid could offer a possible explanation for the lower agreement of vitreous humor screening results with confirmation analysis. Cannabinoids were detected in 33 post-mortem cases and although no confirmatory analysis was undertaken to detect cannabinoids, good agreement was observed between screening results and the decedent's case history where individuals were reported to be cannabis users. Cannabinoids were detected consistently in liver, muscle, blood and urine; however its presence was relatively infrequent in vitreous humor specimens. Only 2 out of 31 cases where vitreous humor was available gave positive screening tests for cannabinoids. In these 2 cases the blood, urine, liver and muscle specimens yielded high semi-quantitative concentrations (greater than the calibration range). This finding was also observed by Jenkins and O'Block [14] who reported positive cannabinoid vitreous humor results only when high concentrations were detected in the related blood specimen.

Conclusion

The Randox DOA I, I+, II and Custom array drug screening panels offer pathologists and toxicologists the opportunity to screen a range of tissue specimens for the presence of common therapeutic and abused drugs, specifically those listed in Table 2. As many drugs on the four arrays were not detected by confirmatory analysis, comments cannot be made on their ability to detect true positives, however the lack of false positives can be commented upon. The screening procedure is simple, sufficiently sensitive to make it appropriate for use in forensic toxicology, and the speed with which the assay can be completed enables a drug screen to be undertaken whilst an autopsy is in progress. The assays may be used to screen

blood, urine, liver, vitreous humor and muscle enabling their use in cases where conventional specimens may be unavailable e.g. fire deaths or decomposed bodies. Excellent agreement for the presence of amfetamines, barbiturates, benzodiazepines, cocaine derivatives, methadone, opioids, paracetamol, salicylate and tricyclic antidepressants in liver, urine, blood and muscle were obtained between the near-body screening test and laboratory analysis of femoral blood specimens. Although confirmatory analysis for the presence of cannabinoids was not undertaken, the cases where cannabinoids were detected by the screening tests were corroborated by case histories. Vitreous fluid showed poorer correlation between the screening test and confirmatory analysis on blood however it is recognised that a number of factors can influence the passage of drugs into eye fluid.

An indication of the presence or absence of drugs in a body whilst the post-mortem is being undertaken could influence the pathologist in deciding whether to remove organs for laboratory analysis, reducing the cost of death investigation and simplifying procedures associated with the Human Tissue Act.

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