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Development and validation of an ESI-LC-MS/MS method for simultaneous identification and quantification of 24 analytes of forensic relevance in vitreous humour, whole blood and plasma†

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Detection and quantification of drugs from various biological matrices are of immense importance in forensic toxicological analysis. Despite the various reported methods, development of a new method for the detection and quantification of drugs is still an active area of research. However, every method and biological matrix has its own limitation, which further encourage forensic toxicologists to develop new methods and to explore new matrices for the analysis of drugs. In this study, an electrospray ionization-liquid chromatograph-tandem mass spectrometry (ESI-LC-MS/MS) method is developed and validated for simultaneous identification and quantification of 24 drugs of forensic relevance in various body fluids, namely, whole blood, plasma and vitreous humour. The newly developed method has been validated for intra-day and inter-day accuracy, precision, selectivity and sensitivity. Absolute recovery shows a mean of 84.5, 86.2, and 103% in the vitreous humour, whole blood and plasma respectively, which is suitable for the screening procedure. Further, the absolute matrix effect (AME) shows a mean of 105, 96.5, and 109% in the vitreous humour, whole blood and plasma, respectively. In addition, to examine the practical utility of this method, it has been applied for screening of drugs in post-mortem samples of the vitreous humour, whole blood and plasma collected at autopsy from ten cadavers. Experimental results show that the newly developed method is well applicable for screening of analytes in all the three matrices. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: vitreous humour; ESI-LC-MS/MS; post-mortem; drugs, forensic

Introduction

Increasing cases of homicide, suicide and accidental death due to consumption of drugs of abuse and other therapeutic drugs is a matter of serious concern around the globe. Easy availability of therapeutic narcotics through modern means of marketing such as the Internet, make their misuse or abuse a serious social problem.[1] In cases where death due to use or abuse of drugs is involved, forensic analysis of drug levels in biological matrices is of immense importance.

Simultaneous screening and quantification of drugs in various matrices is advantageous in the forensic set-up, primarily due to two major reasons: one, the possibility of usage of a wide variety of drugs (chemicals) and the other, rapid disposal of cases. Many researchers have reported methods for simultaneous screening and quantification of drugs, poisons and their metabolites in various body fluids, including blood, plasma, serum[2–4] and urine[5–7] using liquid chromatography-tandem mass spectrometry (LC-MS/MS). One such method has been developed and validated for the determination of 19 drugs of abuse and their metabolites in whole blood by Bjork *et al.*[8] Oiestad *et al.*[9] have developed and validated a method for the screening of multi-drugs in whole blood by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). In a recent study, a method for screening of 70 drugs in vitreous humour and urine samples has been developed using time-of-flight mass spectrometry (TOF-MS).[10] Dresen *et al.*[11] describes a multi-target screening method for simultaneous

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detection and identification of 700 drugs and metabolites in biological fluids using a hybrid triple-quadrupole linear ion trap mass spectrometer. Mueller *et al.*[12] described a multi-target screening procedure for 301 forensically relevant drugs in blood and urine for toxicological analysis. Though several methods have been reported for the screening of drugs in whole blood, systematic studies reporting a screening procedure for drugs in matrices like whole blood, plasma and vitreous humour, are still lacking.

Whole blood is a routine post-mortem sample collected at the time of autopsy for the screening of drugs. Unavailability of whole blood in certain forensic cases has encouraged forensic scientists to explore alternative samples for the screening of drugs. Among the alternative samples, vitreous humour present in the posterior segment of the eye is less liable to be affected by post-mortem changes and appears to be a suitable complementary sample to the whole blood. Despite of the fact, vitreous humour has rarely been used for the screening of drugs due to the complications involved in the interpretation of quantitative results.[13,14]

It is evident from the literature that plasma is the most commonly used sample for clinical purposes while in forensic studies, whole blood is mostly collected and analyzed. It is not justifiable to use the data of clinical literature, which is for plasma, to post-mortem whole blood as during life time an uneven blood to plasma ratio is maintained by active processes, which may decay after death.[15] Thus, there is a need to develop a separate method for both the matrices, i.e., whole blood and plasma.

The present study is aimed to develop an analytical method for the simultaneous identification and quantification of 24 analytes of forensic relevance in the three matrices namely, vitreous humour, whole blood and plasma using LC-MS/MS. In this study, both psychotropic and therapeutic drugs have been included as psychotropic drugs may be administered in combination with other therapeutic drugs to cause death. Drugs are selected from a wide group such as benzodiazepines, analgesics, opiates, stimulants, antihistamines, antipsychotics, anticholinergic, β-blockers, hypnotics, Ca channel blocker and vasodilators. The rationale for selection of these drugs is their frequent occurrence in our laboratory for analysis.

The newly developed method is advantageous over existing methods in two major aspects: one, the simplified sample preparation protocol and the other, requirement of small sample size for analysis. Both aspects are crucial in the forensic scenario, where a lack of sample volume available for analysis may limit the possible number of tests to be conducted. In this study, a small sample volume of 20 μL is sufficient for analysis.

Experimental

Chemicals and reagents

Alprazolam, atropine, clonazepam, cocaine, codeine, flunitrazepam, ketamine, methamphetamine, nicotine, nordiazepam, oxazepam, sulfadimethoxine and zolpidem were obtained from Sigma-Aldrich (St Louis, MO, USA). Norketamine was purchased from Tocris Bioscience (Bristol, Avon, UK). Homatropine hydrobromide was purchased from Boehringer Ingelheim, Germany. Heroin and morphine were obtained from OAW, Neemuch, M.P., India. The rest of the analytes (acetaminophen, amlodipine, chlorpromazine, clonidine, diazepam, olanzapine, pethidine, pheniramine and timolol) were obtained from the pharmaceutical industry. LC-MS grade formic acid, acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Water (18.2 MΩ) was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA). All other chemicals and solvent were of the highest analytical grades available.

UPLC-MS/MS conditions

LC-MS/MS experiments were performed using a 4000 Q-TRAP tandem mass spectrometer (AB Sciex, Foster City, CA, USA) coupled with ultra high performance liquid chromatography (UHPLC, Accela Thermo Fisher, Waltham, MA, USA) with auto-sampler and online vacuum degasser. All the parameters of tandem mass spectrometer and UHPLC were controlled by Analyst software, version 1.4.2 (AB Sciex, Foster City, CA, USA) and ChromQuest software, version 4.5 (Thermo Fisher, Waltham, MA, USA), respectively.

For the analytical separation of 24 drugs, gradient elution was performed on a Hypersil Gold column (50 mm x 2.1 mm, 1.9 μm, Thermo Fisher Scientific, Waltham, MA, USA). Mobile phase consisted of (A) methanol with 0.1 % formic acid and (B) 2 mM ammonium formate in water. All the drugs were eluted with a gradient of 10 % solvent A (0–2 min), 10–25 % solvent A (2–4 min), 25–30 % solvent A (4–6 min), 30–50 % solvent A (6–9 min), 50–80 % solvent A (9–14 min), 80–10 % solvent A (14–16 min) and 10 % solvent A (16–20 min). The mobile phase was pumped at a flow rate of 0.3 mL/min. The column was equilibrated for 4 min between each analysis. The auto-sampler tray and the column were maintained at 25 ± 1°C. Twenty microlitre of sample was injected into the UHPLC.

Electrospray ionization in positive mode was applied using Turbo Ion Spray source (AB Sciex, Foster City, CA, USA). Full scan mass spectra and fragment ion scan spectra of all standards were obtained by flow infusion analysis (FIA). Compound dependent parameters for each analyte such as Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE) and Cell Exit Potential (CXP) were manually optimized by infusing individual standard solutions at 100 ng/mL into the ion source of the mass spectrometer at a flow rate of 5 μL/min using a Harvard pump (Harvard Company, Reno, NV, USA) connected with a Hamilton syringe (Holliston, MA, USA). Quantification was performed using multiple reaction monitoring (MRM) mode based on the molecular/fragment ion transitions for each of the drugs. Detailed parameters for each compound are summarized in Table 1. Source dependent parameters were optimized by FIA: gas 1 (40 psi); gas 2 (60 psi); curtain gas (20 psi); ion spray voltage (5500 V) and temperature (450°C). The dwell time for each MRM transition was set at 50 ms.

Calibration standards and quality control samples

Stock solutions of all the analytes, (acetaminophen, alprazolam, amlodipine, atropine, chlorpromazine, clonazepam, clonidine, cocaine, codeine, diazepam, flunitrazepam, heroin, ketamine, methamphetamine, morphine, nicotine, nordiazepam, norketamine, olanzapine, oxazepam, pethidine, pheniramine, timolol, zolpidem, homatropine and sulphadimethoxine) were prepared separately using methanol to have a concentration of 1 mg/mL. A working solution of 10 μg/mL was prepared as a mixture of the 24 drugs from each of their stock solutions.

Spiking vitreous humour, whole blood, and plasma

For preparation of calibration standards in whole blood and plasma, blank (human) whole blood and plasma, was obtained from the

Table 1. MRM transitions, retention time, relative retention time of 24 analytes (calculated with respect to sulphadimethoxine) and optimized ESI-MS/MS parameters for 24 analytes, homatropine (internal standard 1) and sulphadimethoxine (internal standard 2).

Analyte	Q1 Mass (Da)	Q3 Mass (Da)	Retention time (min)	Relative Retention Time (min)	DP (V)	EP (V)	CE (V)	CXP (V)
Acetaminophen	152.1	**110.0**	1.8	0.2	51	8	20	5
		93.0			37	9	31	16
Alprazolam	309.1	**281.5**	11.4	1.4	100	10	35	6
		205.5			94	10	57	10
Amlodipine	409.0	**238.1**	12.0	1.5	41	4	14	13
		294.1			60	3	17	7
		377.0			73	5	24	9
Atropine	290.0	**124.1**	5.6	0.7	77	5	35	6
Chlorpromazine	319.0	58.0	12.2	1.5	66	9	59	9
		86.0			67	9	28	3
		246.1			70	7	31	5
Clonazepam	316.0	**270.6**	10.8	1.4	108	11	35	13
		205.5			35	12	71	17
Clonidine	230.0	124.0	3.1	0.4	85	10	62	6
		160.0			89	13	46	7
		187.0			93	4	40	8
Cocaine	304.1	**182.1**	6.9	0.9	71	12	28	9
		105.0			59	12	46	4
		150.1			63	12	35	7
Codeine	300.1	**215.0**	2.6	0.3	94	10	35	11
		243.2			89	10	31	5
		282.2			81	10	29	6
Diazepam	285.1	**154.5**	12.5	1.6	98	12	37	7
		193.5			100	10	45	9
		257.5			90	8	30	5
Flunitrazepam	314.1	**268.1**	10.9	1.4	86	12	36	15
		240.1			90	10	37	12
		286.1			80	12	31	14
Heroin	370.0	165.1	6.9	0.9	110	5	66	7
		268.1			85	5	39	15
		211.1			100	4	41	10
Ketamine	238.0	**207.0**	5.9	0.7	57	9	20	4
		220.0			63	9	20	12
		125.0			49	9	35	6
Methamphetamine	150.1	91.0	4.1	0.5	44	10	24	4
		119.1			39	10	15	5
Morphine	286.1	**153.1**	1.1	0.1	90	8	56	7
		157.1			82	10	55	7
Nicotine	163.0	**132.0**	1.0	0.1	48	10	20	10
		106.1			61	10	21	4
		130.1			57	10	27	7
Nordiazepam	271.2	**140.0**	12.2	1.5	109	11	42	6
		208.1			98	9	38	10
		165.0			98	10	40	8
Norketamine	224.1	**125.0**	5.9	0.8	48	5	34	5
		179.0			48	5	22	9
Olanzapine	313.1	**256.1**	6.1	0.8	65	10	30	5
		213.1			74	9	40	11
		282.1			61	6	33	15
Oxazepam	287.0	269.5	11.5	1.4	86	12	21	6
		241.5			80	10	31	5
		231.5			90	11	30	5
Pethidine	248.1	**174.2**	7.8	1.0	78	10	25	9
		131.1			81	12	42	6
Pheniramine	241.0	**196.1**	7.0	0.9	45	4	21	11
		168.0			44	7	48	8

(Continues)

Table 1. (Continued)

Analyte	Q1 Mass (Da)	Q3 Mass (Da)	Retention time (min)	Relative Retention Time (min)	DP (V)	EP (V)	CE (V)	CXP (V)
Timolol	317.0	**261.0**	6.7	0.9	73	8	25	15
		244.0			69	10	29	12
Zolpidem	308.1	**235.0**	7.9	1.0	101	5	46	11
		263.1			89	12	36	15
		221.1			101	13	52	11
Homatropine (IS 1)	276.1	**142.2**	3.7	-	110	5	82	7
Sulphadimethoxine (IS 2)	311.0	**156.0**	8.0	-	50	10	31	7

Note: Transitions in bold were the quantifier ions

blood bank of All India Institute of Medical Sciences (AIIMS), New Delhi, India. Vitreous humour used for the preparation of the calibration standards was collected from autopsies being conducted at AIIMS, New Delhi, India. Vitreous humour for the purpose was collected from the cases, which had neither a history of drug/poison intake nor any positive post-mortem findings indicating that. Blank vitreous humour, whole blood and plasma were verified as blank (drug free) by analyzing each of the matrices separately by LC-MS/MS.

To obtain spiked working standards for calibration in the vitreous humour, whole blood and plasma, a stock solution of 1 μg/mL of 24 drugs (from the working solution of a mixture of 24 drugs) was prepared for each respective matrices. Further, each spiked working standard was serially diluted with its respective matrix (vitreous humour/whole blood/plasma) to reach concentrations ranging from 7.8 to 375 ng/mL for all the analytes. Like the calibration standards, quality control samples for all the 24 drugs were also prepared at three concentration levels: low, medium and high, using vitreous humour, whole blood and plasma for their respective matrices.

Sample preparation

Homatropine was used as the Internal Standard 1 (IS 1), which was used for the quantification of the 24 analytes. Sulphadimethoxine was used as the Internal Standard 2 (IS 2), for the calculation of relative retention time (RRT) for the identification of drugs. Extraction solvent consisted of acetonitrile with 0.1 % formic acid, homatropine (IS 1) at a concentration of 500 ng/mL and sulphadimethoxine (IS 2) at a concentration of 10 ng/mL. Stored post-mortem samples of, vitreous humour, whole blood and plasma were thawed at room temperature. Calibration standards or samples of all the three matrices were prepared by direct protein precipitation. To 20 μL of each standard or sample, 200 μL of the extraction solvent was added, followed by vortexing for 1 min using a cyclomixer and then centrifugation at 7840 g for 10 min. One hundred fifty μL of clear supernatant was then collected and subjected to vacuum evaporation (Rotovac), followed by reconstitution with 200 μL of water containing 0.1% formic acid. Samples then vortexed for 1 min and centrifuged at 7840 g for 5 min with the resulting supernatant subjected to LC-MS/MS analysis. Samples that exceeded the calibration range were appropriately diluted and extracted.

Method validation

The developed analytical method was fully validated for selectivity, linearity, sensitivity, precision, accuracy, recovery and matrix effect according to the currently accepted US Food and Drug Administration (FDA) Bioanalytical Method Validation Guidelines.[16]

Selectivity

For the selectivity experiments, blank samples (6 for each of the vitreous humour, whole blood and plasma) were included and processed without spiking any of the 24 drug standards and internal standard into them. The samples were extracted using the proposed extraction method and analyzed to check the absence of interference from the matrix at the retention time of each analyte and IS. A zero sample for each of the vitreous humour, whole blood and plasma (sample processed with internal standards) was extracted (as described in Sample preparation) and analyzed to make no significant interfering peaks occurred at the expected retention times of the drugs at a concentration near the range of the lower limit of quantification (LLOQ) of the analytes.

Calibration curve and linearity

Six calibration steps from 7.8 to 250 ng/mL were selected for plotting the calibration curve (peak area/IS area ratio) for different matrices (vitreous humour, whole blood and plasma). The linearity of the calibration curves was also calculated, where a correlation coefficient (R^2) of 0.99 or better was selected. The concentration of each drug was calculated from these ratios of area using the appropriate calibration curve. The analyte concentration at which the signal-to-noise ratio was greater than 3 was chosen for the LOD. The sensitivity of the assay was defined as the lowest analyte concentration that could be measured with acceptable accuracy and precision (i.e., LLOQ). The LLOQ was defined as the lowest concentration with accuracy of ± 20 % and precision of a coefficient of variance (% CV) ≤ 20 % was accepted.

Accuracy and precision

Intra-day assays were performed using five replicates during a single day and inter-day assays were performed on five different days. Intra and inter-day assay accuracies were calculated as the closeness of mean test concentration obtained by the method to the actual concentration of analyte and expressed as % accuracy. Percentage accuracy was calculated as, % Accuracy = (Calculated concentration of the analyte using calibration curve equation × 100) / (Actual concentration). Inter-day and intra-day precision was determined at each concentration level and did not exceed 15% of the coefficient of variation (CV). Precision for the LLOQ, did not exceed 20% of the CV. Three concentrations of each drug were evaluated for determining the accuracy and precision for each of the matrices namely, vitreous humour, whole blood, and plasma.

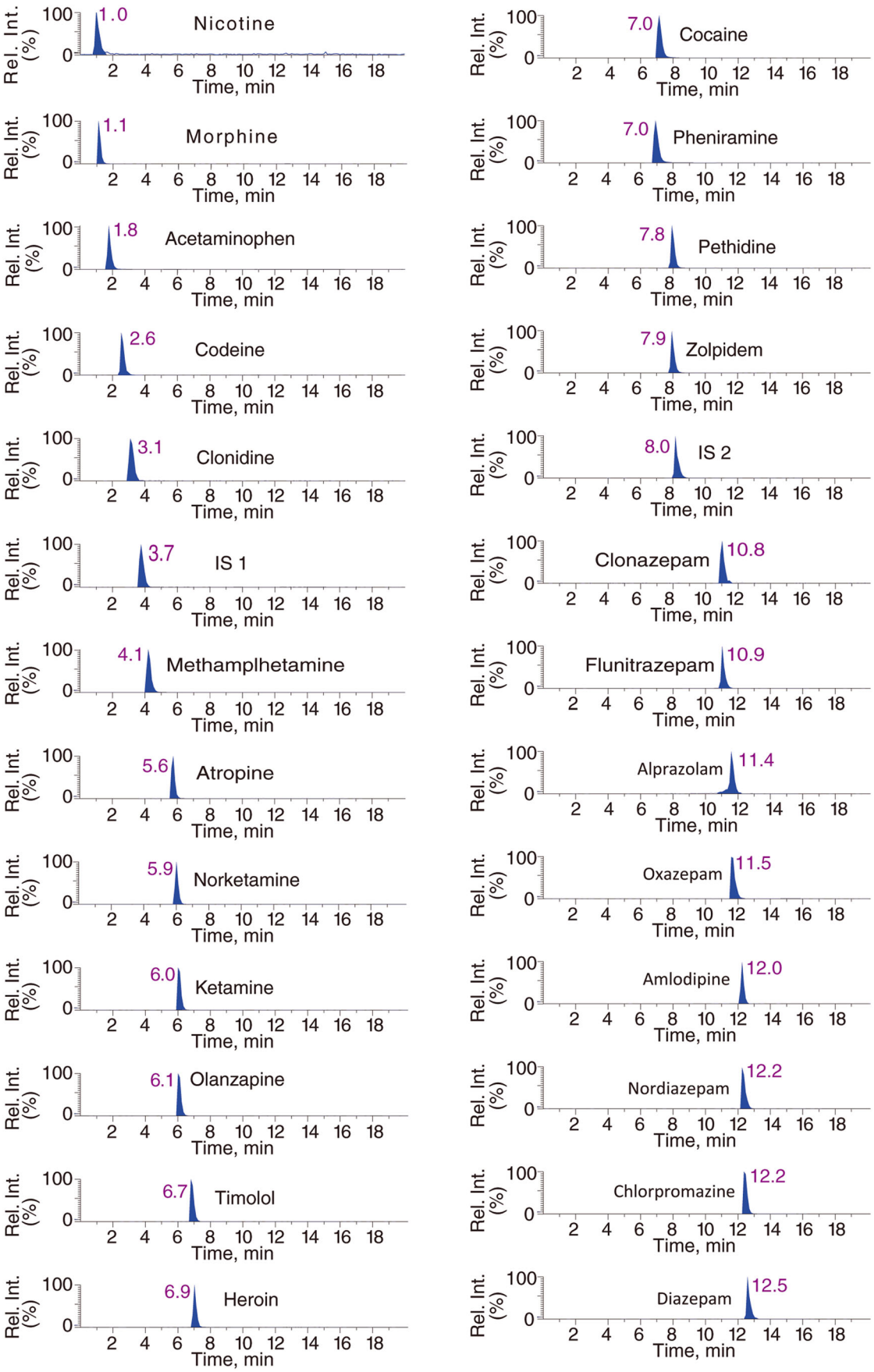

Figure 1. The extracted ion chromatograms in vitreous humor after spiking it with 31.3 ng/ml of 24 analytes. The extracted ion chromatogram of IS 1 and IS 2 is also shown

Absolute recovery, absolute matrix effect (AME), and carry-over effect

Three different concentrations were used for evaluation of recovery and matrix effect for each of the vitreous humour, whole blood and plasma samples. Samples were analyzed with six determinations at each concentration. The pure solution set was prepared by serial dilution of the working standards of the mixture stock solution (24 analytes), with methanol containing 0.1% formic acid. Pre-extraction set was prepared by the proposed preparative procedure. Post-extraction set was prepared by dissolving analytes in the extracted blank matrix (vitreous humour, whole blood and plasma). Absolute recovery was determined by comparing peak areas of 24 drugs spiked in each of the matrices (vitreous humour, whole blood and plasma) to those of pure standard of corresponding concentrations. The absolute matrix effect (AME) was evaluated by comparing the area of peaks of post-extraction blank matrix (vitreous humour, whole blood and plasma) to those of pure standard of corresponding concentrations.

The carry-over effect was studied by analyzing five blank samples after analysis of three samples (for each vitreous, whole blood and plasma) spiked at high concentration.

Ethical approval

Protocol for sample collection was approved by the standing Institutional Human Ethics Committee (IHEC) of All India Institute of Medical Sciences (AIIMS, New Delhi, India).

Screening of post-mortem samples for 24 drugs

The proposed method was applied for the screening and quantification of 24 forensically relevant drugs in the 40 authentic post-mortem samples, wherein post-mortem samples were collected at the time of autopsy during 2011/2012. Samples collected in all the cases included cardiac blood, of which 500 μL in each case was stored as whole blood. From another EDTA added blood sample (approx 500 μL), plasma was separated by centrifugation at 1960 g for 10 min. Vitreous humour from both the eyes (100 μL from each eye in all the cases) was aspirated with a scleral puncture, on the lateral canthus of each eye by using a 20 G needle. All the collected samples were stored in a deep freezer at -80°C till their analysis by LC-MS/MS.

Results and discussion

We have followed a simple sample extraction method for extraction of drugs. As mentioned in the Experimental section, for extraction of drugs from the sample, extraction solvent was added which consisted of acetonitrile with 0.1% formic acid. The addition of acetonitrile (ACN) with formic acid had two major roles; first the addition of acetonitrile induces a protein crash and secondly, it diminishes the losses of volatile basic drugs during the evaporation steps. Once evaporated to dryness, water with formic acid was added. Formic acid was added again presumably because there would have been some loss of formic acid during the evaporation step.

Results obtained on analysis of the samples using newly developed method is placed in subsequent subsections and discussed accordingly.

Method validation

Separation of the 24 analytes, IS 1 and IS 2 was achieved in 20 min. The extracted ion chromatograms of the 24 analytes, IS 1 and IS 2 obtained from vitreous humour spiked at a concentration of 31.3 ng/mL is presented in Figure 1. The relative retention times was calculated for all the 24 analytes with sulphadimethoxine as an internal standard and is shown in Table 1. While a table summarizing the validation parameters, such as correlation coefficients, LOD, accuracy, precision, recovery and AME are shown in Table 2.

Selectivity

Matrix interference was not detected in either of the retention times of the analytes or the internal standard in six different lots of the blank vitreous humour, whole blood and plasma samples or in the zero samples (vitreous humour, whole blood, and plasma). Thus, it may be said that the assay was free of interference from endogenous peaks arising from vitreous humour, whole blood and plasma. Representative chromatogram of the blank vitreous humour, zero sample, and vitreous sample spiked with low concentration (31.3 ng/mL) of all the analytes is presented in Figure 2.

Calibration curve and linearity

All analytes in the three matrices are checked for a linear fit or quadratic fit. The regression coefficients of the calibration curves were higher than 0.99 for all the drugs in the vitreous humour, whole blood and plasma on every occasion. Correlation coefficients of all the analytes are shown in Table 3. A linear fit was used for acetaminophen, atropine, cocaine, flunitrazepam, heroin, methamphetamine, morphine, norketamine, oxazepam, pethidine, timolol and zolpidem. While a quadratic fit was used for alprazolam, amlodipine, chlorpromazine, clonazepam, clonidine, codeine, diazepam, ketamine, nicotine, nordiazepam, olanzapine and pheniramine.

Table 2. Summary of validation data of the 24 analytes in vitreous humour, whole blood, and plasma.

		Vitreous Humour	Whole Blood	Plasma
Correlation Coefficient, R^2		>0.99	>0.99	>0.99
LOD (ng/ml)		0.98 - 3.9	0.98 - 7.8	0.98 - 7.9
Accuracy (%)	**Intra-day**	85.8 - 119	89.2 - 119	80.8 - 119
	Inter-day	87.0 - 120	82.1 - 118	80.5 - 119
Precision (%CV)	**Intra-day**	1.4 - 18.9	0.10 - 19.2	1.3 - 15.5
	Inter-day	1.5 - 20.0	2.5 - 19.6	2.9 - 17.8
Recovery (%)		71.2 - 119	71.7 - 115*	79.2 - 119*
AME (%)		78.5 - 120	77.4 - 119	90.3 - 120

Note* Indicates value for all 24 drugs (except for morphine and heroin)

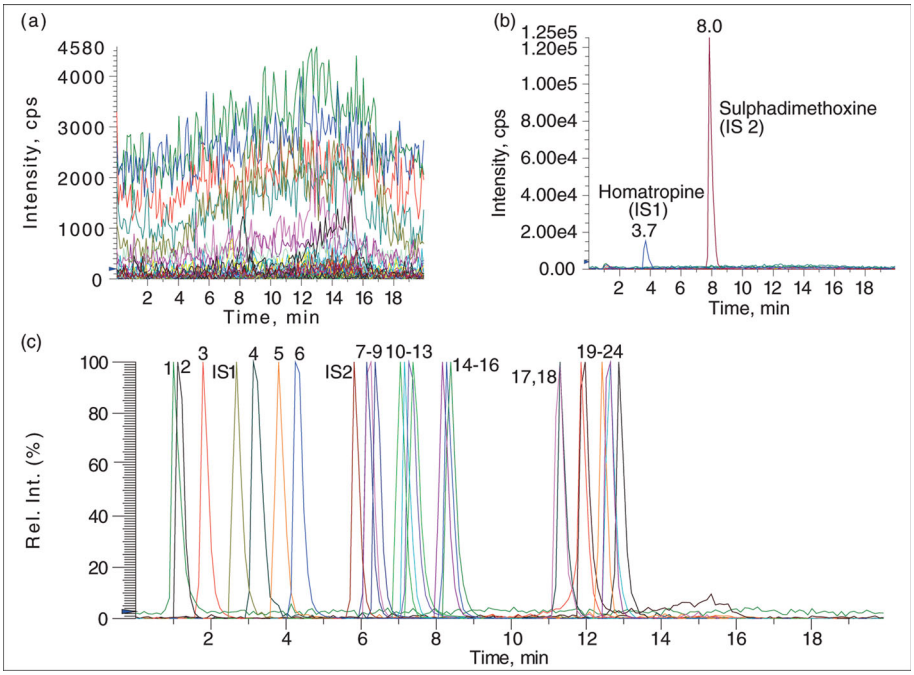

Figure 2. Representative MRM chromatograms resulting from the analysis of (a) blank vitreous humor and (b) zero sample in vitreous humor (c) Extracted ion chromatogram (overlay) of 24 analytes spiked (31.3 ng/ml) in vitreous humor along with IS 1 and IS 2. 1 nicotine, 2 morphine, 3 acetaminophen, 4 codeine, 5 clonidine, 6 methamphetamine, 7 atropine, 8 norketamine, 9 ketamine, 10 olanzapine, 11 timolol, 12 heroin, 13 cocaine, 14 pheniramine, 15 pethidine, 16 zolpidem, 17 clonazepam, 18 flunitrazepam, 19 alprazolam, 20 oxazepam, 21 amlodipine, 22 nordiazepam, 23 chlorpromazine, 24 diazepam

Table 3. Calibration range, correlation coefficient, limit of detection (LOD) and lower limit of quantification (LLOQ) of the 24 analytes in vitreous humour, whole blood and plasma.

Analyte	Calibration Range (ng/mL)	LLOQ (ng/mL)	Vitreous Humour		Whole Blood		Plasma	
			Correlation Coefficient, R^2	LOD (ng/mL)	Correlation Coefficient, R^2	LOD (ng/mL)	Correlation Coefficient, R^2	LOD (ng/mL)
Acetaminophen	11.7-375	11.7	1.00	1.4	0.99	1.4	1.00	1.4
Alprazolam	7.8-250	7.8	1.00	0.9	0.99	0.9	1.00	0.9
Amlodipine	7.8-250	7.8	1.00	1.9	1.00	1.9	0.99	0.9
Atropine	7.8-250	7.8	1.00	0.9	0.99	0.9	1.00	0.9
Chlorpromazine	7.8-250	7.8	0.99	0.9	0.99	0.9	0.99	0.9
Clonazepam	7.8-250	7.8	0.99	3.9	1.00	1.9	1.00	1.9
Clonidine	7.8-250	7.8	1.00	3.9	0.99	1.9	0.99	1.9
Cocaine	7.8-250	7.8	1.00	0.9	0.99	0.9	0.99	0.9
Codeine	7.8-250	7.8	1.00	1.9	0.99	1.9	1.00	1.9
Diazepam	7.8-250	7.8	1.00	0.9	0.99	0.9	1.00	0.9
Flunitrazepam	7.8-250	7.8	0.99	0.9	0.99	0.9	0.99	0.9
Heroin	7.8-250	7.8	1.00	0.9	0.99	7.8	0.99	1.9
Ketamine	7.8-250	7.8	1.00	0.9	0.99	0.9	1.00	0.9
Methamphetamine	7.8-250	7.8	1.00	0.9	0.99	0.9	0.99	0.9
Morphine	7.8-250	7.8	0.99	1.9	0.99	3.9	1.00	3.9
Nicotine	7.9-255	7.8	1.00	0.9	0.99	0.9	0.99	7.9
Nordiazepam	7.8-250	7.8	0.99	0.9	0.99	0.9	1.00	0.9
Norketamine	7.8-250	7.8	0.99	0.9	1.00	0.9	1.00	0.9
Olanzapine	7.8-250	7.8	1.00	0.9	0.99	1.9	1.00	0.9
Oxazepam	7.8-250	7.8	1.00	0.9	0.99	1.9	1.00	0.9
Pethidine	9.7-312	9.7	1.00	1.2	0.99	1.2	0.99	1.2
Pheniramine	8.8-284	8.8	1.00	1.1	0.99	1.1	1.00	1.1
Timolol	7.8-250	7.8	1.00	0.9	1.00	0.9	1.00	0.9
Zolpidem	7.8-250	7.8	1.00	0.9	1.00	0.9	1.00	0.9

Table 4. Intra-day and inter-day accuracy and precision data of the 24 analytes in vitreous humour, whole blood, and plasma.

Analyte	Concentration (ng/mL)	Vitreous Humour				Whole Blood				Plasma			
		Accuracy (%) (n = 5)		Precision (%CV) (n = 5)		Accuracy (%) (n = 5)		Precision (%CV) (n = 5)		Accuracy (%) (n = 5)		Precision (%CV) (n = 5)	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Acetaminophen	11.7	106	106	11.4	17.0	96.5	107	17.7	16.3	88.8	83.7	8.2	4.7
	46.9	93.8	96.9	9.7	10.1	99.2	99.7	14.4	10.6	104	105	7.8	4.2
	375	99.9	99.8	5.2	5.3	101	100	14.3	11.3	100	100	5.1	5.6
Alprazolam	7.8	97.3	101	16.9	19.0	106	83.1	12.6	16.9	91.4	89.9	10.4	10.3
	31.3	101	98.8	6.4	10.7	113	100	8.2	12.2	107	102	5.6	7.1
	250	100	98.6	5.3	6.5	99.4	100	2.7	8.5	100	100	4.7	5.1
Amlodipine	7.8	117	87.0	9.3	8.6	117	87.1	0.1	13.3	97.4	102	11.0	9.8
	31.3	94.7	89.6	6.0	8.9	112	106	10.9	14.1	89.5	96.0	11.2	10.8
	250	99.8	100	2.5	1.5	99.5	99.3	12.7	7.8	100	99.1	13.0	14.0
Atropine	7.8	103	96.1	2.9	14.7	105	83.8	15.0	14.8	95.4	88.6	5.4	10.8
	31.3	99.3	94.9	9.8	7.3	101	101	10.9	14.4	98.1	99.2	5.1	8.0
	250	99.5	99.4	5.2	8.0	100	100	7.2	9.8	99.9	100	5.1	4.8
Chlorpromazine	7.8	102	97.5	6.7	13.7	105	100	18.3	14.2	107	118	15.5	13.1
	31.3	93.7	97.9	1.8	9.2	106	98.1	6.0	14.0	101	107	10.8	12.1
	250	99.9	100	5.5	7.0	101	99.6	9.6	11.9	102	99.9	2.6	13.7
Clonazepam	7.8	110	104	15.6	19.8	110	85.2	15.4	2.5	85.3	110	10.4	12.9
	31.3	97.5	99.4	6.2	10.9	113	106	0.7	4.4	102	112	6.4	9.2
	250	100	100	10.5	9.2	98.8	100	10.2	5.2	100	99.2	5.1	6.5
Clonidine	7.8	99.1	109	11.3	12.8	115	98.3	9.8	16.1	114	89.4	8.5	17.0
	31.3	103	103	2.7	6.5	100	101	13.8	10.4	98.6	101	12.8	5.4
	250	99.9	99.9	3.6	8.0	100	100	6.9	12.7	99.9	99.6	10.3	5.7
Cocaine	7.8	108	105	18.9	7.9	107	115	13.9	16.4	102	90.8	6.2	17.8
	31.3	106	101	10.0	6.0	110	111	6.6	8.1	95.0	94.4	11.6	14.8
	250	100	100	3.8	8.3	99.6	98.8	8.1	11.7	104	101	11.3	10.0
Codeine	7.8	111	116	15.2	17.9	117	89.4	17.8	9.6	117	101	12.0	16.0
	31.3	99.6	94.9	7.9	5.8	93.4	92.7	10.3	10.8	94.8	94.6	4.9	6.1
	250	99.9	100	5.6	6.3	101	99.3	12.2	9.7	101	100	4.5	5.5
Diazepam	7.8	111	107	14.1	14.0	105	85.1	9.1	13.2	88.0	110	13.8	12.4
	31.3	101	107	10.5	7.6	102	102	5.8	9.6	99.4	103	14.7	5.1
	250	99.9	101	4.6	6.7	101	100	7.9	9.3	100	101	1.6	6.6
Flunitrazepam	7.8	108	98.1	12.3	20.0	101	88.1	19.2	19.6	111	107	8.1	8.8
	31.3	102	97.7	10.9	10.6	114	98.8	1.1	15.0	105	101	10.4	6.8
	250	99.8	100	8.1	5.5	101	100	12.9	10.5	101	101	9.4	4.7
Heroin	7.8	109	112	10.3	14.8	107	107	3.8	12.8	83.0	85.9	10.3	8.3
	31.3	96.6	101	10.1	9.0	96.1	112	12.5	15.0	105	97.6	8.6	14.5
	250	99.6	99.9	3.3	8.8	101	98.7	8.7	12.6	100	100	7.1	2.9
Ketamine	7.8	101	114	5.0	11.6	108	101	7.6	11.7	108	119	10.4	6.5
	31.3	105	107	14.4	8.4	98.9	98.6	10.5	15.0	104	98.0	13.6	14.3
	250	100	100	6.7	6.7	100	99.2	7.0	14.4	99.0	101	1.2	6.1
Methamphetamine	7.8	113	90.6	7.8	10.3	118	109	4.8	11.8	118	105	3.6	11.0
	31.3	108	101	8.1	5.0	97.6	112	14.2	7.5	113	101	10.6	14.9
	250	101	99.2	6.0	4.2	101	97.7	14.9	11.8	99.4	101	12.5	7.4
Morphine	7.8	90.6	116	6.1	7.6	89.2	88.5	4.4	8.6	92.9	96.7	11.2	14.8
	31.3	109	96.5	1.4	10.3	99.7	100	11.8	14.3	102	102	5.8	5.7
	250	99.6	100	8.1	8.2	100	100	8.3	9.4	100	100	4.8	5.8
Nicotine	7.9	106	97.5	11.3	4.0	109	107	12.0	15.1	104	83.8	10.9	12.8
	31.9	104	106	5.3	9.5	98.9	102	11.4	5.8	105	95.5	11.6	10.4
	255	100	99.9	5.8	7.9	100	102	10.5	13.2	101	101	12.9	9.6
Nordiazepam	7.8	119	111	8.4	12.8	103	99.3	17.2	16.9	80.8	97.4	5.4	10.5
	31.3	95.5	95.7	8.1	2.0	106	105	8.6	8.4	106	99.4	7.5	5.5
	250	99.9	99.9	10.2	9.4	102	100	15.0	9.4	100	100	6.2	8.0

(Continues)

Table 4. (Continued)

Analyte	Concentration (ng/mL)	Vitreous Humour				Whole Blood				Plasma			
		Accuracy (%) (n = 5)		Precision (%CV) (n = 5)		Accuracy (%) (n = 5)		Precision (%CV) (n = 5)		Accuracy (%) (n = 5)		Precision (%CV) (n = 5)	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Norketamine	7.8	98.6	119	11.9	7.6	111	117	6.7	11.8	89.9	105	11.5	13.8
	31.3	111	105	10.3	7.0	103	112	12.7	8.4	105	107	7.7	13.6
	250	102	100	7.5	8.4	100	101	7.7	9.0	100	102	5.8	4.5
Olanzapine	7.8	119	120	10.2	12.9	119	112	17.9	14.7	108	90.1	4.9	4.3
	31.3	89.9	90.2	5.3	5.4	102	114	13.4	8.7	101	102	12.1	6.6
	250	100	99.9	6.8	9.9	99.1	97.3	7.2	14.7	101	100	6.2	8.1
Oxazepam	7.8	94.9	115	8.8	11.3	115	85.6	18.1	19.6	92.9	83.8	13.1	9.2
	31.3	93.6	98.8	5.7	8.8	109	103	11.6	11.2	103	105	8.6	6.5
	250	99.4	100	4.9	7.6	99.3	100	10.1	6.8	100	101	3.4	4.3
Pethidine	9.7	97.7	112	14.0	6.6	109	116	16.2	15.7	119	106	2.9	12.7
	39.1	110	101	10.0	6.9	112	109	8.1	14.9	114	104	12.7	13.6
	313	100	99.9	2.5	8.2	100	99.2	11.5	13.7	99.5	101	11.7	5.6
Pheniramine	8.8	106	110	15.8	9.0	100	82.1	15.6	11.3	91.4	80.5	9.0	10.4
	35.5	103	100	8.4	4.3	108	104	12.8	12.6	102	102	5.7	6.4
	284.2	99.9	99.9	1.6	7.7	101	100	5.6	11.7	100	100	8.1	5.6
Timolol	7.8	107	94.3	10.0	13.8	117	118	9.9	2.8	95.2	98.9	8.8	6.6
	31.3	102	98.5	9.2	6.3	112	110	8.3	9.3	101	101	5.9	3.3
	250	100	99.4	6.2	8.7	99.5	99.7	1.1	9.6	99.9	101	4.9	3.4
Zolpidem	7.8	85.8	108	14.1	11.3	109	91.1	17.1	16.3	91.6	88.6	7.5	7.0
	31.3	99.6	98.1	6.4	4.6	114	98.8	8.9	13.3	104	103	6.5	4.9
	250	99.1	99.9	4.6	6.1	100	100	10.8	5.6	100	100	3.9	3.8

The LOD and LLOQ for each of the analytes in the vitreous humour, whole blood and plasma is also shown in Table 3. The LODs for vitreous humour ranged from 0.98 ng/mL (for 15 analytes) to 3.9 ng/mL (clonazepam and clonidine) while for whole blood, it ranged from 0.98 ng/mL (for 12 analytes) to 7.8 ng/mL (heroin) and for plasma from 0.98 ng/mL (for 15 analytes) to 7.9 ng/mL (nicotine). On the other hand, the LLOQs for vitreous humour, whole blood and plasma are 7.8 ng/mL for every analyte except for acetaminophen, nicotine, pethidine and pheniramine for which they are 11.7, 7.9, 9.7, and 8.8 ng/mL, respectively.

Accuracy and precision

Table 4 describes the intra-day and inter-day accuracy and precision of the 24 analytes in the vitreous humour, whole blood and plasma. All the results were within the limits of FDA criteria. The intra-day accuracy and precision of the developed method for validating 24 drugs in vitreous humour ranged from 85.8% (zolpidem) to 119% (olanzapine) and 1.4% (morphine) to 18.9% (cocaine) respectively. The inter-day accuracy and precision ranged from 87.0% (amlodipine) to 120% (olanzapine) and 1.5% (amlodipine) to 20.0% (flunitrazepam), respectively.

The intra-day accuracy and precision of the developed method for validating 24 drugs in whole blood ranged from 89.2% (morphine) to 119% (olanzapine) and 0.10% (amlodipine) to 19.2% (flunitrazepam) respectively. The inter-day accuracy and precision ranged from 82.1% (pheniramine) to 118% (timolol) and 2.5% (clonazepam) to 19.6% (flunitrazepam), respectively.

The intra-day accuracy and precision of the developed method for validating 24 drugs in plasma ranged from 80.8% (nordiazepam) to 119% (pethidine) and 1.3% (ketamine) to 15.5% (chlorpromazine) respectively. The inter-day accuracy and precision ranged from 80.5% (pheniramine) to 119% (ketamine) and 2.9% (heroin) to 17.8% (cocaine), respectively.

Absolute recovery, AME, and carry-over effect

Table 5 describes the recovery and AME of the 24 analytes in the vitreous humour, whole blood, and plasma. The absolute recovery of all the 24 analytes in vitreous humour ranged from 71.2 to 119% with a mean of 84.5% and matrix effect was found to be between 78.5 and 120% with a mean of 105%. The absolute recovery of all the 24 analytes in whole blood ranged from 71.7 to 115% (except for morphine and heroin) with a mean of 86.2% and matrix effect was found to be between 77.4 and 119% with a mean of 96.5%. The absolute recovery of all the 24 analytes in plasma ranged from 79.2 to 119% (except for morphine and heroin) with a mean of 103% and matrix effect was found to be between 90.3 to 120% with a mean of 109%.

Low recovery of heroin may be understood from the fact that half life of heroin is 2–5 min in plasma.[17] Further investigation of its recovery might be carried out through analysis of 6-monoacetylmorphine (6-MAM). A weakness of the investigation is that 6-MAM could not be analysed for, as a 6-MAM reference standard could not be obtained in sufficient time before the investigation commenced.

The carry-over effect in vitreous humour was less than 1% for olanzapine, diazepam and heroin and for other drugs it was zero. The carry-over effect in whole blood and plasma was less than 1% for all the studied analytes.

Table 5. Absolute recovery and absolute matrix effect (AME) of the 24 analytes in vitreous humor, whole blood and plasma.

Analyte	Concentration (ng/mL)	Vitreous Humour (n = 6)		Whole Blood (n = 6)		Plasma (n = 6)	
		Recovery % (Mean ± SD)	AME % (Mean ± SD)	Recovery % (Mean ± SD)	AME % (Mean ± SD)	Recovery % (Mean ± SD)	AME % (Mean ± SD)
Acetaminophen	46.9	81.4 ± 0.11	108 ± 0.08	91.2 ± 0.15	99.9 ± 0.07	99.7 ± 0.06	104 ± 0.03
	188	78.5 ± 0.03	110 ± 0.07	83.9 ± 0.15	99.5 ± 0.02	91.9 ± 0.03	106 ± 0.04
	375	84.4 ± 0.02	112 ± 0.01	84.2 ± 0.13	98.7 ± 0.03	99.7 ± 0.04	112 ± 0.02
Alprazolam	31.3	79.3 ± 0.05	94.4 ± 0.13	85.5 ± 0.18	108 ± 0.11	111 ± 0.09	114 ± 0.11
	125	76.2 ± 0.05	92.4 ± 0.22	79.6 ± 0.17	104 ± 0.02	114 ± 0.07	113 ± 0.03
	250	78.9 ± 0.26	119 ± 0.34	86.9 ± 0.20	98.3 ± 0.03	111 ± 0.14	118 ± 0.13
Amlodipine	31.3	74.9 ± 0.11	83.9 ± 0.10	101 ± 0.20	108 ± 0.22	116 ± 0.23	111 ± 0.10
	125	79.4 ± 0.19	93.2 ± 0.13	91.1 ± 0.15	89.5 ± 0.10	103 ± 0.10	117 ± 0.12
	250	71.6 ± 0.45	115 ± 0.27	92.6 ± 0.20	97.8 ± 0.09	107 ± 0.07	106 ± 0.07
Atropine	31.3	91.6 ± 0.01	111 ± 0.15	110 ± 0.21	103 ± 0.03	96.2 ± 0.02	100 ± 0.06
	125	85.1 ± 0.06	115 ± 0.13	71.9 ± 0.20	93.5 ± 0.06	94.7 ± 0.08	105 ± 0.07
	250	93.9 ± 0.02	106 ± 0.03	82.5 ± 0.19	95.7 ± 0.02	102 ± 0.06	110 ± 0.04
Chlorpromazine	31.3	77.5 ± 0.03	103 ± 0.21	115 ± 0.69	95.7 ± 0.29	110 ± 0.20	119 ± 0.07
	125	79.1 ± 0.21	92.3 ± 0.23	91.9 ± 0.18	80.3 ± 0.15	92.1 ± 0.06	107 ± 0.04
	250	81.1 ± 0.28	110 ± 0.36	96.6 ± 0.06	78.8 ± 0.06	102 ± 0.02	113 ± 0.05
Clonazepam	31.3	77.7 ± 0.11	102 ± 0.25	101 ± 0.26	106 ± 0.21	102 ± 0.20	114 ± 0.15
	125	83.9 ± 0.10	116 ± 0.20	86.4 ± 0.14	106 ± 0.04	99.8 ± 0.08	116 ± 0.08
	250	72.3 ± 0.05	108 ± 0.03	97.1 ± 0.28	115 ± 0.08	110 ± 0.14	118 ± 0.12
Clonidine	31.3	105 ± 0.09	112 ± 0.21	83.1 ± 0.15	106 ± 0.06	112 ± 0.17	119 ± 0.12
	125	98.7 ± 0.02	113 ± 0.00	79.4 ± 0.17	102 ± 0.03	107 ± 0.09	115 ± 0.11
	250	104 ± 0.06	114 ± 0.04	80.9 ± 0.14	99.1 ± 0.01	108 ± 0.09	117 ± 0.03
Cocaine	31.3	93.9 ± 0.04	91.0 ± 0.16	90.3 ± 0.15	83.7 ± 0.07	79.2 ± 0.04	108 ± 0.08
	125	82.8 ± 0.03	108 ± 0.15	80.2 ± 0.20	88.9 ± 0.10	79.6 ± 0.04	104 ± 0.01
	250	90.1 ± 0.07	114 ± 0.15	81.1 ± 0.17	82.3 ± 0.05	81.2 ± 0.07	104 ± 0.00
Codeine	31.3	85.5 ± 0.11	113 ± 0.12	74.2 ± 0.08	101 ± 0.18	91.7 ± 0.06	106 ± 0.08
	125	81.2 ± 0.03	116 ± 0.08	79.0 ± 0.08	88.8 ± 0.03	90.5 ± 0.05	104 ± 0.03
	250	87.6 ± 0.05	106 ± 0.06	85.4 ± 0.04	95.0 ± 0.06	106 ± 0.09	117 ± 0.05
Diazepam	31.3	71.2 ± 0.03	78.5 ± 0.13	110 ± 0.13	119 ± 0.04	115 ± 0.07	112 ± 0.10
	125	74.2 ± 0.06	87.3 ± 0.12	101 ± 0.37	97.7 ± 0.04	107 ± 0.19	104 ± 0.09
	250	75.6 ± 0.10	86.9 ± 0.09	100 ± 0.28	113 ± 0.11	109 ± 0.15	104 ± 0.02
Flunitrazepam	31.3	74.1 ± 0.07	104 ± 0.16	97.9 ± 0.22	106 ± 0.12	114 ± 0.13	108 ± 0.14
	125	78.2 ± 0.06	120 ± 0.02	93.7 ± 0.29	101 ± 0.08	103 ± 0.12	106 ± 0.06
	250	79.2 ± 0.03	119 ± 0.04	89.4 ± 0.18	98.1 ± 0.01	109 ± 0.14	103 ± 0.07
Heroin	31.3	77.6 ± 0.08	110 ± 0.10	51.4 ± 0.03	102 ± 0.03	59.7 ± 0.02	115 ± 0.03
	125	81.5 ± 0.07	115 ± 0.11	55.9 ± 0.03	104 ± 0.08	61.6 ± 0.07	115 ± 0.11
	250	86.1 ± 0.05	119 ± 0.08	58.6 ± 0.02	100 ± 0.03	64.0 ± 0.05	111 ± 0.02
Ketamine	31.3	82.7 ± 0.06	94.2 ± 0.06	96.8 ± 0.31	82.3 ± 0.01	116 ± 0.13	108 ± 0.09
	125	89.8 ± 0.21	88.4 ± 0.20	98.9 ± 0.31	85.9 ± 0.07	117 ± 0.03	110 ± 0.04
	250	93.2 ± 0.36	91.6 ± 0.09	94.8 ± 0.25	95.9 ± 0.03	117 ± 0.11	107 ± 0.07
Methamphetamine	31.3	87.7 ± 0.03	92.0 ± 0.10	79.8 ± 0.29	79.8 ± 0.03	119 ± 0.08	90.2 ± 0.07
	125	79.4 ± 0.12	98.7 ± 0.32	74.9 ± 0.18	86.0 ± 0.10	114 ± 0.01	95.4 ± 0.05
	250	80.7 ± 0.04	93.3 ± 0.26	74.2 ± 0.17	97.3 ± 0.05	114 ± 0.09	96.3 ± 0.04
Morphine	31.3	79.3 ± 0.11	116 ± 0.13	64.5 ± 0.08	95.3 ± 0.06	67.5 ± 0.06	100 ± 0.07
	125	75.7 ± 0.05	118 ± 0.06	62.2 ± 0.09	92.8 ± 0.02	63.5 ± 0.02	102 ± 0.03
	250	76.4 ± 0.02	103 ± 0.09	65.1 ± 0.09	94.5 ± 0.03	71.5 ± 0.06	109 ± 0.03
Nicotine	31.9	118 ± 0.34	96.8 ± 0.47	107 ± 0.09	96.9 ± 0.27	115 ± 0.17	111 ± 1.11
	128	107 ± 0.32	94.5 ± 0.44	103 ± 0.41	94.9 ± 0.15	113 ± 0.21	108 ± 1.68
	255	119 ± 0.39	97.6 ± 0.68	109 ± 0.59	95.9 ± 0.26	113 ± 0.29	109 ± 1.69
Nordiazepam	31.3	76.3 ± 0.08	107 ± 0.12	87.5 ± 0.12	102 ± 0.08	102 ± 0.02	119 ± 0.10
	125	79.6 ± 0.04	103 ± 0.03	75.3 ± 0.16	116 ± 0.08	97.4 ± 0.14	117 ± 0.09
	250	73.5 ± 0.02	96.6 ± 0.03	79.0 ± 0.22	116 ± 0.11	99.9 ± 0.03	115 ± 0.12
Norketamine	31.3	77.3 ± 0.08	97.7 ± 0.06	91.2 ± 0.30	82.3 ± 0.01	119 ± 0.03	114 ± 0.05
	125	81.2 ± 0.20	106 ± 0.21	109 ± 0.34	87.3 ± 0.08	111 ± 0.01	117 ± 0.04
	250	76.9 ± 0.18	98.9 ± 0.21	90.2 ± 0.18	85.3 ± 0.06	104 ± 0.11	111 ± 0.00

(Continues)

Table 5. (Continued)

Analyte	Concentration (ng/mL)	Vitreous Humour (n = 6)		Whole Blood (n = 6)		Plasma (n = 6)	
		Recovery % (Mean ± SD)	AME % (Mean ± SD)	Recovery % (Mean ± SD)	AME % (Mean ± SD)	Recovery % (Mean ± SD)	AME % (Mean ± SD)
Olanzapine	31.3	80.4 ± 0.05	102 ± 0.06	76.3 ± 0.18	101 ± 0.06	112 ± 0.12	103 ± 0.07
	125	94.4 ± 0.13	119 ± 0.18	84.0 ± 0.08	87.0 ± 0.09	118 ± 0.20	96.2 ± 0.14
	250	93.2 ± 0.01	117 ± 0.09	75.7 ± 0.10	97.4 ± 0.02	115 ± 0.13	107 ± 0.05
Oxazepam	31.3	79.3 ± 0.11	117 ± 0.13	90.2 ± 0.12	98.1 ± 0.06	108 ± 0.11	101 ± 0.09
	125	82.9 ± 0.07	111 ± 0.15	83.2 ± 0.17	107 ± 0.12	102 ± 0.16	114 ± 0.10
	250	78.1 ± 0.04	112 ± 0.04	92.8 ± 0.26	118 ± 0.03	115 ± 0.08	104 ± 0.10
Pethidine	39.1	92.1 ± 0.07	91.1 ± 0.16	78.7 ± 0.17	82.2 ± 0.06	118 ± 0.09	104 ± 0.05
	156	78.3 ± 0.07	82.8 ± 0.22	82.1 ± 0.20	86.5 ± 0.14	109 ± 0.01	110 ± 0.03
	313	87.8 ± 0.07	86.4 ± 0.20	82.0 ± 0.18	79.6 ± 0.07	114 ± 0.10	110 ± 0.04
Pheniramine	35.5	93.0 ± 0.02	97.2 ± 0.15	99.0 ± 0.07	77.4 ± 0.05	105 ± 0.03	101 ± 0.05
	142	84.7 ± 0.04	111 ± 0.15	77.7 ± 0.18	82.6 ± 0.05	100 ± 0.04	101 ± 0.02
	284	86.5 ± 0.01	118 ± 0.19	82.6 ± 0.14	90.3 ± 0.04	104 ± 0.07	102 ± 0.01
Timolol	31.3	95.6 ± 0.11	116 ± 0.16	98.8 ± 0.18	97.9 ± 0.06	108 ± 0.02	111 ± 0.05
	125	83.7 ± 0.04	115 ± 0.03	86.9 ± 0.15	94.6 ± 0.02	105 ± 0.03	111 ± 0.03
	250	95.0 ± 0.05	106 ± 0.07	78.3 ± 0.19	101 ± 0.02	115 ± 0.10	114 ± 0.06
Zolpidem	31.3	87.6 ± 0.02	104 ± 0.13	82.9 ± 0.15	96.9 ± 0.02	113 ± 0.04	117 ± 0.02
	125	82.8 ± 0.01	116 ± 0.03	71.7 ± 0.13	98.5 ± 0.03	107 ± 0.05	117 ± 0.07
	250	84.9 ± 0.03	119 ± 0.02	79.6 ± 0.20	99.6 ± 0.07	113 ± 0.08	117 ± 0.06

Table 6. Positive findings of several drugs in the vitreous humor, whole blood and plasma in ten autopsy cases after screening them by LC-MS/MS.

Case No.	Age/Gender	Analytes detected	Vitreous Conc. (ng/ml)	Whole Blood Conc. (ng/ml)	Plasma Conc. (ng/ml)
1	30/M	Acetaminophen	92.1	151	135
		Amlodipine	8.8	33.6	27.1
		Atropine	8.4	109	120
		Nicotine	8.1	ND	ND
2	16/M	Atropine	95.7	207	295
		Diazepam	16.8	589	559
		Nordiazepam	8.1	116	334
3	45/F	Atropine	28.9	16.8	17.1
4	40/M	Acetaminophen	69.1	121	70.0
		Atropine	13.5	53.1	45.4
		Olanzapine	66.0	335	276
		Pheniramine	220	207	394
5	50/M	Nicotine	57.2	ND	ND
6	20/F	Alprazolam	8.0	33.9	32.8
7	30/F	Acetaminophen	2265	3880	2720
		Atropine	203	585	923
		Pheniramine	313	65.1	203
8	19/M	Atropine	14.2	242	288
		Diazepam	11.6	216	419
		Nicotine	9.0	ND	ND
		Nordiazepam	ND	19.4	31.6
		Pheniramine	109	45.7	148
9	42/M	Nicotine	161	62.7	126
		Olanzapine	42.3	ND	ND
10	29/M	Alprazolam	18.0	36.1	63.0
		Nicotine	208	41.6	126

Note: ND- Not Detected

Application to post-mortem samples

The method is applied to the screening of the 24 drugs in 40 human cadaver samples. In most of the samples analyzed, a number of drugs are found. The quantitative results for all the three matrices, namely, whole blood, vitreous humour, and plasma are shown in Table 6. Vitreous humour from both the eyes of each cadaver was separately analyzed and mean of right and left eye was taken.

As shown in Table 6, age of the subjects varies from 16 to 50 including both male and female. In most of the cases, multiple drugs were detected on analysis using the newly developed method. The drugs were either a major metabolite of a detected drug or present in a combination with the therapeutic drug. Although screening of 70 drugs in vitreous humour and urine by LC-TOFMS has been reported by A Palender *et al.*[10] but polar analytes had a poor detectability, most likely because of the blood-retinal barrier. Thus paracetamol, temazepam and oxazepam could not be detected in vitreous humour. However, our method was well suited for the detection and quantification of polar drugs too. Moreover, the detected concentration of drugs in most of the cases was promising. This finding seems to be in favour of utilizing vitreous humour in detection and quantification of drugs along with whole blood or plasma.

Conclusion

The paper presents a method for simultaneous screening and quantification of 24 drugs of forensic relevance using LC-MS/MS. The method has been validated for accuracy (intra-day and inter-day), precision (intra-day and inter-day), selectivity and sensitivity. Absolute recovery and absolute matrix effect in the three matrices for the 24 analytes, were suitable for the screening procedure. In addition, this method has been successfully applied for the screening of drugs in post-mortem samples of vitreous humour, whole blood and plasma collected at autopsy. Thus, the developed method may directly be used in the forensic scenario. The incorporation of some more drugs and validation for the same is obvious future work of this study.

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