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Determination of gamma-hydroxybutyrate (GHB), beta-hydroxybutyrate (BHB), pregabalin, 1,4-butane-diol (1,4BD) and gamma-butyrolactone (GBL) in whole blood and urine samples by UPLC–MSMS

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ABSTRACT

The demand of high throughput methods for the determination of gamma-hydroxybutyrate (GHB) and its precursors gamma-butyrolactone (GBL) and 1,4-butane-diol (1,4BD) as well as for pregabalin is increasing. Here we present two analytical methods using ultra-high pressure liquid chromatography (UPLC) and tandem mass spectrometric (MS/MS) detection for the determination of GHB, beta-hydroxybutyrate (BHB), pregabalin, 1,4BD and GBL in whole blood and urine. Using the 96-well formate, the whole blood method is a simple high-throughput method suitable for screening of large sample amounts. With an easy sample preparation for urine including only dilution and filtration of the sample, the method is suitable for fast screening of urine samples. Both methods showed acceptable linearity, acceptable limits of detection, and limits of quantification. The within-day and between-day precisions of all analytes were lower than 10% RSD. The analytes were extracted from matrices with recoveries near 100%, and no major matrix effects were observed. Both methods have been used as routine screening analyses of whole blood and urine samples since January 2010.

1. Introduction

Gamma-hydroxybutyric acid (GHB) and its precursors 1,4-butane-diol (1,4BD) and gamma-butyrolactone (GBL) (Fig. 1) are increasingly abused drugs in Norway and other Scandinavian countries [1,2]. 1,4BD and GBL are rapidly converted to GHB in the body. GHB as well as its precursors induce euphoria, relaxation and anxiolysis with a similar mechanism of action as ethanol and benzodiazepines [3,4]. All three compounds are listed as drugs of abuse in Norway. GHB is easily prepared from commercially available chemicals, as powder or as a colorless and odorless fluid. It is often abused in combination with other drugs [3,5,6] and reports state that it is mixed into drinks and used as a party rape or "knock out" drug. Being a precursor of the endogenous neurotransmitter gamma-aminobutyric acid (GABA) and as a metabolic product, GHB is present in small amounts in the body [7–9]. Recently, the pharmacodynamics, pharmacokinetics, toxic effects, addiction, analytical methods, and interpretation of results were reviewed by Andresen et al. [10].

Pregabalin (Fig. 1), a lipophilic analog of GABA, is an antiepileptic drug used to treat neuropathic pain, generalized anxiety disorder and seizures [11–13]. It is reported to be a safe and efficacious drug for the discontinuation of benzodiazepine dependence [14]. Hence, it is speculated whether pregabalin has gained in popularity as a drug of abuse among long-term benzodiazepine users, even though the European Medicines Agency (EMEA) and the Drug Enforcement Administration (DEA) have considered pregabalin to have a low potential of abuse [15,16]. As side-effects of pregabalin include dizziness and sleepiness, driving is not recommended after the administration of the drug.

Beta-hydroxybutyrate (BHB; Fig. 1) is an endogenous ketone body and was included in the whole blood method in order to give analytical evidence of ketoacidosis. Fatty acids are utilized as an alternative fuel pathway and are converted via the β-oxidative pathway to acetoacetate which undergoes further reactions to form acetone (via decarboxylation) and BHB (via reduction) [17]. Increasing BHB levels occur with ketoacidosis, a biochemical disturbance in the body. Ketoacidosis may be a result of extensive alcohol abuse or diabetes. Analytical evidence of ketoacidosis, e.g. high concentrations of BHB, might indicate a pathological diagnosis or a possible cause of death [18–21].

In Norway, GHB is gaining popularity revealed by increased numbers of seizures [22], increased numbers of young people using GHB [23] and GHB being the fifth most common cause of admission

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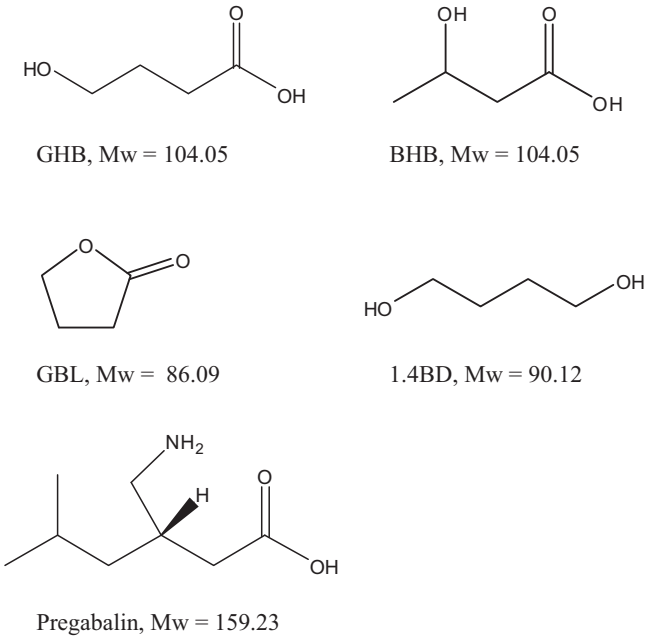

Fig. 1. Structural formula and molecular weight of the analytes.

to hospitals [24]. An increased sale of pregabalin (Lyrica) has been reported as well [25]. Thus, it was desirable to map the abuse of these substances among drivers suspected to be under the influence of drugs. The Division of Forensic Medicine and Drug Abuse Research (DFM) at the National Institute of Public Health (NIPH) is analyzing GHB, BHB and pregabalin in whole blood in cases where the police suspects driving under the influence of drugs, drug abuse, and in samples from forensic autopsies. GHB and pregabalin in urine are determined in cases where the penitentiary or the police suspect drug abuse, and in workplace, medical or social drug testing cases. With an average sample amount of more than 500 whole blood samples and more than 200 urine samples per month, robust high-throughput methods were needed for routine analysis of these substances.

Previously at the DFM, GHB in whole blood and urine was determined by gas chromatography with flame ionization detection (GC-FID). In these methods, GHB was converted to GBL and liquid–liquid extraction with dichloromethane/hexane was used as sample pre-treatment. These methods were labor-intensive and put high strain to the analyst, hence they were not suitable for sample amounts of over 100 samples per week. Additionally, the GC methods could not distinguish between GHB, 1,4BD and GBL. Despite this fact, GC was the most abundant separation technique used in the determination of GHB between 2001 and 2010 [26].

High performance liquid chromatography (HPLC) methods overcome most disadvantages mentioned above for GC methods and several publications report the use of HPLC coupled to mass spectrometry (MS) [27–29] or ultra violet spectrometry (UV) [30,31] for the determination of GHB in urine, serum and hair. Using HPLC–MS methods, time consuming and labor-intensive derivatization steps are not required. The use of toxic solvents may be reduced and all analytes can be determined simultaneously, since conversion of GHB to GBL is not required. However, with a retention time of 2–5 min for GHB, these methods still are time consuming. Ultra-high performance liquid chromatography with MS detection (UPLC–MS) or tandem MS detection (UPLC–MS/MS) is characterized by higher efficiency compared to conventional HPLC. Thus, shorter analysis time and higher sensitivity can be achieved using UPLC methods. A couple of methods using UPLC–MS/MS have been proposed for the determination of GHB, BHB, 1,4BD and GBL in urine or whole blood [32,33]. However, none of these methods include pregabalin. Additionally, a selective sample preparation step using SPE and still maintaining high throughput has not been reported previously.

Here, we report a UPLC–MS/MS method for the simultaneous determination of GHB, BHB, pregabalin, 1,4BD and GBL in whole blood and urine. The sample preparation of the blood method utilizes a 96-well format, and hence is capable of screening many hundred samples per week. The urine method utilizes solely a dilution and filtration step which makes it easy, fast, and less prone to errors. With a total run-time of only 5 min per injection, including washing and reconditioning of the column, a lot of time is saved compared to GC and HPLC methods.

2. Materials and methods

2.1. Reagents and standards

HPLC grade acetonitrile (ACN) and methanol (MeOH) were obtained from LabScan (Dublin, Ireland). Formic acid (FA) was purchased from BDH Prolabo (Briare, France). Type 1 water (18.2 Ω) was obtained from an in-house Milli-Q Biocel from Millipore (Billerica, MA, USA) with an Ultrapore Quantum Organex cartridge. GHB, BHB, 1,4BD and GBL were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pregabalin was obtained from Pfizer (New York, NY, USA). GHB-d_6 and pregabalin-d_6 were purchased from Cerilliant (Round Rock, TX, USA).

Blank whole blood was supplied by the blood bank of Oslo at the Oslo University Hospital, Ullevål, Norway. Blank urine was obtained from the lab staff at the DFM, NIPH.

Stock solutions with a concentration of approximately 10.4 g/L GHB, BHB, GBL and 1,4BD and 1.6 g/L pregabalin were prepared with MeOH and stored at −21 °C. Calibration and control solutions were prepared by appropriate dilutions with type 1 water (Table 1) and stored at 4 °C.

Authentic samples were stored in polypropylene vials (Sarstedt, Nümbrecht, Germany) at 4 °C until analysis.

2.2. Sample preparation – blood

100 μl of calibration solutions or control solutions was added to 100 μl blank whole blood. Unknown samples had a volume of 100 μl blood and 100 μl water was added to each sample to compensate for the volume of the calibration/control solutions. Internal standard solution (50 μl) was added to each vial to compensate for variations during sample extraction and to compensate for matrix effects.

The samples were precipitated with 400 μl ice-cold acetonitrile:MeOH (85:15, v/v), immediately mixed on a whirlimixer for 10–20 s and, to obtain complete precipitation, the tubes were stored in a freezer for at least 10 min before centrifugation (4750 rpm at 4 °C, 10 min). Supernatants were added to 250 μl 0.4% formic acid solution and loaded on a preconditioned (MeOH and water) 96-well plate containing 30 mg OASIS HLB stationary phase (Waters, Milford, MA, USA). The eluate was collected, excess organic modifier was removed by N_2 (40 min, 70 °C, 40 sf/min), the remaining eluate was diluted further with 750 μl 0.4% formic acid solution and stored in an Acquity sample manager (Waters) at 10 °C. Samples were injected using the partial loop with overfill technique with an injection volume of 3 μl.

2.3. Sample preparation – urine

100 μl of calibration solutions or control solutions was added to 100 μl blank urine. Unknown samples had a volume of 100 μl urine and 100 μl water was added to each sample to compensate for

Table 1
Concentrations [μg/ml] of calibration and control solutions.

	GHB		BHB		Pregabalin		GBL		1,4BD	
	b	u	b	u	b	u	b	u	b	u
Calibration LOQ	2.6	–	–	–	0.4	–	2.1	–	2.3	–
Calibration 1	5.2	–	31.1	–	0.8	–	4.3	–	4.6	–
Calibration 2	10.4	–	–	–	1.6	–	8.6	–	9.2	–
Calibration 3	52	–	–	–	4.0	–	43.0	–	46	–
Calibration 4	104.1	104.1	104.1	–	8.0	–	86.0	–	92.1	–
Calibration 5	208.2	–	–	–	15.9	15.9	172.2	–	184.2	–
Calibration 6	312.3	–	312.3	–	39.8	–	258.3	–	276.3	–
Control 1 (C1)	7.8	7.8	26	–	1.2	1.2	6.5	–	6.9	–
Control 2 (C2)	260.3	104.1	260.3	–	39.8	15.9	215.2	–	230.3	–
Control 3 (C3)	–	260.3	–	–	–	39.8	–	–	–	–

b, blood method; u, urine method.

the volume of the calibration/control solutions. Internal standard solution (50 μl) was added to each vial to compensate for variations during sample extraction and to compensate for matrix effects.

Each sample was diluted with 1.75 ml 0.2% formic acid solution and an aliquot of 500 μl was transferred to a mini-UniPrep filter chamber and filtered using a mini-UniPrep plunger containing a 0.2 μm filter membrane (Whatman, Springfield Mill, UK). The samples were stored in an Acquity sample manager (Waters) at 10 °C until injection which was performed using the partial loop with overfill technique with an injection volume of 2 μl.

2.4. Instrumentation

Chromatographic separation was obtained by an Acquity UPLC (Waters) using an HSS T3 column (2.1 mm id × 100 mm, dp = 1.7 μm) with a mobile phase containing 0.2% formic acid (A) and methanol (B) at a flow rate of 0.5 ml/min. A linear gradient from 0 to 55% B in 1.5 min followed by a step to 90% B (2 min) was used to wash the column and a re-equilibration step (1 min, 100% A) was also performed. The column was held at a temperature of 65 °C. A solvent delay program directed interferences eluting during the first 0.5 min of the gradient and the first minute of the washing step into waste.

Mass spectrometric detection was performed using either a Quattro Premier XE tandem mass spectrometer (Waters) or a XEVO TQ tandem mass spectrometer (Waters). Electrospray ionization (ESI) in positive mode and multiple reaction monitoring (MRM) was performed on both instruments. Ideal MS tune settings and MRM conditions were found for each compound by direct infusion of the analytes. Data was handled by the TargetLynx software v4.1 (Waters).

3. Results and discussion

3.1. Chromatographic separation and mass spectrometric detection

GHB, BHB and 1,4BD are highly polar compounds. Thus, hydrophilic interaction chromatography (HILIC), where the retention is determined by reversed polarity compared to traditional reversed phase applications would be the separation method of choice. In HILIC however, separation is obtained by using acetonitrile which, compared to MeOH, has higher toxicity and is more expensive and should therefore not be utilized in a screening method with over 6000 samples per year. Furthermore, in our routine lab, all UPLC–MS/MS methods utilize reversed-phase columns. Thus, reversed phase chromatography was chosen for practical reasons, as for example instrumental back-up.

HSS T3 columns are designed to retain and separate polar organic compounds in reversed-phase chromatography; they also

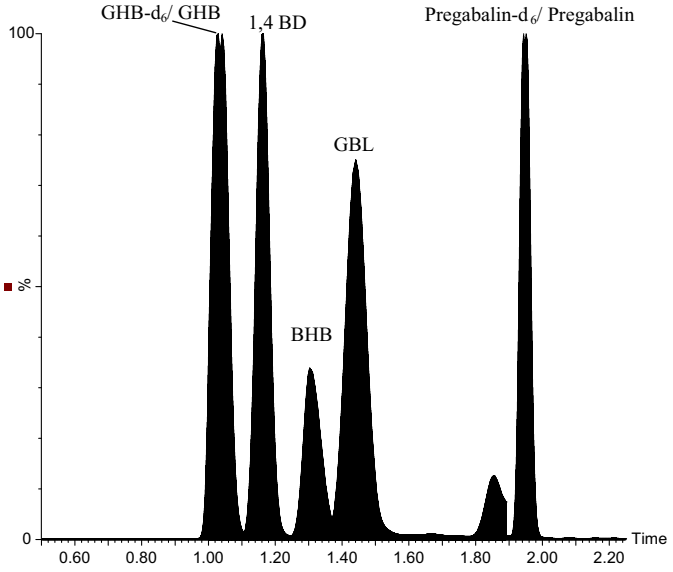

Fig. 2. MRM chromatogram of the quantifier ions for GHB, BHB, pregabalin, GBL, 1,4BD and the internal standards from an injection of calibration level 3.

are compatible with 100% aqueous mobile phases [34]. Using an HSS T3 column with 100% aqueous mobile phase, GHB and 1,4BD showed sufficient retention. GHB, BHB and pregabalin are weak acids with pK_a values of 4.7 (GHB/BHB) and 4.2 (pregabalin). Thus, in an acidic mobile phase with pH 2, the analytes are protonated and hence show greater retention in reversed-phase separations. A solution of 0.2% FA has sufficiently low pH and thus was used as aqueous mobile phase, while MeOH was used as organic modifier. Using an HSS T3 column and a mobile phase as described above, baseline separation of GHB, BHB and 1,4BD was achieved (Fig. 2).

The optimalization of the mass spectrometric detection was performed using the auto-tune function of the MassLynx software. Each compound was tuned individually by direct infusion of standards. Several adequate MS/MS transitions were found and transitions with the highest intensity were chosen as quantification and target transitions (Table 2). Thus, the identity of each compound was determined by retention time and at least three MS/MS transitions.

3.2. Sample preparation

Sample preparation is an important and critical part of an analytical method. Biological samples may contain a large number of interferents at high concentrations compared to the analyte of interest. Matrix effects such as ion suppression or ion enhancement are critical factors which may lead to uncertain quantifications,

Table 2
MS/MS transition, cone voltage, collision energy and dwell time.

Analyte	$[M+H]^+$	Daughter ion	Cone voltage (V)	Collision energy (V)	Dwell time (s)
GHB/BHB	105.06	87.05[a]	10	6	0.025
	105.06	43.05	10	10	0.025
	105.06	42.3	10	12	0.025
GHB-d_6	111.09	93.08[a]	10	6	0.025
	111.09	49.05	10	10	0.025
Pregabalin	160.13	142.12[a]	18	10	0.025
	160.13	97.10	18	14	0.025
	160.13	55.05	18	18	0.025
Pregabalin-d_6	166.13	148.12[a]	18	10	0.025
	166.13	103.10	18	14	0.025
1,4BD	91.08	97.10	12	6	0.025
	91.08	73.07	12	8	0.025
	91.08	55.05[a]	12	6	0.025
GBL	87.04	45.03[a]	20	8	0.025
	87.04	44.95	20	10	0.025

[a] Quantifier ion.

especially in ESI-UPLC–MS/MS methods. An optimal sample preparation should reduce the amount of interfering matrix introduced into the mass spectrometer and should further extract the analyte(s) with high recovery and specificity.

Utilizing previous methods, we have found GHB concentrations up to 5.2 g/L in urine samples and up to 1.25 g/L in blood samples. The cut-off of both methods is 10.4 mg/L, which is well above the limit of quantification. Thus dilution of the samples was a necessary step of the sample preparation.

3.2.1. Blood samples

Whole blood contains a variety of interferences such as blood cells, proteins, glucose, hormones and lipids which should be removed prior to UPLC–MS/MS analysis. Since the plasma-protein binding of GHB and pregabalin is reported to be low [35,36], protein precipitation is an adequate technique as the first step in sample preparation. Three different precipitation solutions were tested; MeOH/1% FA (v/v) (room temperature), ice cold ACN/MeOH/FA (84.5/14.5/1, v/v/v) and ice cold ACN/MeOH (85/15, v/v). The agents containing formic acid did not give clear supernatants, while ACN/MeOH (85/15, v/v) gave a clear supernatant. Hence, ice cold ACN/MeOH (85/15, v/v) was chosen as precipitation agent. The amount of precipitation agent was found by adding 250, 300, 350, 400 and 500 μl to 100 μl whole blood sample diluted with 100 μl H_2O. Complete precipitation was obtained using 400 μl of precipitation agent.

Interferents are not completely removed by protein precipitation. Thus, an additional sample clean-up step using solid phase extraction (SPE) was desirable. Many SPE materials are available in 96-well format which is well suited for large numbers of samples. Additionally, SPE may be automated and the use of toxic solvents is limited.

Since both acidic compounds (GHB, BHB, pregabalin) and neutral compounds (1,4BD, GBL) should be possible to extract, the SPE protocol could not be based on an ion-exchange mechanism. However, as mentioned above, the hydrophilic compounds GHB, BHB and 1,4BD did not show sufficient retention on a reversed phase SPE material to follow a standard SPE protocol (based on concentration of the analytes on the SPE phase, followed by a washing step and elution of the analytes). Hence, it was decided to elute the analytes during the sample loading step and retain hydrophobic interferences. In this approach, it is crucial to adjust the percentage of organic modifier; the analytes of interest were completely eluted from the SPE, while as many interferences as possible were retained by the reversed phase mechanism. Pregabalin and GBL, which are more hydrophobic compounds, required 50% organic solvent to be completely eluted during the loading step. Thus, 250 μl 0.4% formic acid was added to each vial after precipitation to adjust both organic modifier percentage and pH.

Further reduction of the organic modifier content prior to injection onto the chromatographic system was necessary to avoid band broadening leading to high variation in relative retention time. Removal of the organic modifier from the eluate was thus performed, followed by further dilution with 0.4% formic acid.

3.2.2. Urine samples

Urine contains mainly dissolved inorganic and organic ions such as urea, chloride, sodium, potassium and creatinine. Additionally, it may contain particles and small amounts of inorganic and organic compounds. In general however, urine is a less complex matrix compared to whole blood. Since water-soluble toxic waste products are excreted from the body by urine, the concentration of illicit drugs and their metabolites is often high compared to their concentration in blood. Appropriate dilution and filtration of the urine samples was considered to be sufficient sample preparation.

3.3. Method validation

Performance of the developed methods was evaluated and is described in further detail below. The MRM transitions for BHB, GBL and 1,4BD were included in both MS methods, however these analytes were not included in the calibration or control samples of the urine method and therefore were not included in the validation.

The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the concentration giving a S/N of 3 and 10 respectively, and were found by appropriate dilution of a control solution. With the developed methods an LOD and LOQ according to Table 3 was obtained for GHB, pregabalin, 1,4BD and GBL. The cut-off values for these compounds were 10.4 mg/L (GHB, 1,4BD, GBL) and 0.8 mg/L (pregabalin) for both blood and urine samples. The cut-off values were set by the DFM to ensure discrimination of endogenous and exogenous analytes and to avoid false interpretation of the results. Hence the LOD and LOQ obtained with the current methods were satisfactory in order to quantify concentrations at the given cut-off concentrations. BHB had a cut-off value of 52 mg/L in blood samples obtained in forensic autopsies, while BHB was not analyzed in urine samples. Thus, the LOD and LOQ were not determined for this compound.

Table 3
LOD and LOQ; within-assay ($n = 10$) and between-assay precision given as RSD [%]; matrix effects (ME) and recovery (RE) given in % ($n = 6$).

	GHB	BHB	Pregabalin	1,4BD	GBL
LOD [μg/ml]	0.65	–	0.004	0.23	1.1
LOQ [μg/ml]	1.3	–	0.008	0.37	2.15
C1, within, blood	8	–	10	9	9
C2, within, blood	4	–	4	5	4
C1, within, urine	4	–	6	–	–
C2, within, urine	3	–	4	–	–
C3, within, urine	2	–	5	–	–
C1, between, blood	10 ($n = 267$)	17 ($n = 73$)	9 ($n = 244$)	23 ($n = 10$)	19 ($n = 10$)
C2, between, blood	9 ($n = 250$)	8 ($n = 97$)	8 ($n = 244$)	9 ($n = 10$)	14 ($n = 10$)
C1, between, urine	9 ($n = 190$)	–	8 ($n = 144$)	–	–
C2, between, urine	5 ($n = 185$)	–	7 ($n = 144$)	–	–
C3, between, urine	6 ($n = 141$)	–	9 ($n = 145$)	–	–
ME, C1, whole blood	111	–	109	120	107
ME, C1, forensic autopsy blood	101	–	100	104	102
ME, C2, whole blood	96	–	100	96	98
ME, C2, forensic autopsy blood	97	–	100	99	97
ME, C1, urine	103	–	97	–	–
ME, C2, urine	105	–	100	–	–
ME, C3, urine	104	–	101	–	–
RE, C1, whole blood	117	–	98	91	103
RE, C1, forensic autopsy blood	140	–	100	89	96
RE, C2, whole blood	97	–	96	104	105
RE, C2, forensic autopsy blood	104	–	105	105	105
RE, C1, urine	99	–	95	–	–
RE, C2, urine	107	–	99	–	–
RE, C3, urine	98	–	98	–	–

–, analyte not included.

Calibration curves were made using spiked samples. GHB-d_6 was used as internal standard for GHB, BHB, 1,4BD and GBL, while pregabalin-d_6 was used as internal standard for pregabalin. All calibration curves showed acceptable linearity ($R^2 > 0.95$).

Carry-over was tested by injecting a blank sample after the calibration sample with highest concentration and during routine analysis, several authentic samples with concentration exceeding the calibration range were found; up to 832 mg/L GHB and 95 mg/L pregabalin were found in blood samples, while up to 5.2 g/L of GHB and 1.3 g/L pregabalin were found in urine samples. Neither blank samples injected after a high calibration sample, nor negative authentic samples injected after authentic samples containing high concentrations did show carry-over.

The within-assay precision was obtained by injecting 10 replicates of the control samples at the same day. The within-assay ($n = 10$) precisions (given in RSD %) of both methods were below 10% for all analytes, which was acceptable (Table 3). However, for routine analysis, the methods' performance over a long time range was more important than the within-assay performance. The between-assay precisions for GHB, BHB and pregabalin were calculated by the lab data system (StarLIMS Client, Ver. 9.383) using approved control samples (<3 standard deviations from the theoretical value) over a time range of 16 months. In Table 3, the numbers shown in brackets in the between-assay rows indicate the number of control samples included in the calculation. According to bioanalytical method validation guidelines published by the Food and Drug administration (FDA), precisions below 15% (20% at the LOD level) are acceptable [37]. The between-assay precision (given in RSD %) of both methods was below 10% for GHB, pregabalin and BHB (C2, Table 3), while the between assay precision of BHB (C1) was 17%. Only high concentrations of BHB (>52 mg/L in forensic autopsies) were reported, thus a precision of 17% at a low level was acceptable. Precisions of up to 23% were obtained for 1,4BD and GBL, which was acceptable.

To test the matrix effects and extraction recovery, three sets of samples were prepared: aqueous samples (set A, $n = 6$), samples spiked after sample preparation (set B, $n = 6$) and samples spiked before sample preparation (set C, $n = 6$) [38]. Matrix effects were obtained by comparing set A and set B; $\mathrm{ME}(\%) = (\text{set B/set A}) \cdot 100$, while recovery was obtained by comparing set B and set C; $\mathrm{RE}(\%) = (\text{set C/set B}) \cdot 100$.

Since whole blood obtained from healthy volunteers and blood obtained in forensic autopsies could be regarded as different matrices, the matrix effect and recovery test was carried out using both blood types as matrix. The autopsy blood was obtained from cases where GHB abuse prior to death was not probable. Table 3 shows the matrix effects given in % at different concentration levels and with different matrices. Values less than 100% indicate ion suppression, while values higher than 100% indicate ion enhancement by matrix components. All analytes showed values around $100 \pm 10\%$; hence no major matrix effects were effecting the quantification. Table 3 also shows the extraction recoveries at different concentration levels from different matrices. All analytes showed extraction recoveries of $100 \pm 10\%$, hence the analytes were extracted without great loss from the matrix. At the low concentration level, GHB showed a recovery of 117% (whole blood) and 140% (autopsy blood) which might have been caused by endogenous GHB present in low concentrations in blood samples, especially autopsy samples where small amounts GHB may have formed post mortem [39].

The stability of solutions under different storing conditions has been evaluated. Aqueous solutions were stable for at least one year stored in the fridge (4 °C). Extracted samples were stable in the auto sampler (10 °C) for at least one night, while they were stable at both 4 °C and −21 °C for one week. The supernatant obtained after protein precipitation was stable at 4 °C overnight.

The specificity of the developed methods was tested by injecting calibration solutions from other in-house methods containing high concentrations of possible interfering compounds. These calibration solutions contained about 30 legal and illicit drugs. An MS scan in the range of m/z 50–1000 and an MRM scan with the transitions of the present methods' analytes were monitored. The MS scan did not reveal any interferences co-eluting with the analytes.

The MRM scan did not reveal interferences of other compounds at the chosen transitions.

3.4. Application

The developed UPLC–MS/MS methods have been used at the NIPH since January 2010 for the quantitative screening of GHB, pregabalin and BHB in more than 9000 whole blood samples and the qualitative screening of GHB and pregabalin in more than 3000 urine samples. The MS methods contain MRM functions for 1,4BD and GBL, which thus might be revealed even though they were not included in calibration and control samples. Each blood assay contains two blank whole blood samples, two replicates of three calibration levels (1, 4 and 6), two replicates of the control levels and up to 84 authentic samples. Each urine assay contains two blank urine samples, three replicates of the calibration level, two replicates of the control levels and up to 61 authentic samples. Quality control samples are accepted when the found concentrations are within 3 standard deviation from the theoretical concentrations. The cut-off concentrations used are 10.4 mg/L for GHB in whole blood and urine, 312 mg/L for GHB in autopsy samples, 52 mg/L for BHB in autopsy samples and 0.8 mg/L for pregabalin in whole blood and urine. Since January 2010, we have found 408 cases with a GHB concentration above the cut-off in whole blood, with a mean concentration of 91 mg/L (range 13–868 mg/L). 167 cases with a pregabalin concentration above the cut-off with a mean concentration of 8.5 mg/L (range 0.8–105 mg/L) have been found. Screening of BHB in forensic autopsies was initiated in November 2010 and since then 197 cases with a BHB concentration above the cut-off have been found, with a mean concentration of 237 mg/L (range 52–1270 mg/L). In urine, we have found 157 cases with a GHB concentration above the cut-off, with a mean concentration of 403 mg/L (range 10.4–4737 mg/L), while 56 cases with a pregabalin concentration above the cut-off with a mean concentration of 125 mg/L (range 0.9–1264 mg/L) have been found.

4. Conclusion

Fast and simple UPLC–MS/MS methods for the determination of GHB, BHB, pregabalin, 1,4BD and GBL in whole blood and urine samples were developed. The analytical performance of both methods was documented. Satisfactory linearity and acceptable limits of detection and limits of quantification were obtained. Both methods performed very well in routine analysis, showing within- and between-assay precisions lower than 10% RSD, extraction recoveries of 100% and no major matrix effects.

The developed UPLC–MS/MS methods have been used at the DFM since January 2010 for the quantitative screening of GHB, pregabalin and BHB in whole blood samples and the qualitative screening of GHB and pregabalin in urine samples. In whole blood samples, 408 cases with positive GHB and 167 cases with positive pregabalin have been found. In urine samples, 157 cases with positive GHB and 56 cases with positive pregabalin have been found. Since November 2010, 197 forensic autopsy cases with positive BHB have been found.

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