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A hydrophilic interaction liquid chromatography electrospray tandem mass spectrometry method for the simultaneous determination of γ-hydroxybutyrate and its precursors in forensic whole blood

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ABSTRACT

A liquid-chromatography–tandem-mass-spectrometry method using pneumatically assisted electrospray ionisation (LC–ESI-MS/MS) was developed for the simultaneous determination of γ-hydroxybutyric acid (GHB), γ-butyrolactone (GBL) and 1,4-butanediol (1,4-BD) in human ante-mortem and post-mortem whole blood. The blood proteins were precipitated using a mixture of methanol and acetonitrile, and the extract was cleaned-up by passage through a polymeric strong cation exchange sorbent. Separation of the analytes and their structural isomers was obtained using a column with a zwitterionic stationary phase. Matrix-matched calibrants, combined with isotope dilution, were used for quantitative analysis. GHB was determined in both positive and negative ion modes. The relative intra-laboratory reproducibility standard deviations were better than 10% and 6% for blood samples at concentrations of 2 mg/L and 20–150 mg/L, respectively. The mean true extraction recoveries were 80% for GHB and greater than 90% for GBL and 1,4-BD at concentration levels of 20–50 mg/L. The limits of detection were approximately 0.5 mg/L for GHB and GBL, and 0.02 mg/L for 1,4-BD in ante-mortem blood. The corresponding lower limits of quantification were less than 1 mg/L for GHB and GBL, and less than 0.1 mg/L for 1,4-BD. GBL was unstable in whole blood freshly preserved with a sodium fluoride oxalate mixture, but the stability could be improved significantly by preservation with a sodium fluoride citrate EDTA mixture.

1. Introduction

The drug γ-hydroxybutyric acid (GHB) is a short-chain hydroxylated fatty acid that is endogenously present in various mammalian tissues, blood and urine. It is a powerful central nervous system depressant and was used in legal medicine as an anaesthetic agent prior to being phased out because of its very steep dose-response curve and various side effects. Today, GHB is mainly used for the treatment of narcolepsy and less frequently during the treatment of alcohol dependence [1]. However, GHB is also abused for recreational purposes and is available as both a colourless, odourless liquid and as a solid material on the illicit market. GHB related fatalities have been reported [2,3] and the drug has been found in both the body fluids and hair from suspected victims of drug-facilitated sexual assaults [4,5].

Although GHB is a controlled drug, the pharmacologic effects of GHB may be obtained indirectly from the intake of the commercially available and legal chemicals γ-butyrolactone (GBL) and 1,4-butanediol (1,4-BD). Both are used as industrial solvents and they may be constituents in various cleaners or used during the synthesis of other chemicals (e.g. 1,4-BD is used during the production of tetrahydrofuran). Following the direct intake of GBL, the substance is rapidly converted to GHB by the lactonase enzymes present in the blood of mammals. However, GBL can also be easily converted to GHB by treatment with lye in a simple non-industrial (kitchen) process. In humans, 1,4-BD is converted to GHB through the combined actions of alcohol and aldehyde dehydrogenases. GHB is rapidly and extensively metabolised in the body. Approximately 1% is excreted unchanged in urine [6].

In forensic toxicology, body fluids are monitored for substances that may have been abused, for instance, by vehicle drivers. However, testing for GHB in biological matrices is challenging because of its polar properties, its equilibrium state with GBL favouring GBL at low pH, and the complete conversion of GLB to GHB at high pH values. Determination of GHB in biological fluids is typically performed by gas chromatography mass spectrometry (GC–MS) after derivatisation of the substance, such as silylation. The use of LC–MS/MS allows for omission of the derivatisation step, but poor retention on analytical columns is often observed. Methods based on LC–MS/MS of the native

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molecule have been published for the determination of GHB in plasma/serum, [7,8] and for the simultaneous determination of GHB, GBL and 1,4-BD in whole blood and urine [9,10]. These methods use reverse phase chromatography for the separation of the components and acidified mobile phases to obtain some retention of GHB, which restricts the electrospray ionisation (ESI) to be performed in the positive ion mode where only a single abundant product ion is produced. To improve the chromatographic and retention properties, derivatisation has also been applied in the determination of GHB in urine and serum using LC–MS [11]. However, the chromatographic separation of small polar molecules can often be directly improved through the use of hydrophilic interaction liquid chromatography (HILIC) instead of classical reverse phase chromatography. The HILIC conditions allow the chromatography to be performed under neutral conditions.

The present LC–MS/MS method is based on the HILIC analysis of extracts that have been cleaned-up using cation-exchange solid phase extraction (SPE) for adsorption of interfering substances. The method was validated as a robust technique, suitable for the determination of GHB, GBL and 1,4-BD in both ante-mortem and post-mortem whole blood samples. Several abundant transition products of GHB were obtained for the proper identification of the substance.

2. Materials and methods

2.1. Chemicals

α-Hydroxybutyric acid (AHB) sodium salt, β-hydroxybutyric acid (BHB) sodium salt, GHB, GBL, 1,4-BD and 2,3-butanediol (2,3-BD) were obtained from Sigma–Aldrich (Schnelldorf, Germany). The isotope analogues, GHB-D6 and GBL-D6, were obtained from Cerilliant (Round Rock, Texas) and 1,4-BD-D4 was obtained from Cambridge Isotope Laboratories (Andover, MA). Whole blood samples, used for calibration, were obtained from the Blood Bank, Aarhus University Hospital (Skejby, Denmark). Formic acid, ammonium acetate and sodium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). Acetonitrile (MeCN) and methanol (MeOH) of LC–MS grade were purchased from Sigma–Aldrich. Water was purified using a Direct-Q 3 apparatus (Millipore, Bedford, MA).

2.2. Samples

Samples of ante-mortem and post-mortem whole blood, used for validation of the method, were obtained from the Department of Forensic Medicine, Aarhus University. Ante-mortem blood samples that were used in the validation study were preserved in Venosafe VF-053SFC32 tubes that contained 6.8 mg of sodium fluoride (NaF) and 15.7 mg of citrate-EDTA buffer ingredients (FC mixture) for a 3 mL draw volume of blood (Terumo Europe, Leuven, Belgium). Post-mortem blood samples were preserved with 200 mg of NaF per 30 mL of blood. Venosafe VF-109SFX07 tubes (Terumo Europe) that contained 100 mg of NaF and 22.5 mg of potassium oxalate (FO mixture) for a 9-mL draw volume of blood and Venosafe VF-052SDK tubes (Terumo Europe) that contained 3.9 mg K_2EDTA for a 2-mL draw volume of blood were included in a stability study on GHB and its precursors in ante-mortem whole blood.

2.3. Standards

Separate stock solutions containing 1 mg/mL of the active substances were prepared in MeOH and stored at -20 ± 2 °C. The combined standard solutions for the fortification of the samples and preparation of the calibrants were prepared by diluting the stock solutions with MeOH. An internal standard solution (IS) containing 0.1 mg/mL of the deuterated analogues of GHB, GBL and 1,4-BD was prepared in MeOH.

2.4. Equipment

The liquid chromatography system was a Waters Acquity UPLC system that consisted of a binary pump, an autosampler with a 10-μL sample loop thermostated at 7 ± 2 °C and a column oven thermostated at 30 ± 2 °C (Waters, Milford, MA). The mass spectrometer was a Waters Xevo TQMS triple-quadrupole instrument with an ESI ion source and a programmable infusion pump. The separation was performed using a SeQuant ZIC HILIC (5 μm, 200 Å, 2.1 mm I.D. × 100 mm) column (Merck SeQuant, Umeå, Sweden). Solid phase extraction (SPE) was performed on a 3-mL Strata-X-C cartridge containing 60 mg of a polymeric strong cation exchange (SCX) sorbent (Phenomenex, Torrance, CA). A VacMaster-20 vacuum manifold (Biotage, Uppsala, Sweden) was used during the SPE procedure. Disposable 2-mL polypropylene Safe-Lock tubes (Eppendorf, Hamburg, Germany) were used for the extractions. Autosampler vials made of glass (Mikrolab, Aarhus, Denmark) were used for storage of the final extracts. Other equipment used included pipettes (Biohit, Helsinki, Finland) and a Heraeus Biofuge Pico (Thermo Scientific, Langenselbold, Germany).

2.5. Extraction and clean-up

A 200-μL volume of sample was transferred to a disposable 2 mL centrifuge tube. Then, 100 μL of MeOH and 100 μL the IS were added, and the tube contents were mixed gently. Shortly thereafter, a 600-μL volume of MeCN was added and the tube was immediately closed and vigorously vortex-mixed for a few seconds. After a standing time of 5–10 min, the mixture was centrifuged at 10,000 × g for 5 min. A volume of 600 μL of the supernatant was mixed with 250 μL of water and was then passed through a CX SPE cartridge with a flow rate of max 0.5 mL/min. The cartridge was previously conditioned sequentially with 1 mL of MeOH, 1 mL of water, 1 mL of a 1 M sodium dihydrogen phosphate solution and 1 mL of water and then dried under full vacuum suction for few seconds. The eluate was collected, and 200 μL of the eluate was mixed with 600 μL of MeCN in an autosampler vial. The total sample dilution factor was 28.

2.6. Calibration

The calibrants for both the ante-mortem and post-mortem samples were based on blank donor blood from single persons preserved with FC mixture. The samples were treated using the same procedure with the exception that the 100 μL of MeOH was replaced by 100 μL of the mixed standards of the drug substances. Sample concentrations were obtained at 5, 50, 100, 150 and 200 mg/L of GHB, and 2.5, 25, 50, 75 and 100 mg/L of GBL and 1,4-BD in the original blood sample. In addition, a blank sample (a processed matrix sample without analyte and without IS) and a zero sample (a processed matrix sample with IS) were included to verify the absence of detectable concentrations of the analytes. The calibration curves were created from weighted ($1/x$) linear regression analysis of the IS-normalised peak areas (analyte area/IS area) and were forced through the origin.

2.7. LC–MS/MS analysis

The sample extracts were maintained at 7 ± 2 °C prior to analysis. A 10-μL volume was injected onto a SeQuant ZIC HILIC column running 5% mobile phase A (1 mM ammonium acetate) and 95% mobile phase B (MeCN). The mobile phase was changed through a linear gradient to 50% A and 50% B over 4 min. Then, the gradient was changed to 95% A over 0.2 min. Five minutes after injection, the gradient was returned to 5% A over 0.5 min, and the column was equilibrated for 4.5 min before the next injection resulting in a total runtime of 10 min. The column flow rate was 200 μL/min and the column temperature was maintained at 30 ± 2 °C. During the interval of 0.5–2.5 min, a reagent containing 0.1% formic acid was infused post column with a flow rate of 100 μL/min. The eluent was diverted to waste during the time interval of 4–9 min after injection by using a post-column switch. The source and desolvation temperatures were set at 150 °C and 600 °C, respectively, and the cone and desolvation nitrogen gas flows were set at 50 L/h and 800 L/h, respectively. The mass spectrometer was operated in positive ion mode with a probe voltage of 3 kV and in negative ion mode with a probe voltage of 2.5 kV. The dwell time was 50–64 ms depending on the number of ion transitions processed during the same time period. At least 12 data points were obtained across the peaks. Selected reaction monitoring (SRM) was applied using the conditions shown in Table 1. Argon was used for collision-induced dissociation (CID). The data acquisition and processing were performed using MassLynx 4.1 (Waters).

2.8. Limits of detection and quantification

The limits of detection (LODs) were determined using a random selection of 20 different samples of ante-mortem whole blood and 20 different samples of post-mortem blood. The samples were spiked prior to extraction with the individual substances in order to obtain concentrations that were approximately three to five times the signal/noise ratio. The LODs were calculated as $2 \times t_{0.95} \times SD_B$ ($t_{0.95}$ = 1.645), where SD_B is the standard deviation of the results obtained from the spiked samples. The lower limits of quantification (LLOQs) were calculated as 10 times the SD_B of the quantifier ions.

2.9. Precision, trueness and recovery

The repeatability standard deviation (SD_r) (i.e., the variability of independent analytical results obtained by the same operator using the same apparatus under the same conditions on the same test sample and in a short interval of time) and the intra-laboratory reproducibility standard deviation ($SD_{R,intra\text{-}lab}$) (i.e., the variability of independent analytical results obtained on the same test sample in the same laboratory by different operators on different days) were determined on blank control samples of ante-mortem whole blood spiked to levels of 2, 20 and 75 mg/L of GBL and 1,4-BD, and 2, 20 and 150 mg/L of GHB and from post-mortem samples with an endogenous content of GHB. Duplicate analyses were performed on eight