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Determination of γ-hydroxybutyric acid in human urine by capillary electrophoresis with indirect UV detection and confirmation with electrospray ionization ion-trap mass spectrometry

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Abstract

γ-Hydroxybutyric acid (GHB), a minor metabolite or precursor of γ-aminobutyric acid (GABA), acts as a neurotransmitter/neuromodulator via binding to GABA receptors and to specific presynaptic GHB receptors. Based upon the stimulatory effects, GHB is widely abused. Thus, there is great interest in monitoring GHB in body fluids and tissues. We have developed an assay for urinary GHB that is based upon liquid–liquid extraction and capillary zone electrophoresis (CZE) with indirect UV absorption detection. The background electrolyte is composed of 4 m*M* nicotinic acid (compound for indirect detection), 3 m*M* spermine (reversal of electroosmosis) and histidine (added to reach a pH of 6.2). Having a 50 μm I.D. capillary of 40 cm effective length, 1-octanesulfonic acid as internal standard, solute detection at 214 nm and a diluted urine with a conductivity of 2.4 mS/cm, GHB concentrations ≥2 μg/ml can be detected. Limit of detection (LOD) and limit of quantitation (LOQ) were determined to be dependent on urine concentration and varied between 2–24 and 5–60 μg/ml, respectively. Data obtained suggest that LOD and LOQ (both in μg/ml) can be estimated with the relationships 0.83 κ and 2.1 κ, respectively, where κ is the conductivity of the urine in mS/cm. The assay was successfully applied to urines collected after administration of 25 mg sodium GHB/kg body mass. Negative electrospray ionization ion-trap tandem mass spectrometry was used to confirm the presence of GHB in the urinary extract via selected reaction monitoring of the m/z 103.1→m/z 85.1 precursor–product ion transition. Independent of urine concentration, this approach meets the urinary cut-off level of 10 μg/ml that is required for recognition of the presence of exogenous GHB. Furthermore, data obtained with injection of plain or diluted urine indicate that CZE could be used to rapidly recognize GHB amounts (in μg/ml) that are ≥ 4 κ.
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Keywords: Hydroxybutyric acid

1. Introduction

γ-Hydroxybutyric acid (GHB) is a minor metabolite or precursor of γ-aminobutyric acid (GABA), an inhibiting neurotransmitter. It acts as a neurotransmitter/neuromodulator via binding to GABA receptors and to specific presynaptic GHB receptors. The

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half-life in plasma is about 0.2–1 h and only 2 to 5% is believed to be excreted unchanged in urine. GHB has been shortly applied for the treatment of narcolepsy and used as an anesthetic, but was withdrawn due to its scarce anesthetic power and due to serious side-effects. It was also used as an anabolic substance and for weight control and is currently considered for the treatment of alcohol and opioid addiction and other clinical applications. Furthermore, as GHB can produce stimulatory effects if administered at dosages of 1–2 g, it is currently being widely abused and consumed illegally, often together with 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) and alcohol. Because of its ability to induce a rapid loss of consciousness when administered at dosages of about 3–4 g or higher, GHB is also known as a date-rape drug [1–7]. In a recent study in which 670 antemortem urines of presumably drug and GHB free subjects were analyzed, endogenous urinary GHB concentrations were found to be between 0.34 and 5.75 μg/ml (mean and median of 3.08 and 3.00 μg/ml, respectively). For proper recognition of the presence of exogenous GHB, a 10 μg/ml cut-off level could thus be established [8].

Due to the increasing abuse of GHB and of its potential clinical use, it is necessary to be able to identify and quantitate this analyte in a variety of biological matrices. For analysis of GHB in urine, rapid colorimetric screening tests capable of recognizing GHB concentrations ≥100 μg/ml were reported [9,10]. Quantitative data on a μg/ml or lower level are typically obtained via acidic conversion of GHB to its lacton γ-butyrolactone (GBL), followed by liquid–liquid or solid-phase extraction of GBL and its analysis by gas chromatography–mass spectrometry (GC–MS) [11,12]. Alternatively, GHB can be extracted and derivatized for analysis with GC–MS [4,13,14] and GC with flame ionization detection [15] or can be derivatized prior to extraction and analysis by GC–MS [16].

Recently, capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) have been shown to be attractive approaches for analysis of drugs in body fluids, including urine [17–19]. Except for one paper reporting the simultaneous analysis of GHB, GBL and 1,4-butanediol and the determination of GHB in seizure samples using MEKC with indirect UV absorption detection [20], no electrokinetic capillary assays for GHB have been reported thus far. The determination of urinary GHB via CZE with indirect detection was investigated in our laboratory. In the present paper, the developed CZE-based assay for GHB in urine is described. Data obtained for analysis of urinary liquid–liquid extracts are compared to those obtained by direct injection of plain or diluted urine. Furthermore, the presence of urinary GHB has been confirmed by negative electrospray ionization ion-trap tandem mass spectrometry (ESI–MS), an approach that appears to be new as well.

2. Experimental

2.1. Chemicals, reagents, standard solutions and origin of urines

All chemicals used were of analytical or research grade. Nicotinic acid, sodium GHB, sodium 1-octanesulfonic acid (OSA) and Triton X-100 were purchased from Fluka (Buchs, Switzerland). L-Histidine, spermine tetrahydrochloride and hydroxypropylmethyl cellulose (product H-7509) were obtained from Sigma (St Louis, MO, USA). Ethyl acetate, benzoic acid and $Na_2HPO_4 \cdot H_2O$ were from Merck (Darmstadt, Germany). Water prepared with the Purelab Option-E apparatus (ELGA Vivendi Water Systems, High Wycombe, UK) was employed. Stock solutions of sodium GHB and sodium OSA were prepared by dissolving 10 mg of each salt in 10 ml of water. They were kept at 4 °C and standards were prepared freshly every day. Urines analyzed stemmed from one GHB-naive volunteer who gave his informed consent and participated in a controlled study conducted after the approval of the local ethics committee for clinical research. A dose of 25 mg sodium GHB per kg body mass was administered and urine samples were collected before and 30, 60, 120, 240, 360, 720, and 1440 min after drug administration. Our own urines were employed as blank urines and all urines were stored at −20 °C until analysis. The preparation of the calibrator urines is discussed in Results and discussion. All GHB and OSA concentrations given in this paper refer to the concentrations of their sodium salts.

2.2. Sample preparation

All urines were filtered prior to analysis using disposable 0.22 μm Millex-GS syringe filters (Millipore, Molsheim, France). For extraction, urine samples (0.1 ml, plain or diluted urine) were placed in 5 ml glass tubes (Schott GL 14) and spiked with 25 μl of an aqueous solution containing 1 mg/ml sodium OSA (internal standard). Then, 500 μl of a saturated aqueous solution of $NaH_2PO_4 \cdot H_2O$ and 4 ml of ethyl acetate were added. After shaking with a horizontal shaker (5 min) and centrifugation at about 1500 *g* (3000 rpm) for 3 min, the organic phase (upper phase) was transferred into a new glass vial and evaporated in a water bath under a gentle stream of air at about 40 °C. The residue was redissolved in 100 μl of water (for CE analysis) or in 100 μl of a mixture of methanol–water (80:20, v/v) containing about 0.1% of concentrated ammonia solution (for ESI–MS analysis).

2.3. Capillary electrophoresis

The experiments were performed on the Beckman P/ACE System 5510 (Beckman, Fullerton, CA, USA). A capillary of 40 cm length to the detector (47 cm total length) with an I.D. of 50 μm (Polymicro Technologies, Phoenix, USA) was mounted into the cartridge (Beckman). The capillary temperature was maintained at 25 °C and the sample carousel was at ambient temperature. Solute detection was performed via indirect UV absorption at 214 nm with the reversed absorbance signal being stored. Samples were introduced by hydrodynamic injection for 6 s with 0.5 p.s.i., followed by injection (1 s, 0.5 p.s.i.) of a plug of running buffer (1 p.s.i.=6894.76 Pa). All operations were computer controlled using the Beckman P/ACE station software (version 1.1). If not stated otherwise, the running buffer consisted of an aqueous solution of 4 m*M* nicotinic acid and 3 m*M* spermine, the pH value was adjusted to 6.2 by adding histidine powder. The voltage applied was −18 kV (current about 12 μA; power level of about 0.46 W/m). Each morning the capillary was rinsed with 0.1 *M* NaOH (10 min), ultrapure water (5 min) and running buffer (5 min). Prior to each run, the capillary was rinsed with running buffer for 2 min. Data evaluation was based upon relative peak heights, i.e. peak heights divided by detection time.

2.4. Electrospray ionization ion-trap mass spectrometry

MS analyses were performed on a Finnigan LCQ ion trap instrument (Finnigan MAT, San Jose, CA, USA) equipped with an ESI interface for liquid chromatography (Finnigan) that was run in the negative ion mode (3.9 kV). Sheath gas (N_2) pressure was set at 40 arbitrary units. Samples were introduced via the syringe inlet using a 500 μl Hamilton syringe and a flow-rate of 5 μl/min. The temperature of the heated capillary was at 200 °C. The instrument was computer controlled using the XCalibur 1.0 software (Finnigan). For each sampling, five mass spectra were acquired in the mass range of 50–250 Th and data evaluation was based upon the mean of the five spectra. Automatic gain control (AGC) was employed using three microscans and a maximum injection time of 200 ms. MS–MS was performed using an isolation width of 1 or 2 and a relative collision energy of 39%. Selected reaction monitoring (SRM) of the m/z 103.1→m/z 85.1 precursor–product ion transition was performed with an isolation width of 1 or 2 and data evaluation was based upon the mean of five readings.

2.5. Determination of urine properties

Osmolality was determined using the Advanced Model 3900 Multi-Sample Osmometer (Advanced Instruments, Norwood, MA, USA) that is based upon the freezing point method. The conductivity was measured with a conductivity meter model 101 (Orion Research, Cambridge, MA, USA) equipped with a model PW 9510/65 cell (Philips, Eindhoven, The Netherlands). The density was determined by weighing a 4.864 ml pycnometer flask on a Model AG 245 analytical balance (Mettler Toledo, Greifensee, Switzerland). Creatinine was determined enzymatically using the ILab 300 Plus Clinical Chemistry System (Instrumentation Laboratory, Milan, Italy) and the creatinine test kit of Wako (Wako Chemicals, Neuss, Germany).

2.6. Computer simulations

The simulator used was the same as described previously [21,22]. The program was executed on a Pentium III 600 MHz computer. The pK_a values of nicotinic acid, GHB, butyric acid and histidine were 4.82, 4.72, 4.81 and 6.04, respectively [23]. The mobilities used were 3.46, 3.20, 3.39 and 2.02×10^{-8} $m^2\ V^{-1}\ s^{-1}$, respectively. The mobility of Na^+ was 5.19×10^{-8} $m^2\ V^{-1}\ s^{-1}$. The simulation was performed with a 15 cm separation space divided into 6667 segments of equal length and an applied constant voltage of 1800 V (initial current density: 238.4 A/m^2; current density after 10 min: 241.5 A/m^2). The buffer was composed of 4 m*M* nicotinic acid and 9.5 m*M* histidine (pH 6.21, conductivity 0.02036 S/m). The sample comprised 30 μ*M* sodium GHB, 100 μ*M* sodium butyrate and 10-fold diluted buffer and was placed at the anodic column end (sample plug length: 1.5% of column length). The electroosmotic flow (towards the cathode) was taken as a constant 676.8 μm/s.

3. Results and discussion

3.1. UV absorbing coanion, dynamic column conditioning and detection wavelength

The choice of possible background electrolytes was carried out according to the requirement that the analyte of interest and the coionic substance responsible for indirect UV absorption detection should have a similar electrophoretic mobility. Based upon the work of Foret et al. [24], the first substance that was tested was benzoic acid (concentration range of 2.5 to 20 m*M*), buffered with histidine to pH 6.2, and having 0.1% (v/v) Triton X-100 or 0.05 to 0.5% (w/v) hydroxypropylmethylcellulose as flow modifiers. Using 50 and 75 μm I.D. capillaries and negative polarity, these approaches did not lead to reproducible results. The same was found to be true using 5 m*M* nicotinic acid, a compound that dissolves more easily in water than benzoic acid. Column conditioning, i.e. incompletely controlled suppression of the electroosmotic flow (EOF), was identified as the reason for malfunction. Without the two flow modifiers and positive polarity, however, expected electropherograms and acceptable reproducibility were obtained. This is illustrated with the data presented in Fig. 1A that were obtained employing a 50 μm I.D. capillary of 47 cm total length and having a buffer containing 4 m*M* nicotinic acid and histidine (pH 6.2). For that configuration with the detector being placed at 85% of column length, Na^+, electroosmotic flow (marked with EO), GHB and butyrate were detected after 1.80, 3.15, 6.38 and 6.58 min, respectively. The electroosmotic flow was calculated to be 5.64×10^{-8} $m^2\ V^{-1}\ s^{-1}$. All detected peaks exhibit a lower absorbance than the buffer. The signal, however, was reversed as is customary in CZE with indirect detection.

Simulation data provide insight into the composition of the detector signals in CZE with indirect detection. For the simulation presented here, a constant electroosmotic flow (676.8 μm/s) that is based upon the experimentally determined flow value was employed instead of the in situ calculated electroosmotic flow that is a function of ionic strength and temperature and thus a challenge to quantitatively match with experiments (predicted EOF of 719.6 μm/s for 35 °C which corresponds to a mobility of 6.0×10^{-8} $m^2\ V^{-1}\ s^{-1}$, for model refer to Ref. [22]). Furthermore, the electric field applied was 120 V/cm, a value that is 3.19-fold lower compared to that employed in the experiment. As the capillary length is shorter by about the same factor, the time scales of simulation and experimental data should and were indeed found to be comparable. A graph representing the simulated detector response at 85% of column length is presented in Fig. 1B. The graph represents the sum of the nicotinic acid concentration and 69% of the histidine concentration. The unequal composite is based on the fact that the absorbance of histidine is about 31% lower compared to that of nicotinic acid (assessed with 0.125 m*M* solutions at 214 nm). The thereby constructed computer predicted electropherogram (Fig. 1B) was found to compare well with the electropherogram monitored experimentally (Fig. 1A). The simulation data further reveal that the detector signal for the anionic part is almost entirely based upon the changes of nicotinic acid (Figs. 1C and D). For the cationic part, however, the histidine change is strongly contributing and is even overcompensating the

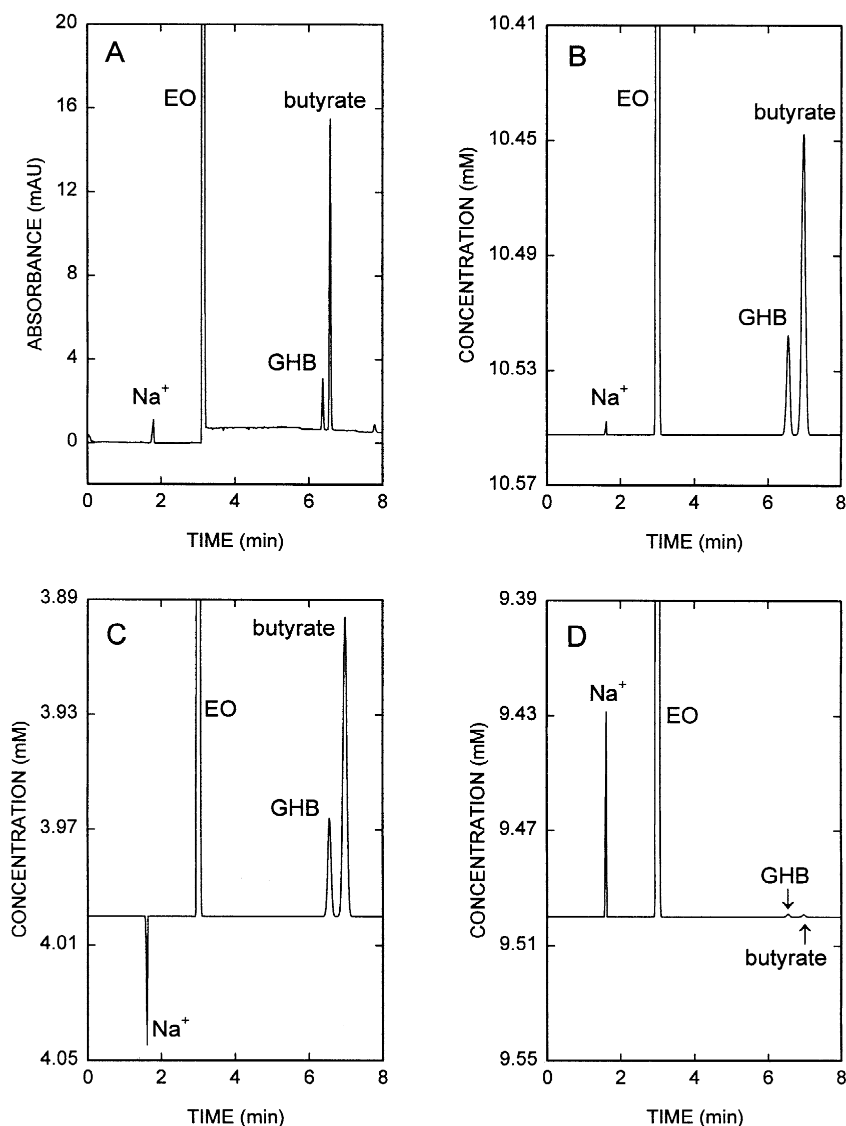

Fig. 1. Comparison of (A) experimental and (B–D) computer predicted electropherograms. Panel A depicts the electropherogram obtained with a sample containing 10 μg/ml sodium GHB and 50 μg/ml sodium butyrate in a buffer composed of 4 m*M* nicotinic acid and histidine (pH 6.2). The applied voltage was 18 kV and the current was 2.2 μA. Sample was injected for 6 s at 0.5 p.s.i. and detection was effected at 214 nm. Panels B–D represent simulated detector responses based upon (B) a 1.00:0.69 composite of the nicotinic acid and histidine signals, (C) the nicotinic acid change and (D) the histidine change. For the simulation conditions refer to text and Section 2.6. EO refers to the initial sample fluid element that is transported across the point of detection by electroosmosis.

positive nicotinic acid change (compare Na^+ signals of Figs. 1C and D).

The oligoamine spermine [*N*,*N*′-bis(3-aminopropyl)1,4-butanediamine] is not only known to be an effective quencher of protein adsorption to the capillary walls [25], but also to strongly reduce or reverse EOF. Addition of 3 m*M* spermine to the nicotinic acid buffer (pH 6.2) was found to reverse EOF and thereby permitted the direct anionic determination of GHB via application of reversed polarity (Fig. 2). Although the EOF was found to vary from capillary to capillary and also with

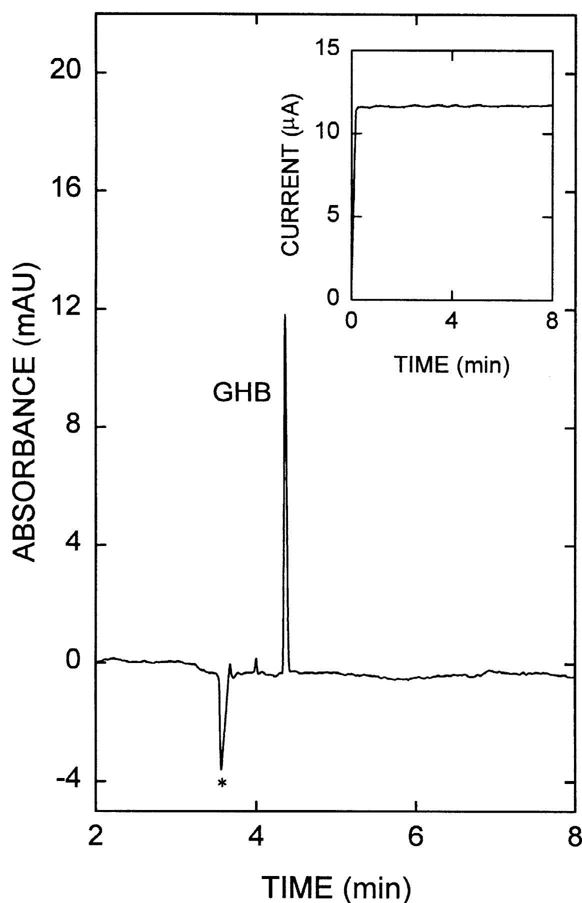

Fig. 2. Analysis of a sample containing 100 μg/ml sodium GHB using a buffer composed of 4 m*M* nicotinic acid, 3 m*M* spermine and histidine (pH 6.2). The applied voltage was −18 kV and the current was 11.7 μA (see insert). Sample was injected for 6 s at 0.5 p.s.i. and detection was effected at 214 nm. The asterisk marks a peak of unknown origin.

prolonged use of a capillary (EOF mobility ranged between $-0.77\cdot10^{-8}$ and $-2.13\cdot10^{-8}$ m^2 V^{-1} s^{-1}), this configuration was found to be robust and reproducible. For the detection time of GHB applied as standard and in liquid–liquid extracts of diluted urines (see below), intra-day RSD values were determined to be <1% ($n=5$).

Detection wavelengths of 200, 214, 232 and 254 nm were evaluated. For overall sensitivity, 200 nm was found to be the best choice. For urine extracts, however, GHB was better recognized using the 214 nm filter. No advantages were noted at 232 nm, whereas high selectivity but insufficient sensitivity was obtained with the 254 nm filter. Thus, all determinations were made at 214 nm.

3.2. Sample preparation, internal standard and running buffer

Using liquid–liquid extraction with ethyl acetate and a saturated salt solution, a procedure that is similar to that reported in Ref. [14], GHB was determined to ineffectively transfer into the ethyl acetate phase. The use of saturated NH_4Cl as suggested by Elian [14] resulted in a poor recovery whereas saturated NaH_2PO_4 (500 μl added to 100 μl urine) was determined to provide reasonable CZE

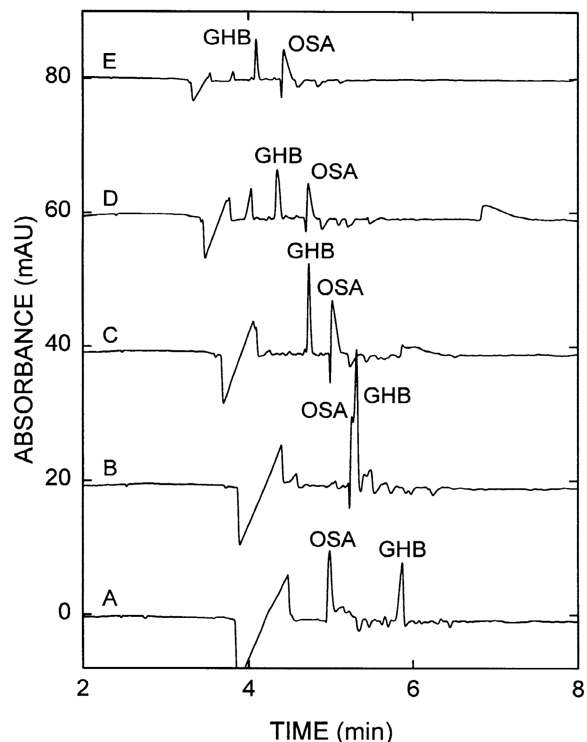

Fig. 3. Electropherograms obtained after liquid–liquid extraction of undiluted blank urine "RT" that was fortified with 100 μg/ml sodium GHB and 250 μg/ml sodium OSA and using spermine (3 m*M*) containing nicotinic acid buffers at (A) pH 4.7, (B) pH 5.2, (C) pH 5.7, (D) pH 6.2 and (E) pH 6.7. The applied voltage was −18 kV in all cases and the currents were around 12 μA. For the sake of presentation, successive electropherograms were plotted with a *y*-axis shift of 20 mAU.

data with indirect detection (Fig. 3). For 40 μg/ml sodium GHB in diluted urine (see below), the mean recovery was determined to be 52.7% (n=5, RSD 15.6%). Sample preparation with solid-phase extraction using disposable Clean Screen® ZSGHB020 (United Chemical Technologies, Bristol, PA, USA) as were employed in Ref. [4], SAX Isolute (International Sorbent Technology IST, Hengoed, UK) and HAX Isolute (IST) cartridges did not provide promising results. For blank urines, the baselines were not as clean as those obtained after liquid–liquid extraction (data not shown).

Experiments performed using a running buffer comprising 4 mM nicotinic acid and histidine (pH 6.2) indicated that butyric acid could be a suitable internal standard (Fig. 1A). Unfortunately, butyric acid could not be extracted by liquid–liquid extraction as described above. Experiments performed with various weak acids revealed the necessity of an additional hydroxylic group. However, some compounds tested did not provide a suitable peak (including glycolic acid and thioglycolic acid which had a poor recovery) or were found to be unsuitable due to their presence in urine (such as lactic acid which was found to extract well) and others could not be separated from GHB (including 3-hydroxybutyric acid). Alternatively, OSA was found to be extractable, to separate well from GHB, to form an acceptable peak and was thus employed as internal standard (Fig. 3).

To find the best running buffer, blank urine was fortified with 100 μg/ml of sodium GHB and 250 μg/ml of sodium OSA, extracted as described in Section 2.2 and analyzed at a range of pH values using 4 mM nicotinic acid buffers that contained 3 mM spermine and were titrated to the desired pH with histidine. Buffers with pH values ranging from 4.2 to 6.7 (0.5 pH interval) were employed and, except for the lowest pH value, electropherograms are presented in Fig. 3. Both, peak position and magnitude were determined to change as function of pH. For both analytes, sensitivity was found to be best around pH 5.2, a pH value that did not permit the complete separation of the two compounds. The separation of GHB and OSA increased as the buffer pH was increased, with GHB being detected ahead of OSA. At a pH lower than 5.2, GHB was detected behind OSA. This is in agreement with the fact that the mobility of GHB is strongly pH dependent (pK_a=4.72). OSA is fully dissociated within the entire pH range and its electrophoretic mobility is therefore constant. With the buffers below pH 5.2, OSA was found to comigrate with endogenous compounds (Fig. 3), whereas at higher pH values, the internal standard was essentially squeezed in between two "negative" peaks and comigrated with a small "positive" peak (Figs. 3 and 4). Least interferences between the two substances of interest, GHB and OSA, and endogenous compounds were

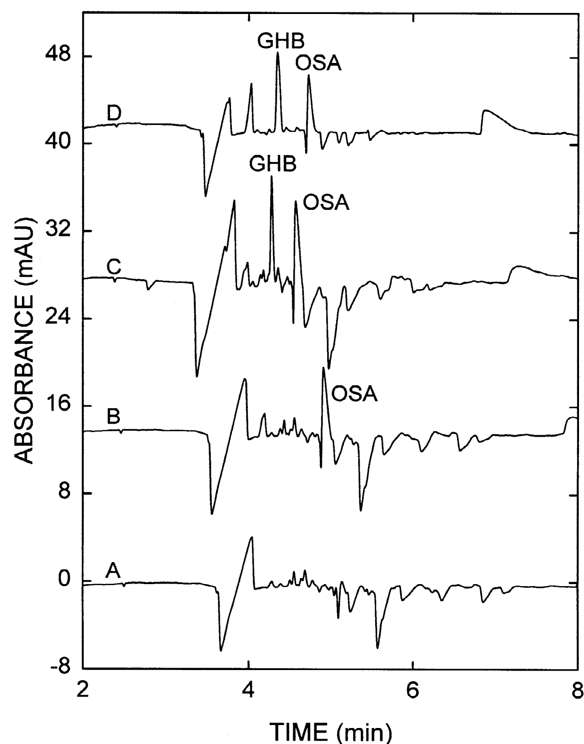

Fig. 4. Electropherograms obtained after liquid–liquid extraction of (A) undiluted blank urine "AB", (B) undiluted blank urine "AB" that was fortified with 250 μg/ml sodium OSA, (C) undiluted blank urine "AB" that was fortified with 100 μg/ml sodium GHB and 250 μg/ml sodium OSA and (D) undiluted blank urine "RT" that was fortified with 100 μg/ml sodium GHB and 250 μg/ml sodium OSA. The spermine (3 mM) containing nicotinic acid buffer at pH 6.2 was employed. The applied voltage was −18 kV in all cases and the currents were around 12 μA. Electropherograms are presented with a y-axis shift of 14 mAU.

observed for a buffer pH around 6.2. Thus, the pH 6.2 buffer was employed for all further studies.

3.3. Urine dilution, assay validation, analysis of volunteer urines and direct urine injection

The data presented in Figs. 3 and 4 were generated with different blank urines. Although the overall patterns monitored are comparable, the data indicate that the blank urine of volunteer "AB" (Fig. 4, panels A–C) was more concentrated than urine of subject "RT" (Fig. 3; Fig. 4, compare panels C and D). With increased urine concentration, increased pattern complexity and thus decreased sensitivity for GHB were noted. Analysis of the 60 min volunteer sample revealed that this urine was considerably less concentrated than the urine of the same person collected prior to GHB intake (Table 1). Thus, the blank urine "AB" was reanalyzed at different dilutions. With a 12-fold dilution (Fig. 5A), background data similar to those obtained with the 60 min urine (Fig. 5C) were obtained. At this dilution, interferences comigrating with GHB became hardly detectable, and the interfering peaks around OSA were also significantly smaller than those observed in the data generated with the undiluted urine (Fig. 4). The endogenous peak marked with an asterisk in the electropherograms of Fig. 5 was employed as a ruler to match the impact of the urinary matrix. Moreover, the state of dilution can simply be assessed via osmolality, conductometry and the creatinine concentration (Table 1). Good CZE data were obtained

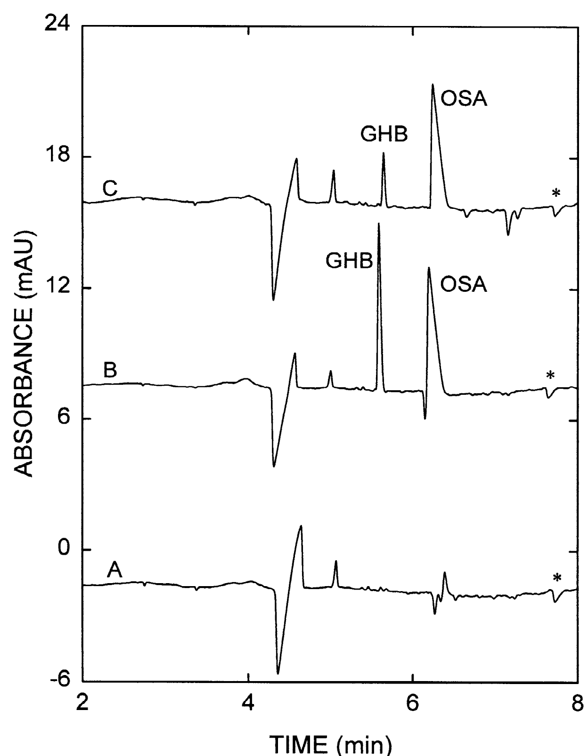

Fig. 5. Electropherograms obtained after liquid–liquid extraction of (A) 12-fold diluted blank urine "AB", (B) 12-fold diluted blank urine that was fortified with 100 μg/ml sodium GHB and 250 μg/ml sodium OSA, and (C) undiluted 60 min volunteer urine that was fortified with OSA. The spermine (3 m*M*) containing nicotinic acid buffer at pH 6.2 was used. The applied voltage was −18 kV in all cases and the currents were around 12 μA. Electropherograms are presented with a *y*-axis shift of 9 mAU. The asterisks mark the peak of an endogenous compound that was used to match urine dilution.

Table 1
Properties of study urines "AB"

Urine collection (min)	Osmolality (mOsm/kg H_2O)	Conductivity (mS/cm)	Density (g/ml)	Creatinine (m*M*)
0	956	28.90	1.022	16.60
30	678	19.60	1.008	11.40
60	95	2.37	0.995	1.40
120	67	1.92	0.993	0.86
240	164	5.44	1.002	2.60
360	340	10.20	1.006	6.00
720	525	15.30	1.012	11.60
1440	671	16.00	1.016	18.30

with urines having an osmolality ≤100 mOsm/kg H_2O, a conductivity of ≤2.5 mS/cm and a creatinine concentration of ≤1.5 m*M* (Table 1, 60 and 120 min urines). The osmolality, conductivity and creatinine values of the 60 min urine specimen were indeed noted to be about 12-fold lower compared to those obtained for the blank urine ''AB'' (0 min urine of Table 1). Due to the small differences, the use of the density as a ruler for urine dilution is not convenient.

The CZE assay for GHB with indirect detection was validated employing the 12-fold diluted blank urine ''AB'' that was fortified with GHB and OSA. The assay was found to have a GHB detection limit (LOD) of 2 μg/ml whereas the limit of quantitation (LOQ) was taken as 5 μg/ml. For calibration and assessing repeatability, 100 μl of 12-fold diluted blank urine was spiked with 25 μl of the 1 mg/ml sodium OSA solution and with an amount of the GHB stock solution providing sodium GHB concentrations of 100, 50, 20, 10, and 5 μg/ml. These samples were extracted and analyzed as described in Sections 2.2 and 2.3, respectively, and data were evaluated based upon the ratio of the relative peak heights of GHB and OSA. The assay was found to be reproducible (Tables 2 and 3) and characterized by a linear response (Table 3). Analysis of aqueous standard solutions covering the 5–100 μg/ml sodium GHB concentration range was also found to provide a linear relationship ($y=0.017x+0.088$, $r=0.9974$). The LOD was determined to be 1 μg/ml. Thus, this assay without extraction could also be employed for analysis of seized GHB solutions and powders.

Table 3
Calibration data ($n=5$)[a]

	Mean	RSD (%)	Range
Slope	0.0132	10.96	0.0114–0.0151
y-Intercept	0.0447	40.57	0.0138–0.0592
r	0.9975	0.29	0.9939–0.9996

[a] Obtained with 12-fold diluted blank urine ''AB'' that was fortified with sodium GHB (range: 5–100 μg/ml) and OSA. Concentrations and relative peak height ratios were taken as x-axis and y-axis, respectively.

The urines collected 30, 60, 120, 240, 360, 720 and 1440 min after GHB intake were first analyzed without dilution. The 30 min urine was found to be very concentrated (Table 1, electropherogram not shown) and its GHB content could not be determined. The 60 min urine revealed a nice GHB peak (Fig. 5C) that was verified by spiking the extract and reanalysis (data not shown). The GHB concentration in that urine was determined to be 30.3 μg/ml (Table 2). The electropherogram obtained with the 120 min urine showed a small GHB peak whose size was comparable to that obtained at LOQ (data not shown). A peak at LOD was detected for the 240 min urine and no GHB peaks were monitored for the urines collected 360, 720, 1440 min after drug intake. Furthermore, 6-fold and 12-fold dilutions of the 30 min urine prior to extraction did not reveal a GHB peak as well. With the data of Table 1 and

Table 2
Typical reproducibility data for GHB ($n=5$)

Sample	GHB conc. (μg/ml)	Inter-day (analysis on different days)		Intra-day (same day analysis)	
		Mean of rel. peak height ratio	RSD (%)	Mean of rel. peak height ratio	RSD (%)
Spiked blank urine[a]	100	1.347	10.06	1.310	2.55
Spiked blank urine[a]	50	0.762	8.85	–	–
Spiked blank urine[a]	20	0.305	8.33	0.264	5.67
Spiked blank urine[a]	10	0.173	7.32	0.225	11.90
Spiked blank urine[a]	5	0.098	12.16	–	–
Volunteer urine[b]	30.3	0.440	14.93	0.407	5.51

[a] Twelve-fold diluted blank urine AB that was fortified with GHB and OSA.
[b] Urine collected 60 min after GHB intake. The concentration value given represents the inter-day mean ($n=5$) determined by CZE (for calibration data see Table 3). The corresponding intra-day value was determined to be 30.9 μg/ml.

comparison to the 60 min urine, the GHB content of this urine can be assumed to be <16 μg/ml (see below).

For completeness, the possibility of analyzing GHB via direct urine injection was also investigated (Fig. 6). Again, concentrated urines such as the blank urine "AB" (Fig. 4) and the 30 min volunteer urine were found to provide overloaded electropherograms (data not shown). Upon dilution with water, however, reasonable data were obtained (Figs. 6A and B). GHB could be monitored in the 60 min study urine (Figs. 6C and D) without dilution. Comparison of the electropherograms depicted in Fig. 6 with those of Fig. 5 reveals that extraction is clearly simplifying the sample matrix. Despite that GHB recovery after liquid–liquid extraction is about 50% only, direct urine injection was not found to provide improved sensitivity (compare electropherograms Fig. 5C and Fig. 6C). Thus, no assay validation with direct urine injection was performed. For analysis of the 30 min study urine, GHB could not be detected after injection of undiluted, 6-fold diluted and 12-fold diluted urine. Nevertheless, injection of plain or diluted urine could be an interesting and rapid approach to recognize the large GHB concentrations often found in forensic case work (>100 μg/ml [4,9,10]).

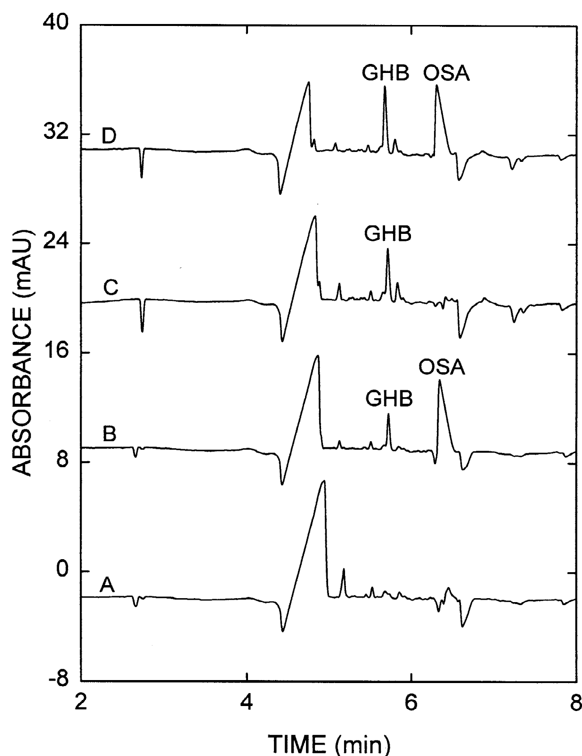

Fig. 6. Electropherograms obtained after injection of (A) 12-fold diluted blank urine "AB", (B) 12-fold diluted blank urine fortified with GHB and OSA (about 15 and 185 μg/ml, respectively), (C) undiluted 60 min volunteer urine and (D) the 60 min volunteer urine fortified with GHB and OSA (about 15 and 185 μg/ml, respectively). Electropherograms are presented with a *y*-axis shift of 11 mAU. Other conditions as for Fig. 5.

3.4. Confirmation with electrospray ionization ion-trap mass spectrometry

As was previously discussed for the analysis of urinary opioids [26], the LCQ ESI ion-trap MS was also applied for the confirmation of urinary GHB. Samples were introduced via the syringe of the infusion pump into the liquid chromatography ESI interface of the instrument. Application of an aqueous standard solution containing 10 μg/ml sodium GHB revealed a parent ion with m/z of 103.4 (inset in panel A of Fig. 7). Isolation of this ion and fragmentation led to the MS–MS mass spectrum with an m/z 85.1 ion (data not shown). Direct urine injection is not possible as the high salt content would tremendously pollute the source and the heated capillary. Introduction of our extracts, however, was found to lead to interesting data. With infusion of an extract prepared from the 12-fold diluted blank urine "AB", endogenous GHB could not be detected (Fig. 7A and B). However, with an extract prepared from a 12-fold diluted blank urine "AB" that was fortified with 20 μg/ml sodium GHB, the presence of GHB could unambiguously be recognized in the mass spectrum (Fig. 7C) and confirmed by MS–MS (data not shown) and selected reaction monitoring of the m/z 103.1→m/z 85.1 precursor–product ion transition (Fig. 7D). The same was found to be true for a urinary drug level of 5 μg/ml. Signal magnitudes, however, for that sample were quite low, indicating that 5 μg/ml is close to the detection limit. Not surprisingly, GHB could be unambiguously detected in the extract of the 60 min volunteer urine (Fig. 7E and F). These data confirm the validity of the CZE assay with indirect detection. Furthermore, analysis of an extract prepared from the undiluted 30 min urine also

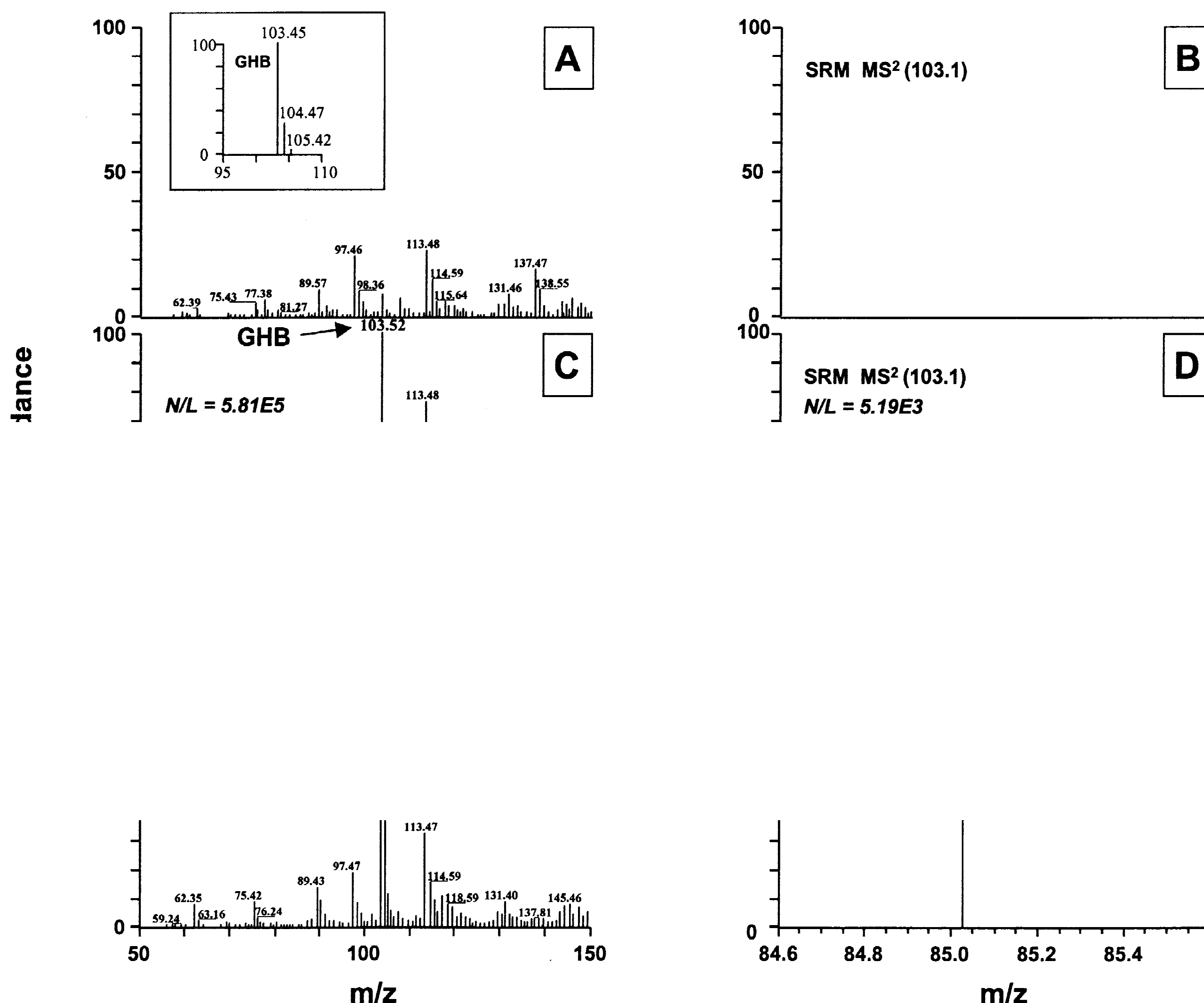

Fig. 7. (A,C,E) Mass spectra and (B,D,F) product ion mass spectra (SRM data for the m/z 103.1→m/z 85.1 precursor–product ion transition) of extracts prepared from (A,B) 12-fold diluted blank urine "AB", (C,D) 12-fold diluted blank urine that was fortified with 20 μg/ml sodium GHB and (E,F) 60 min volunteer urine. The inset in panel A depicts the mass spectrum of a standard containing 10 μg/ml sodium GHB.

revealed the presence of GHB, suggesting a GHB content >5 μg/ml. The GHB concentration in that urine is thus believed to be between 5 and 16 μg/ml (see above). ESI–MS appears to provide the better selectivity and thus better sensitivity than CZE with indirect detection.

4. Conclusions

The data presented in this paper demonstrate the successful use of CZE for the recognition and determination of urinary GHB. The developed assay is based upon a liquid–liquid extraction procedure that was found to have a recovery for GHB of about 50% followed by CZE analysis of the reconstituted extract with indirect UV absorption detection at 214 nm. LOD and LOQ were noted to be dependent on urine concentration. LOD and LOQ for a fortified diluted urine with a conductivity of 2.4 mS/cm were determined to be 2 and 5 μg/ml, respectively. For more concentrated urines which have to be diluted for proper analysis of GHB, corresponding values are higher (up to 24 and 60 μg/ml, respectively, for

the urines of Table 1). Alternatively, the data suggest that LOD and LOQ (both in μg/ml) can be estimated with the relationships 0.83 κ and 2.1 κ, respectively, where κ is the conductivity of the urine in mS/cm. Similar relationships could be constructed employing the osmolality or the creatinine concentration instead of the conductivity. The sensitivity based upon LOQ meets the required cut-off level of 10 μg/ml for diluted urines with $\kappa \leq 4.8$ mS/cm only. To increase the assay's sensitivity, a more selective and more efficient extraction procedure will have to be developed. Nevertheless, the assay could successfully be applied to some of the urines collected after administration of 25 mg sodium GHB/kg body mass. Furthermore, negative electrospray ionization ion-trap tandem mass spectrometry was used to confirm the presence of GHB in the urinary extracts via selected reaction monitoring of the m/z 103.1→m/z 85.1 precursor–product ion transition. This approach appears to provide better selectivity and thus better sensitivity (including the required cut-off level of 10 μg/ml) than CZE with indirect detection. Analysis of untreated urines by CZE was noted to provide a somewhat lower sensitivity than after extraction and could be an interesting approach for rapidly detecting urinary GHB at concentrations (in μg/ml) that are ≥ 4 κ (i.e. >100 μg/ml for the concentrated urines of Table 1). The CZE-based assay cannot be employed for the monitoring of GBL. An MEKC configuration would have to be used.

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