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Rapid and direct analysis of γ-hydroxybutyric acid in urine by capillary electrophoresis–electrospray ionization ion-trap mass spectrometry

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Abstract

The present work was aimed at the development of a capillary electrophoretic analysis of γ-hydroxybutyric acid (GHB) using electrospray ion trap mass spectrometry to achieve the direct and unequivocal detection of this analyte in human urine. Optimized capillary electrophoretic conditions were: injection, 20 s at 0.5 psi (1 psi = 6894.76 Pa); buffer electrolyte, 12.5 mM ammonium formate adjusted to pH 8.35 with diethylamine; fused silica capillary: 100 cm × 50 μm i.d.; separation voltage, 25 kV (forward polarity) + 0.5 psi; room temperature. Electrospray and mass spectrometric conditions were: drying gas and nebulizing gas (nitrogen) at flow rate 3 l/min, temperature 250 °C, nebulizer pressure: 10 psi; sheath liquid solution: methanol–water (90:10) containing 0.1% ammonia delivered at 3 μl/min; spray voltage 3.5 kV. Mass spetrometric detection was carried out in the selected ion monitoring mode of negative molecular ions at 103 *m/z* for GHB and 115 *m/z* for maleic acid (I.S.). Under these conditions the baseline separation of GHB and the I.S. was obtained. The selectivity of the analysis allowed for direct injection of unextracted urine, previously diluted 1:4 with water. Linearity was assessed in the GHB concentration range from 80 to 1280 μg/ml in urine. Analytical sensitivity (as limit of detection) resulted about 5 μg/ml in water and 20 μg/ml in original urine. Analytical precision was fairly acceptable with R.S.D. values lower than 5% for migration times and 18% for quantitation in real samples, in both intra day and day-to-day experiments. On these grounds, the developed method can be adopted for rapid identification of acute intoxications from GHB in humans.

Keyword: γ-Hydroxybutyric acid

1. Introduction

γ-Hydroxybutyric acid (GHB) was synthesized in 1960 with the aim of its therapeutic use as a γ-aminobutyric acid (GABA) analogue. However, because of its moderate pharmacological potency and lack of specificity, the therapeutic use of GHB has been limited to the treatment of narcolepsy and to the pharmacological control of the alcohol withdrawal syndrome. In addition to an interaction with GABA receptors, GHB has also been found to affect dopaminergic and colinergic transmission and growth hormone secretion [1]. Moreover, an endogenous production of GHB has been reported, leading to detectable concentrations of this compound in plasma and urine in the low microgram per milliliter range [2,3].

In recent times, a recreational use of GHB, because of its inducing effects on euphoria, sedation and disinhibition, has spread in many countries [4,5]. GHB has also been reported in drug facilitated sexual assaults [6]. Finally, on the basis an alleged GHB-mediated increase of the growth hormone secretion, GHB has been used as a doping agent to enhance muscle growth [7].

On this basis, in the European Union (EU), following EU Council conclusions adopted on 15 March 2001, GHB has been listed under schedule IV of the 1971 UN Convention on psychotropic substances [8]. Nevertheless, GHB and its analogues [γ-butyrolactone (GBL) and 1,4-butanediol (1,4-BD)] are widely publicized through the Internet and easily available in the illicit market in tablets/capsules, powder or liquid forms.

Following unadverted or intentional intake of GHB or its congeners, numerous Emergency Room reports have pointed

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out acute GHB intoxications with seizures, coma, respiratory and cardiovascular depression and, possibly, death [1,9–12]. In fatal cases, blood concentrations of GHB up to 837 μg/ml and urine concentrations up to 5430 μg/ml have been reported [13].

In solution, GHB is in equilibrium with its lactone, GBL, in dependence of the pH of the medium (the lactone form predominates at pH values < 4.7). After ingestion, 1,4-BD is enzymatically converted to the corresponding acid (GHB) by alcohol dehydrogenase and aldehyde dehydrogenase. Consequently, after intake of GHB, GBL or 1-4 BD, the major compound present in biological fluid is in any case GHB, which exerts the biological activity, with a minor percentage of GBL and 1-4BD [13].

GHB is rapidly metabolized by GHB dehydrogenase (half life ranges from 20 to 60 min); only about 5% of the ingested dose is eliminated unchanged in urine, which is the favorite biological specimen for GHB determination [10].

The analytical determination of GHB and analogues in biological fluids is mainly based on gas chromatography (GC) with flame ionization detection (FID) and on gas chromatography–mass spectrometry (GC–MS). However, because of its nature of polar molecule/anion and its thermal instability, GHB is not directly suitable to CG. Consequently, some authors apply a conversion of GHB to GBL [14,15] in strong acids before injection. However, most of authors using GC–MS apply silylation [bis(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane (BSTFA/TMCS)] of the hydroxy and carboxy groups of GHB [16–18].

Reported extraction methods include liquid–liquid extraction of GBL (after chemical conversion of GHB in sulfuric acid) with organic solvents [15,17] and solid-phase extraction of GHB [16,19,20]. Also, solid-phase microextraction (SPME), after GHB conversion to GBL or hexylchloroformate derivatization, has been used in association with GC–MS [21,22]. More recently, Villain et al. reported a ultra rapid sample pretreatment based on blood or urine deproteinization with acetonitrile followed by evaporation of the supernatant under nitrogen stream, BSTFA/TMCS derivatization and GC–MS analysis [23].

Quite surprisingly, in recent literature high-performance liquid chromatography (HPLC) has been reported only by deVriendt et al. [20] who used reversed-phase separation on a C-18 column with UV detection at 220 nm. Unfortunately, after solid-phase extraction on a strong anion exchanger of rat plasma, the HPLC separation generated very complex chromatograms in which the GHB peak eluted in a crowd of matrix related peaks, thus showing a poor analytical selectivity.

The first method reporting the use of capillary electrophoresis (CE) was based on micellar electrokinetic capillary chromatography (MECC) with indirect UV detection and was applied to the analysis of GHB, GBL and 1-4 BD only in clandestine preparations [24]. Capillary zone electrophoresis (CZE), also with indirect UV detection, was recently used by Baldacci et al. [25] and by Bortolotti et al. [26] for the quantitative determination of GHB in urine and serum and urine, respectively. CZE with indirect detection did not require sample pretreatment, but just 1:8 dilution with water before analysis.

For confirmation of CZE analyses, in the mentioned paper [25], Baldacci et al. tested preliminarily the application of electrospray ionization ion trap mass spectrometry (ESI-ion trap-MS). This approach was based on the direct infusion of the urine sample extracts into the ESI interface, without a preliminary separation step.

In view of the advantages of the coupling of capillary electrophoresis with mass spectrometry, the aim of the present work was to test this hyphenation for rapid and selective determination of GHB in untreated human urine at potentially toxic concentrations.

2. Materials and methods

2.1. Standards and chemicals

Standards of GHB and maleic acid (used as internal standard, I.S.) were obtained from Sigma (St. Louis, MO, USA) (in an early stage of method development, small amounts of GHB were kindly donated by Dr. G. Frison, University of Padua). Water, methanol and chemicals (diethylamine, ammonium formate, ammonia) used for the preparation of CZE buffers and of the solution for the ESI sheath liquid were of HPLC or analytical grade and were purchased from Carlo Erba (Milan, Italy). The electrophoretic electrolyte solution was composed of 12.5 mM ammonium formate, adjusted to pH 8.35 with diethylamine; before use it was filtered through 0.45 μm cellulose membranes and degassed under vacuum (water pump).

2.2. Instrumentation and analytical conditions

A P/ACE 5500 automated capillary electropherograph (Beckman, Fullerton, CA, USA) equipped with a UV absorbance detector (not used in the present work, because of the poor UV absorbance of GHB) was used throughout the present study. Untreated fused-silica capillaries (50 μm i.d., 100 cm total length, Beckman) were used, directly connected to the ESI needle at their cathodic end. For this purpose, an external detector adaptor (Beckman Coulter) was used in combination with the standard capillary cartridge. The CE instrument was placed on a platform that was adjustable in height and position to avoid siphoning effects. The used CZE conditions were: injection, 20 s at 0.5 psi; buffer electrolyte, 12.5 mM ammonium formate adjusted to pH 8.35 with diethylamine; separation, 25 kV (forward polarity) + 0.5 psi; temperature, 20 °C (because of steric problems, only 20 cm of the capillary were thermostated, whereas the remaining part of the capillary was exposed to the room temperature).

The capillary electropherograph was interfaced with a MSD ESI-ion trap mass spectrometer, model SL, from Agilent Technologies (Palo Alto, CA, USA). The on-line

coupling of the capillary electropherograph with the mass spectrometer was achieved with a commercial coaxial sheath liquid interface (Agilent) which was orthogonally positioned to the MS ion source. Nitrogen was used as both drying gas and nebulizing gas (drying gas flow rate: 3 l/min, drying gas temperature: 250 °C, nebulizer pressure: 10 psi, 1 psi = 6894.76 Pa). A coaxial sheath liquid consisting of a mixture of methanol–water (90:10) added with 0.1% ammonia was delivered at 3 μl/min by syringe pump (KdScientific, Holliston, MA, USA). MS detection was carried out in the selected ion monitoring (SIM) mode of negative molecular ions at 103 *m*/*z* for GHB and 115 *m*/*z* for maleic acid (I.S.). The spray voltage was set at 3.5 kV.

Quantification was carried out on the basis of peak areas by using the internal standard method.

2.3. Sample collection and preparation

Standards of GHB were diluted in water for the preparation of the standard curves at the following concentrations: 20, 40, 80, 160, and 320 μg/ml, in the presence of a fixed concentration of I.S. (100 μg/ml).

Blank urine samples were collected from the authors of the present work and from the laboratory staff and stored in plastic vials frozen at −20 °C until analysis. Blanks were spiked with GHB at concentrations in the range from 80 to 1280 μg/ml and, before injection, diluted 1:4 with water containing a fixed concentration of I.S. (100 μg/ml) to construct standard curves. In addition, real samples from subjects undergoing therapy with GHB (Alcover, CT Laboratorio Farmaceutico, Sanremo, Italy) during alcohol detoxication treatment were analysed.

Sample pretreatment was dilution 1:4 with water containing the I.S. (maleic acid, 100 μg/ml).

3. Results and discussion

CZE, providing the separation of ions in solution on the basis of their mass-to-charge ratio, looks, in principle, the ideal method for the analysis of a small organic acid such as GHB. On the other hand, the poor absorbance of the UV light of GHB hampers the development of sensitive CE methods unless indirect detection is used. Fairly recently CZE with indirect detection has been applied to GHB analysis in biological fluids with encouraging results [24–26]. However, especially for forensic applications, a more specific determination is required in order to offer the possibility of an unambiguous confirmation. To this aim, mass spectrometry is undoubtedly the technique of choice and the possibility of easy coupling with capillary electrophoresis made attractive the development of CE–MS method for GHB determination in biological samples.

Because of the restrictions in the running electrolyte composition for CE–MS, the buffer adopted in the CE–indirect UV method previously developed by our group was substantially changed by using volatile salts (ammonium formate/dietylamine) which precluded indirect UV detection. The choice of a basic pH (8.35), as in the CE method, was necessary for hindering lacton formation from GHB. The resulting electroosmotic flow (EOF), directed toward the detector, was unfortunately insufficient to draw the anionic GHB molecule, having a counter-EOF mobility, to the mass spectrometer in a reasonable time. For this reason a pressure of 0.5 psi was applied at the injection end of the capillary throughout the separation. Under these conditions GHB migrated at about 9 min and the I.S. at about 13 min (a baseline separation was obtained although the discrimination of the two analytes was also possible on the basis of the different *m*/*z*) (Fig. 1). Unfortunately, the separation efficiency was much lower than in the CE–indirect UV method, accounting for about 4-5000 plates/m for both GHB and I.S. This unusually low efficiency for CZE can be ascribed mainly to flaws in our home made CE–MS coupling and namely to excessive capillary length (100 cm), lack of thermostating of 80% of the capillary, addition of pressure to speed up the separation, thus introducing a detrimental laminar flow. The mass detector conditions were optimized in terms of spray voltage value (3.5–6 kV), sheath liquid composition and rate (2–6 μl/min), temperature (150–350 °C) and flow rate (3–6 l/min) of drying gas on the basis of direct infusion experiments of GHB dissolved in the electrolyte buffer. Because of the nature of small organic acid of maleic acid and GHB, negative ion monitoring mode was chosen, selecting molecular ions of GHB and I.S. at *m*/*z* 103 and 115, respectively. An attempt to fragment the molecular ions of GHB gave a product at *m*/*z* 85 originating from GHB by loss of water. However this conversion, which otherwise is poorly specific, reduced the analytical sensitivity to a level not acceptable for our purposes. Because of the

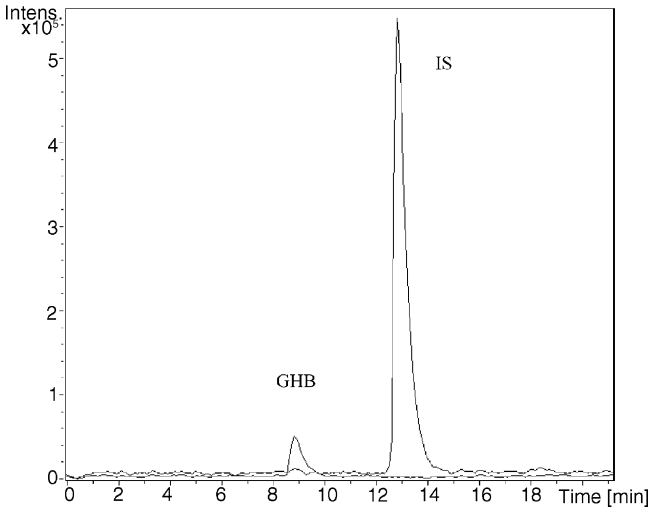

Fig. 1. Superimposed electropherograms of extracted ions of GHB at *m*/*z* 103 (40 μg/ml) and I.S. (maleic acid) at *m*/*z* 115 (100 μg/ml) in water. *Analytical conditions*: electrolyte buffer, 12.5 ammonium formate pH 8.35; separation +25 kV and 0.5 psi, injection 0.5 psi, 20 s; detection SIM in the negative mode; sample treatment 1:4 dilution with water. For details see text.

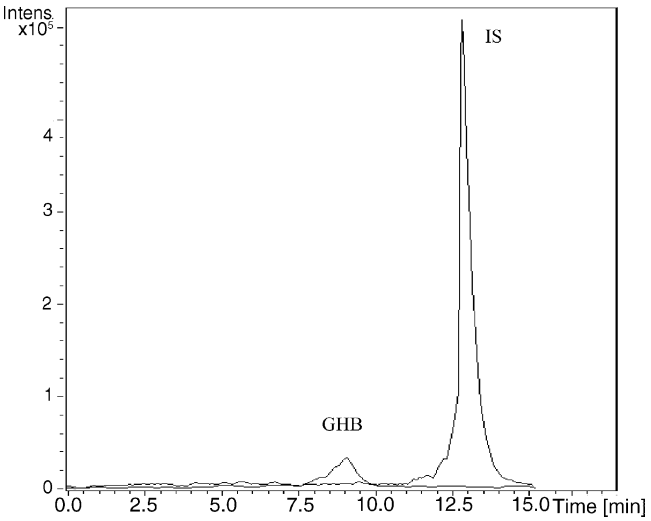

Fig. 2. Superimposed electropherograms of extracted ions of GHB at *m*/*z* 103 (160 μg/ml) and I.S. (maleic acid) at *m*/*z* 115 in spiked urine. Analytical conditions as in Fig. 1.

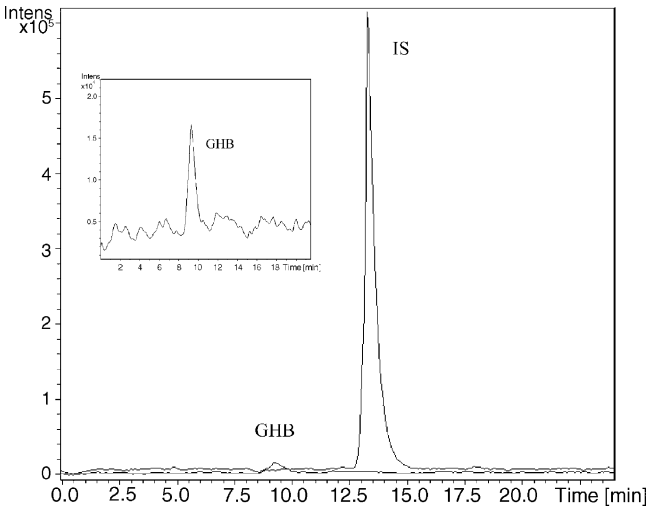

Fig. 3. CE–ESI-ion trap MS analysis of an urine sample from a subject under therapeutic treatment with GHB (Alcover). GHB concentration: 47 μg/ml. Insert: expanded view of electropherogram at the migration time of GHB.

simple structure of the GHB molecule, no other fragments at suitable *m*/*z* were obtained, and consequently the determination was based only on the mass of the molecular ion and on its migration time.

Notwithstanding these limitations, the method proved very selective, showing no interferent peaks in the electropherogram at the selected *m*/*z*, even after injection of unextracted urine (Fig. 2). In addition no interferences were observed from the most common drugs of abuse including: opiates, cocaine and benzoylecgonine, barbiturates, THC and its acid metabolite, benzodiazepines.

The method validation included a study of linearity of response in both water and urine calculated on the correlation between the area ratio of GHB/I.S. and GHB concentrations. The studied concentrations ranged from 20 to 320 μg/ml for water and from 80 to 1280 μg/ml for urine.

The two correlations were described by the following equations: $y = 0.0023x + 0.0036$ ($R^2 = 0.9994$) for pure standard solutions in water and $y = 0.0027x + 0.0103$ ($R^2 = 0.9994$) for spiked urine. The similarity of the regression lines exclude matrix interferences on GHB and I.S. ionization. The intercept on the *y*-axis slightly higher in the standard curve in urine than that in water can be ascribed to the small endogenous GHB concentration (<10 μg/ml) naturally present in human urine.

Analytical sensitivity (as limit of detection) was calculated as the lowest GHB concentration in urine matrix giving a signal-to-noise ratio ≥3. Under these conditions limits of detection (LODs) resulted of about 5 μg/ml in water and 20 μg/ml in original urine. This sensitivity, although limited by the peak spreading related to the poor efficiency, on the basis of literature data, is sufficient for identification of acute intoxication. Unfortunately during the development of the present study, no real intoxication cases came to our observation. However, we had the opportunity to determine GHB in the urine of subjects treated with therapeutical doses of GHB (8.6 g/day), thus supporting indirectly the validity of the assay to detect acute intoxication (Fig. 3).

Analytical reproducibility has been studied by repeating five times injections of water solutions at GHB concentrations of 40 and 160 μg/ml and spiked urine at concentrations of 160 and 640 μg/ml on the same day and on three different days. Results are shown in Table 1.

In conclusion, the coupling of CZE with ESI ion trap mass spectrometry proved to be an easy and effective technical hyphenation, which can be performed also in non specialized analytical environments, such as that of forensic toxicology. In particular, the ability of CZE to deal with miniaturized amounts of samples allows for ion trap mass spectrometry application also to biological matrices, which otherwise would interfere with the ionization process, when higher amounts of samples are injected such as in traditional liquid chromatography.

Although directly applicable to acute intoxications, the present method is not sensitive enough for determining the

Table 1
Intra-day and day-to-day analytical precision (R.S.D. of relative migration times and peak areas)

	Intraday (R.S.D., %) (n = 5)		Day-to-day (R.S.D., %) (n = 3)	
	Time	Area	Time	Area
Water (μg/ml)				
40	0.98	4.44	1.70	13.50
160	2.38	4.17	1.37	13.55
Urine (μg/ml)				
40	1.04	5.22	1.93	15.07
160	4.21	6.84	3.82	17.00

endogenous GHB concentrations, which are below 10 μg/ml. However, improvements of interfacing conditions leading to increased efficiency and, possibly, sample enrichment techniques (e.g. field amplified sample stacking), should reasonably lead to the possibility of determining GHB concentrations in the low μg/ml range.

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