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Determination of γ-hydroxybutyric acid in biological fluids by using capillary electrophoresis with indirect detection

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Abstract

γ-Hydroxybutyric acid (GHB) is a central nervous system (CNS) depressant and hypnotic which, in recent times, has shown an increasing abuse either as recreational drug (due to its euphoric effects and ability to reduce inhibitions) or as doping agent (enhancer of muscle growth). Analogues of GHB, namely γ-butyrolactone (GBL) and 1,4-butanediol (1,4-BD), share its biological activity and are rapidly converted in vivo into GHB. At present, GHB and analogues are placed in the Schedules of Controlled Substances. Numerous intoxications in GHB abusers have been reported with depressive effects, seizures, coma and possibly death. The purpose of the present work was the development of a rapid analytical method based on capillary zone electrophoresis for the direct determination of GHB in human urine and serum at potentially toxic concentrations. Analytical conditions were as follows. Capillary: length 40 cm (to detector), 75 μm i.d.; buffer: 5.0 mM Na_2HPO_4, 15 mM sodium barbital adjusted to pH 12 with 1.0 M NaOH; voltage: 25 kV at 23 °C; indirect UV detection at 214 nm; injection by application of 0.5 psi for 5 s. α-Hydroxyisobutyric acid was used as internal standard (IS). Sample pretreatment was limited to 1:8 dilution. Under these conditions, the sensitivity was ∼3.0 μg/ml (signal-to-noise ratio >3). Calibration curves prepared in water, urine and serum were linear over concentration ranges 25–500 μg/ml with $R^2 \geq 0.998$. Analytical precision was fairly good with R.S.D. < 0.60% (including intraday and day-to-day tests). Quantitative precision in both intraday and day-to-day experiments was also very satisfactory with R.S.D. ≤ 4.0%. No interferences were found neither from the most common "drugs of abuse" nor from endogenous compounds. In conclusion, capillary electrophoresis can offer a rapid, precise and accurate method for GHB determination of biological fluids, which could be important for screening purposes in clinical and forensic toxicology.

Keywords: Direct injection; γ-Hydroxybutyric acid

1. Introduction

γ-Hydroxybutyric acid (GHB) is a central nervous system (CNS) depressant and hypnotic compound, which was synthesized in 1960 as a γ-aminobutyric acid (GABA) analogue. In addition to a weak agonist activity on GABA receptors, GHB has been reported to have specific receptors in the central nervous system.

GHB was reported to affect dopaminergic and colinergic transmission as well as growth hormone and prolactine secretion. Outside the CNS, GHB has also shown depressing effects on the cardiovascular system.

An endogenous production of GHB has been reported causing physiological concentrations of this compound in plasma and urine in the low microgram per milliliter range. On this basis, cutoffs of 4 μg/ml and 10 μg/ml are generally accepted in plasma and urine, respectively, for demonstrating GHB abuse [1].

Although potential applications of GHB in anesthesia and in the treatment of neurological disorders were soon devised, the therapeutic use of GHB, because of a moderate pharmacological potency and sometimes unpredictable responses, is limited to the treatment of narcolepsy and to the pharmacological control of the alcohol withdrawal syndrome.

In recent times, an illicit use of GHB for recreational purposes has been reported for its inducing effects on euphoria, sedation and disinhibition: for the same reasons GHB has been used as a "date rape drug" [2]. Also, GHB is currently

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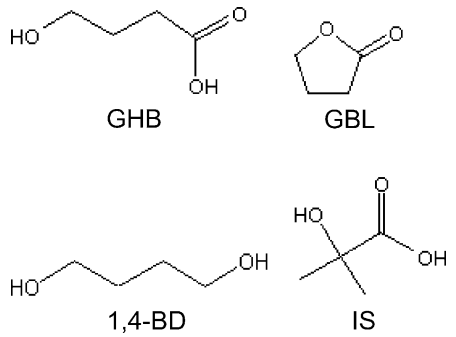

Fig. 1. Chemical structures of γ-hydroxybutyric acid (GHB), γ-butyrolactone (GBL), 1,4-butanediol (1,4-BD) and α-hydroxyisobutyric acid (IS).

abused as muscle growth enhancer among body builders, because of an alleged GHB-mediated increase of the growth hormone secretion [3].

On this basis, worldwide GHB and analogues are placed in the Schedules of Controlled Substances.

GHB is widely publicized through the Internet and is easily available in the illicit market in tablets/capsules, in powder or in liquid forms. Also, GHB analogues, namely γ-butyrolactone (GBL) and 1,4-butanediol (1,4-BD), are also easily available (Fig. 1). In solution, GHB is in equilibrium with its lactone, GBL, in dependence of the pH of the medium (the lactone form predominates at pHs < 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, with a minor percentage of GBL [4].

Numerous Emergency Room reports have pointed out acute intoxications with GHB with seizures, coma, respiratory and cardiovascular depression and, possibly, death [5]. In intoxications, urine concentrations ranging from 80 to 140,000 are reported [6].

Half life time of GHB in serum is fairly short ranging from 20 min to 1 h. On the other hand, GHB is rapidly metabolized by alcohol dehydrogenase and only about 5% of the ingested dose is eliminated unchanged in urine, which, because of the rapid disappearance of this drug in serum, is the favorite biological specimen for GHB determination [6].

The analytical determination of GHB and analogues in biological fluids is mainly based on gas-chromatography (GC) with FID detection and on gas-chromatography-mass spectrometry (GC-MS). However, because of its nature of small polar molecule/small anion and its thermal instability, GHB is not directly suitable to gas-chromatography. Consequently, some authors apply a conversion of GHB to GBL [7,8] in strong acids before injection. Silylation (BSTFA) of the hydroxy and carboxy groups of GHB is also frequently used [9–11]. In addition, the nature of GHB and its similarity to other biological organic acids hamper its extraction from the biological matrices. Reported extraction methods include liquid–liquid extraction of GBL (after chemical conversion of GHB) with organic solvents [8,10] and solid phase extraction of GHB by anion exchangers [9,12,13]. More recently SPME has also been used for sample extraction in association with GC-MS [14,15]. RP-HPLC with UV detection at 220 nm has also been applied for the determination of GHB in rat plasma [13].

Fairly recently has capillary electrophoresis been used for GHB determination. To the best of our knowledge, the first method reported was based on micellar electrokinetic capillary chromatography (MECC) with indirect detection and was aimed at the determination of GHB, GBL and 1,4-BD in clandestine preparations [16]. Capillary zone electrophoresis, also with indirect detection, was recently used by Baldacci et al. for the quantitative determination of GHB in urine [17].

The aim of the present work was the development of a rapid analytical method based on capillary zone electrophoresis for the determination of GHB in untreated human serum and urine at potentially toxic concentrations for application in emergency toxicology and in deaths from GHB overdosing.

2. Materials and methods

2.1. Standards and chemicals

Standards of γ-hydroxybutyric acid, α-hydroxyisobutyric acid (used as internal standard, IS) and barbital were obtained from Sigma–Aldrich (St. Louis, MO, USA); other solvents, chemicals and reagents, all of analytical grade or better, were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation and analytical conditions

All the experiments were carried out on a PACE MDQ capillary electropherograph (Beckman Coulter, Fullerton, California, USA); 40 cm long (up to the detection window) bare fused silica capillaries (75 μm i.d.) were used throughout the study; the running buffer was composed of 5.0 mM Na_2HPO_4 and 15 mM sodium barbital adjusted to pH 12 with 1.0 M NaOH; the applied voltage was 25 kV at 23 °C with a resulting current of ∼160 mA; detection was by indirect UV absorption at 214 nm wavelength corresponding to the absorption maximum of barbital, the UV absorbing co-ion. Injection was by pressure application (0.5 psi for 5 s). Between runs the capillary was rinsed by flushing with 1.0 M NaOH for 7 min, 10 mM sodium citrate for 7 min and running buffer for 5 min (20 psi).

2.3. Sample collection and preparation

Blank urine and serum were obtained directly from the authors and added with standards at the needed concentrations. Real urine samples were obtained from subjects undergoing treatment for alcohol dependence with a

pharmaceutical preparation (syrup) of GHB containing the active principle at a concentration of 17.5% (Alcover®, CT Laboratorio Farmaceutico, Sanremo, Italy) and were kindly provided by Dr. E. Manzato, Drug Dependence Unit, Hospital of Zevio, Verona, Italy.

Before analysis, urine and serum samples were added with α-hydroxyisobutyric acid (IS) to obtain a final concentration of 1000 μg/ml; sample pretreatment was limited to dilution 1:8 with 3 mM NaOH, in order to convert completely GBL into GHB.

3. Results and discussion

Under the applied pH and voltage, GHB and α-hydroxyisobutyric acid (IS), migrating as anions in the separation buffer, were drawn towards the detector (i.e. towards the cathodic end of the capillary) by the prevailing cathodic mobility of EOF. Notwithstanding GHB and IS had equal number of charges and the same molecular weight, they were completely separated, being α-hydroxyisobutyric acid a faster anion than GHB and consequently reaching the detector later (Fig. 2). Admitting negligible lactone (GBL, uncharged) formation from GHB at the basic pH of the running buffer, the reason of the observed mobility difference is unclear. However, a reasonable explanation could be found in a different solvation of the molecules due to their different structure (Fig. 1), which leads to different Stokes radii and consequently different mobilities.

While the GHB peak is reasonably symmetric, the IS peak showed a neat tailing. The reason of this asymmetry is also unclear and, only tentatively, could be attributed to a mobility difference between the IS and the running buffer co-ions. However, for the IS peak magnitude, the observed peak tailing did not hamper the precise measurement of its area (from 10 integrations of the same IS peak the resulting R.S.D. was 0.303%).

Typical electropherograms from blank urine and serum and from the same samples after spiking with GHB and IS are depicted in Figs. 3 and 4, respectively. No significant matrix interferences were found at the migration times of both GHB and IS.

By adopting a criterion based on a signal-to-noise ratio >3, the sensitivity of the present method was ∼3.0 μg/ml in pure solution and ∼24.0 μg/ml in real samples (which had to be diluted 1:8 before injection). Though not suitable for the determination of physiological levels of GHB, this sensitivity is sufficient for the diagnosis of acute intoxications, in which concentrations in the order of hundreds or thousands of micrograms per milliliter have been reported [4].

Calibration curves were prepared in water in the GHB concentration ranges from 0 to 500 μg/ml (seven points) and in blank urine and blank serum in the GHB concentration ranges from 25 to 500 μg/ml (six points) (IS concentration: 1000 μg/ml). Each point was then diluted 1:8 with 3 mM NaOH and injected. Linear relationships were found between GHB concentrations (on the x axis) and the respective peak area ratios GHB/IS (y axis).

The corresponding equations were as follows:

$$\text{water}: \ y = 0.0008x - 0.003 \quad R^2 = 0.999$$
$$\text{urine}: \ y = 0.0013x + 0.0049 \quad R^2 = 0.998$$
$$\text{serum}: \ y = 0.0006x - 0.0061 \quad R^2 = 0.999$$

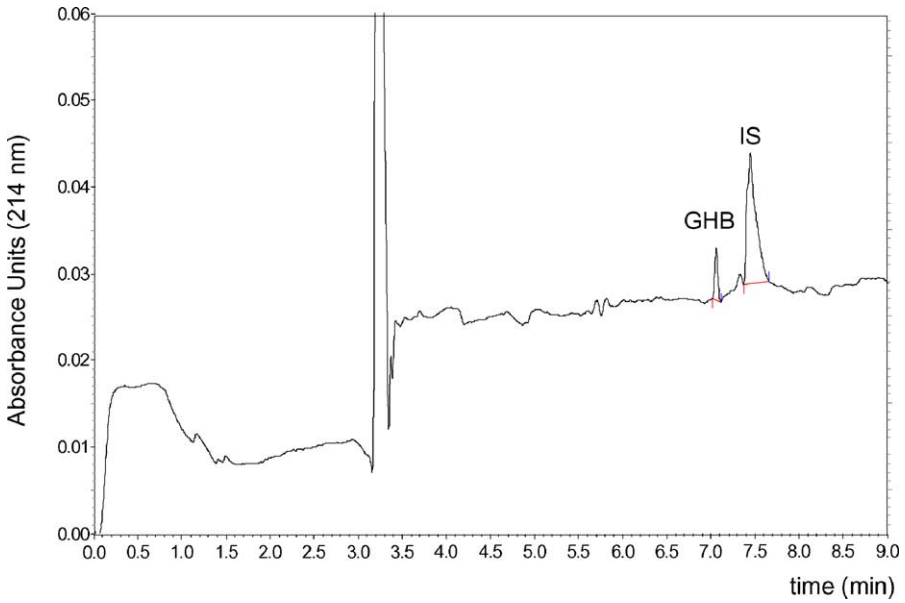

Fig. 2. Electropherogram of the capillary zone electrophoretic separation of GHB (200 μg/ml) and IS (1000 μg/ml) in pure solution. Analytical conditions: capillary: 40 cm (length to detector), 75 μm i.d.; buffer: 5.0 mM Na_2HPO_4, 15 mM sodium barbital adjusted to pH 12 with 1.0 M NaOH; voltage: 25 kV; current: 160 μA; temperature: 23 °C; indirect UV detection at 214 nm; injection by application of 0.5 psi for 5 s.

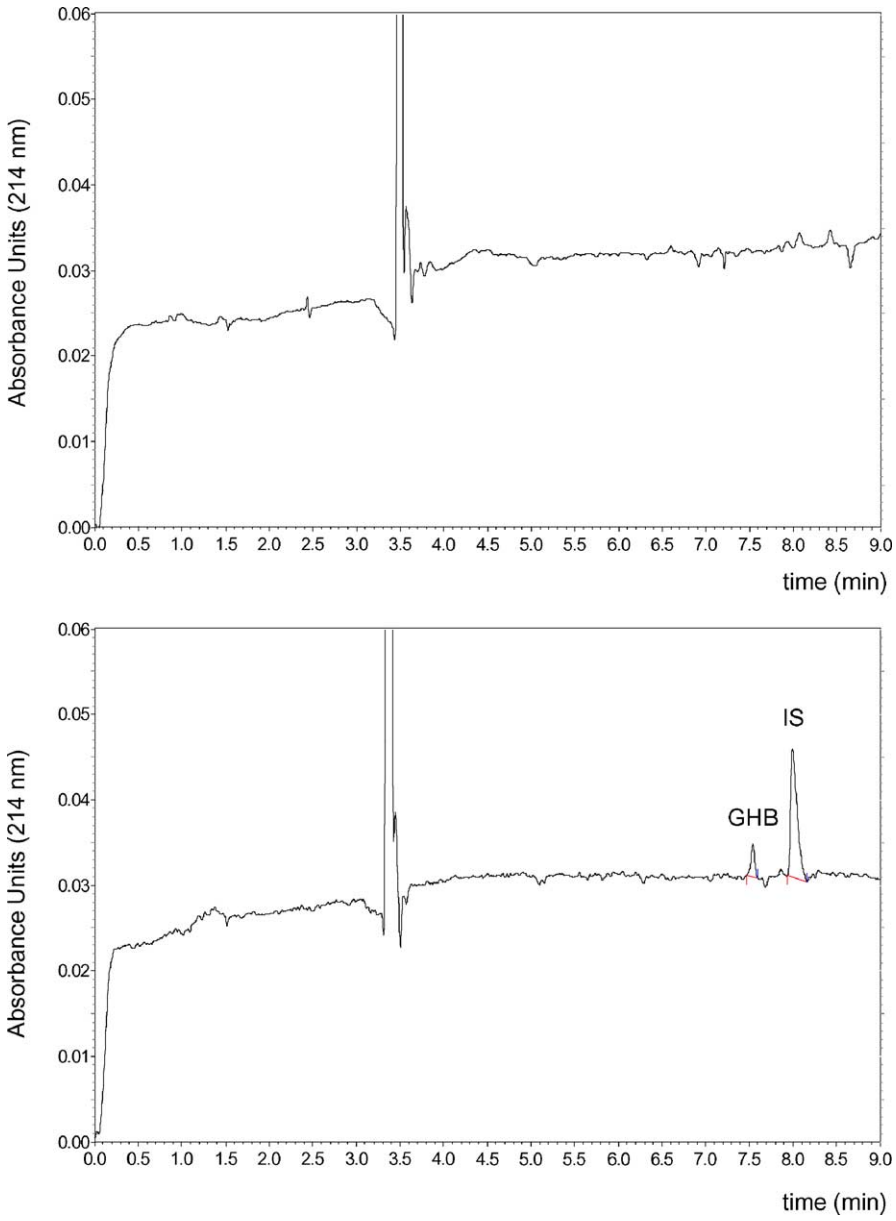

Fig. 3. Electropherograms of (top) blank urine and (bottom) blank urine spiked with GHB (100 μg/ml) and IS (1000 μg/ml). Analytical conditions as in Fig. 2.

Analytical precision was tested in urine and serum at GHB concentrations of 100 and 500 μg/ml. Relative migration times (GHB migration time/IS migration time) were very reproducible with R.S.D. values <0.50% in intraday experiments ($n = 5$) and <0.60% in day-to-day experiments ($n = 5$). Quantitative precision in both intraday and day-to-day experiments was also very satisfactory as described in Table 1.

Analytical accuracy was calculated in urine and serum samples spiked with GHB to obtain concentrations of 100 and 500 μg/ml by comparison between theoretical and measured concentrations (see Table 1). As shown in Table 1, recoveries were always slightly higher than 100%. This can be explained with the presence in biological fluids of low physiological GHB concentrations of endogenous origin.

Table 1
Precision and recovery of GHB analysis

	Intraday R.S.D., $n = 5$ (%)	Day-to-day R.S.D., $n = 5$ (%)	Recovery, mean ± S.D.; $n = 5$ (%)
Urine (μg/ml)			
100	3.23	4.01	107.5 ± 2.380
500	1.02	1.41	102.6 ± 0.721
Serum (μg/ml)			
100	1.05	2.53	107.1 ± 0.322
500	1.23	3.24	101.1 ± 0.322

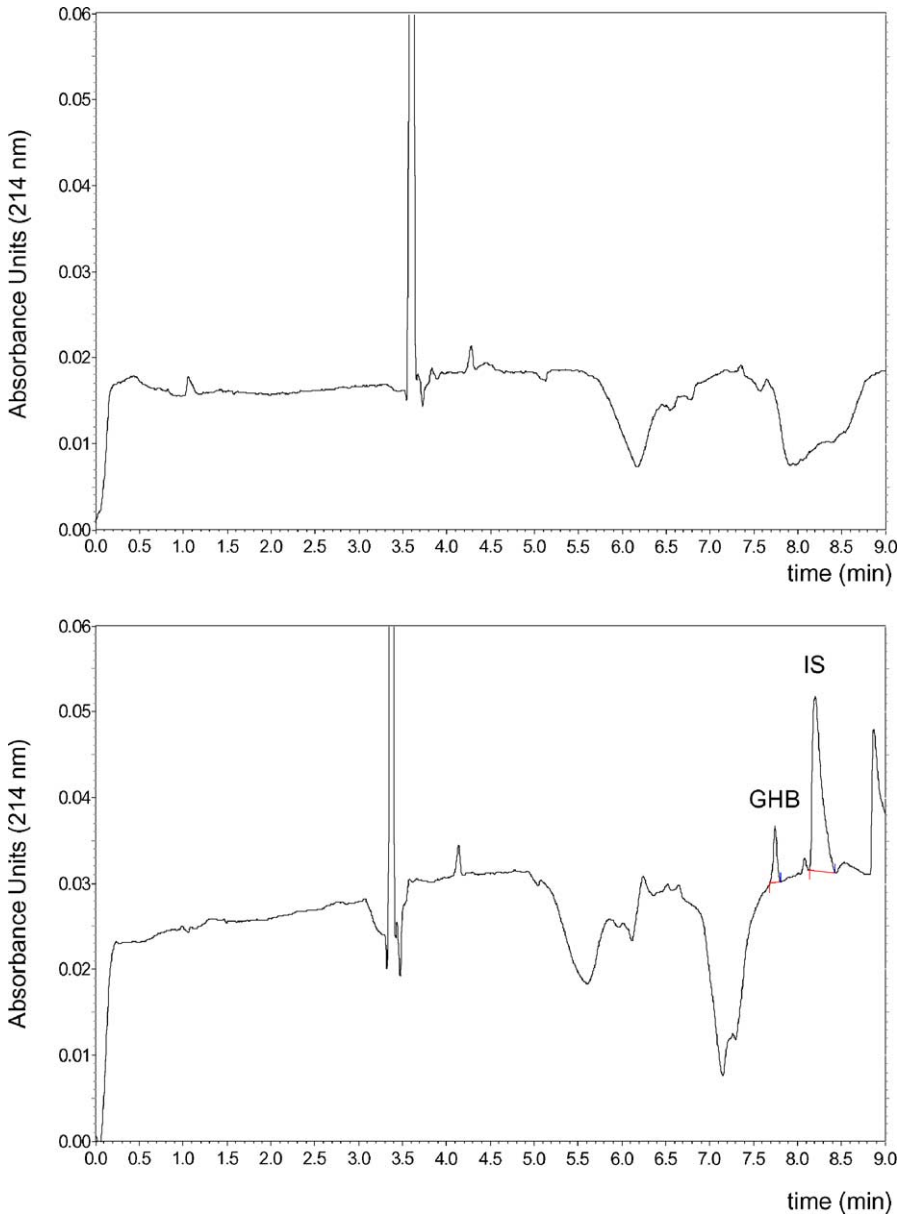

Fig. 4. Electropherograms of (top) blank serum and (bottom) blank serum spiked with GHB (200 μg/ml) and IS (1000 μg/ml). Analytical conditions as in Fig. 2.

Fig. 5 shows a typical electropherogram from urine of a subject under therapy with Alcover® 50 ml/day (corresponding to 8.6 g of GHB).

No interferences were found neither from the most common "drugs of abuse" (opiates, amphetamines, cocaine, cannabinoids) nor from endogenous compounds (hundreds of blank samples did not show interfering peaks). This specificity can be attributed on one side to the selectivity of indirect detection, and on the other on the high concentrations of GHB, which are relevant from a toxicological point of view.

In comparison with the other two published CE methods, the present is substantially simpler than the MECC approach adopted by Dahlen and Vriesman [16]. Although suitable for the simultaneous detection also of the uncharged GBL and 1,4 BD, the MECC method was applied only to confiscated materials and consequently is not yet ready for toxicological analysis. The method recently published by Baldacci et al. [17] and based, as the present one, on capillary electrophoresis with indirect detection, but using substantially different analytical conditions (co-ion, pH, etc.), shows similar performances. However, the authors failed to find a suitable IS, did not apply the method to serum (the most important biological sample in emergency toxicology) and reported frequent interferences from concentrated urines.

All these flaws may limit the usefulness of these methods in the real practice, but the two papers above mentioned have

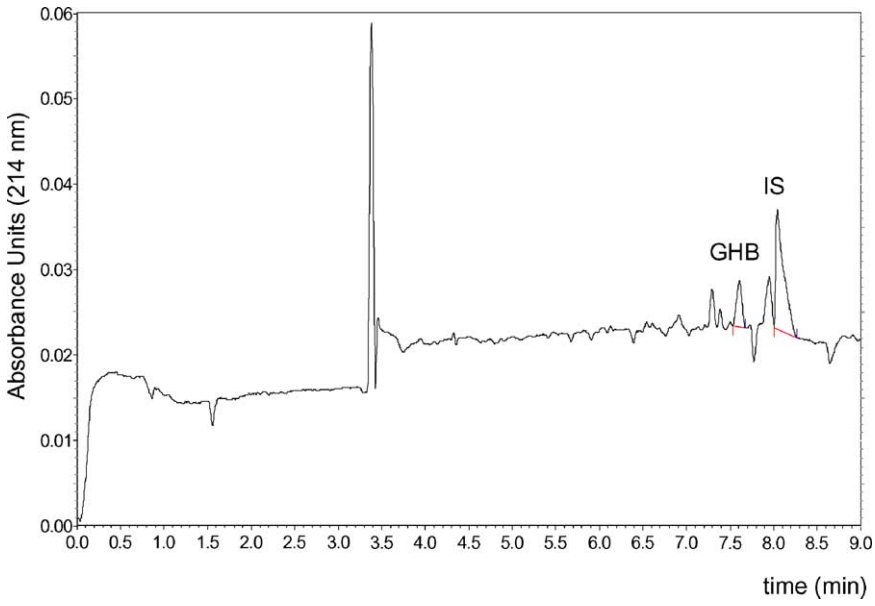

Fig. 5. Typical electropherogram from the urine of a subject under therapy with Alcover® (8.6 g per day) in which GHB was determined at a concentration of 184 μg/ml. Analytical conditions as in Fig. 2.

pointed of the feasibility of a capillary zone electrophoretic analysis of GHB, which was refined, improved and validated in the present work.

4. Conclusions

Capillary zone electrophoresis with indirect detection has proved to be an excellent tool for the rapid and direct determination of GHB in biological samples. This is particularly important because of the lack of immunoassays for screening purposes, which hampers rapid analysis.

In particular, the present method, avoiding sample extraction and derivatization, overcomes the well known extraction and gas chromatographic problems of small anions such as GHB, which still hamper the traditional approaches, mainly based on GC and GC-MS.

In conclusion, capillary electrophoresis can offer a simple, precise and accurate method for GHB determination, which could be precious for screening of GHB overdosing in both clinical and forensic environments.

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