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TITOLO TESI

ORIGINAL ANALYTICAL METHODS FOR THE DETERMINATION OF PSYCHOACTIVE COMPOUNDS IN COMPLEX MATRICES

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“There is a crack in everything. That's how the light gets in.”

Leonard Cohen

Dedicated to my family

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1. INTRODUCTION

1.1. Drug abuse, drug addiction and doping

Drug abuse is an important global problem with a strong economic, personal and social impact. In the "World Drug Report" 2014 the United Nations Office on Drugs and Crime (UNODC) estimates that, in 2012, between 162 and 324 million people, that is 3.5% to 7.0% of the population aged 15-64, used illicit substances at least once in the previous year and that about half of that number used illicit drugs at least once during the month before the assessment[1]. The problem is not limited to illicit substances, but concerns also legal psychoactive drugs, such as alcohol, tobacco and medications. Recently, there has been an increase in the non-medical use of prescription drugs, such as a number of opioid analgesics (used in the treatment of pain), tranquillizers and sedatives (employed to treat anxiety and sleep disorders) and prescription stimulants (prescribed for the treatment of attention deficit hyperactivity disorder (ADHD)). While in Europe the extent of this phenomenon has not been fully evaluated (though some European countries have reported non-medical use of prescriptions drugs), in the USA the problem has dramatically increased over the last years[1,2].

Drug abuse can lead to the phenomenon of addiction, defined as a chronic, relapsing brain disease characterized by compulsive drug seeking and use, despite harmful consequences[3,4]. Addiction arises from the changes in the brain electrical structure that the drugs produce over time. Drugs of abuse activate the brain's reward system, particularly the mesocorticolimbic dopaminergic pathway, producing positive reinforcing effects that powerfully compel people to keep using drugs, thus leading to addiction[4]. Moreover, long-term abuse causes neurobiological changes that impair cognitive function and may trigger mental disorders[3]. Besides effects on the brain, drug abuse has other serious health consequences, such as chronic diseases and organ failure associated with repeated use, blood-borne bacterial and viral infections (among injecting drug users, 17.9% is HIV positive and 50% is infected with HCV) and acute toxic effects, including overdose. Each year between 104,000 and 263,000 deaths are related to or associated with the use of illicit drugs and over half of these deaths are fatal overdose cases occurring mainly in young people in the mid-

thirties. In addition, drug abuse strongly affects society on multiple levels. For example, illicit drug use generates criminal activities, people under the influence of drugs can cause accidents and the risk of spreading infectious diseases is increased[1,3]. Different strategies can be employed to reduce drug abuse and limit its negative consequences. Prevention programs aimed to increase the awareness of drug-induced harms have proved to be effective in decreasing drug abuse[3]; controls carried out on people that perform particular tasks, such as those requiring high attention (e.g. driving), in order to ascertain drug intake can prevent accidents; proper medical treatment helps patients improve their health conditions and the quality of their lives and limits the social harm related to drug abuse.

2. AIM OF THE THESIS WORK

The aim of this thesis work was to develop original analytical methods for the determination of drugs with a potential for abuse, of substances used in the pharmacological treatment of drug addiction in biological samples and of potentially toxic compounds added to street drugs.

In fact reliable analytical methods can play an important role in this setting. They can be employed to reveal drug intake, allowing the identification of drug users and to assess drug blood levels, assisting physicians in the management of the treatment. Pharmacological therapy needs to be carefully monitored indeed in order to optimize the dose scheduling according to the specific needs of the patient and to discourage improper use of the medication.

In particular, methods have been developed in this thesis work for the detection of gamma-hydroxybutiric acid (GHB), prescribed for the treatment of alcohol dependence; glucocorticoids, one of the most abused pharmaceutical class to enhance sport performance; and adulterants, pharmacologically active compounds added to illicit drugs for recreational purposes. All the presented methods are based on capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) coupled to various kinds of detectors (diode array detector, mass spectrometer); biological sample pre- treatment was carried out using different extraction techniques, namely liquid-liquid extraction (LLE) and solid phase extraction (SPE). Different kind of matrix have been considered: human plasma, dried blood spots, human urine, simulated seized powders. The developed analytical methods will be individually described and discussed in the following chapters.

3. ANALYSIS OF GAMMA-HYDROXYBUTIRIC ACID (GHB) IN HUMAN PLASMA BY CE-DAD WITH INDIRECT DETECTION

3.1. Introduction

Alcohol is one of the most frequently abused drugs and it has an high tendency to cause mental disorders and death as a result of chronic use[1,5]. According to a recent study, it appears to be more harmful than cannabis, lysergic acid diethylamide and ecstasy[6]. A substantial number of individuals are co-dependent on these drugs and this leads to an increased risk of medical complications and negative health consequences. Alcohol dependence still represents a significant global problem affecting millions of people in the world[5] and it is responsible for an high demand of treatment, thus reflecting the considerable harm associated with alcohol abuse[1,7]. The combination of psychosocial support and pharmacological therapy showed to be the most effective approach to alcohol dependence[7].

γ-Hydroxybutyric acid (GHB) is naturally occurring in the mammalian brain tissue where it is believed to act as a neurotransmitter[8]. It is also found in heart, liver, kidney, muscle and brown fat, but its function is still unknown. GHB has been found to be useful in the treatment of narcolepsy[9] and of alcohol withdrawal[7]: it reduces craving and prevents withdrawal symptoms relieving the patient of the need to use alcohol, it has a low potential for abuse and a low risk of overdose fatalities.

On the other hand body builders have consumed the compound as it stimulates the secretion of growth hormone[10].

Furthermore, it has recently become a public health issue as a club drug (called *liquid ecstasy*) and appears occasionally in drug-facilitated sexual assaults. This is due to its effects and chemical properties as it is colourless and odourless liquid, which can be easily mixed with drinks[11].

The principal clinical effect of exogenously administered GHB is Central Nervous System depression, effect that may be mediated by a specific GHB receptor, binding to γ-aminobutyric acid (GABA) receptors, modulation of GABA levels or interactions with other neurotransmitters. The

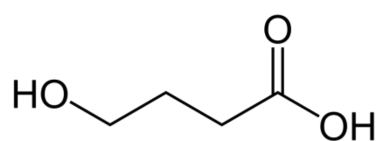
range between the therapeutic effects and toxic ones is narrow, frequently resulting in headache, nausea, vomiting, respiratory depression and coma[12].

Consequently, the correct determination of GHB is important in both forensic and clinical toxicology. Many previously reported analytical methods detect GHB in different biological matrices and involve the use of gas chromatography (GC)[13,14,15,16,17,18,19], high performance liquid chromatography (HPLC)[20,21,22] with different detector and capillary zone electrophoresis (CE)[23,24,25].

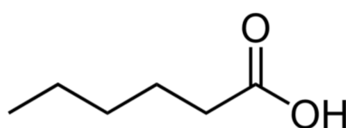
The qualitative techniques include infrared (IR) spectroscopy[15], nuclear magnetic resonance (NMR) spectroscopy[16] and colorimetry[17].

The outcome of GHB is highly variable depending on different factors, including individual metabolism. For this reason, it would be advisable to carefully monitor plasma levels of GHB in order to tailor the dose to each patient’s needs. To these purposes, it is desirable to have at disposal high-sensitivity, low-cost analytical methods to determine plasma levels of GHB.

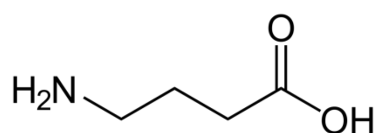
The aim of the present work was to develop a CE-DAD method with indirect detection for the determination of GHB in human plasma in order to perform therapeutic drug monitoring in patients under treatment for alcohol abuse.



γ-Hydroxybutyric acid (GHB)



Hexanoic acid (IS)



γ-Aminobutyric acid (GABA)

Figure 1. Chemical structures of GHB and hexanoic acid, used as internal standard (IS), compared to GABA structure.

3.2. **Experimental**

Chemicals

GHB (gamma-hydroxybutyric acid) has been provided by LGC Standards Srl (Sesto San Giovanni, Milan).

Hexanoic acid used as internal standard (IS) was provided by Janssen Chimica (Beerse, Belgium).

Methanol, used for dilutions, for the precipitation of the proteins and for the preparation of the background electrolyte (BGE), benzoic acid, used for the BGE, were supplied by Sigma Aldrich (Steinheim, Germany).

CTAB (cetyl-trimethylammonium bromide), used for the preparation of BGE, sodium hydrogen phosphate, used to prepare the phosphate buffer, and sodium hydroxide, were supplied by Carlo Erba (Milan, Italy).

The ultrapure water (18.2 MΩ cm) was obtained using a Milli-Q system supplied by Millipore (Milford, Massachusetts, USA)

Preparation of stock and working solutions

The stock solution (1 mg/mL) of GHB was purchased from the pharmaceutical manufacturer. The stock solutions (1 mg/mL) of hexanoic acid (SI) were prepared by diluting the solution of hexanoic acid with a density of 0.927 g / mL . the exact volume of 10.8 µL of this solution were diluted with MeOH in a 10 mL volumetric flask.

Working solutions were prepared, daily, by diluting stock solutions to desired concentrations, with a mixture of phosphate buffer 25 mM, pH 7.4 / MeOH (25:75 v/v). All solutions were stored in the freezer at a temperature of -20 ° C.

The backgound electrolyte (BGE) is constituted by a benzoate buffer 15 mM at pH 5.5 containing 0.5 mM CTAB and MeOH to 30% (v / v).

This buffer is prepared as follows: 91.5 mg of benzoic acid is weighted ad dissolved with approximately 25 mL of water, agitated on a plate heated to 37 ° C. let the solution col avoiding the

formation of benzoate cristals. CTAB (9.1 mg) and 15 mL of MeOH are added to the clear solution obtained. The solution is adjusted to pH 5.5 with 1 M NaOH, appropriately diluted, and then transferred to a 50 cc flask and brought to volume with water.

Sample collection

Blood samples (3 mL) were drawn from patients subjected to therapy with GHB at Drug Addiction Treatment Centres (Ser.T.) in Bologna (Italy). Blood samples were usually collected in the morning from fasting patients, 2-3 hours after the last drug administration. The study was approved by local review board and informed consent was obtained for experimentation with human subjects. Blood was stored in glass tubes containing ethylenediaminetetraacetic acid (EDTA) as the anticoagulant and then centrifuged (within 2 hours from collection) at 4000 rpm for 15 minutes at 5°C. The supernatant (plasma) was then transferred to polypropylene tubes and stored at -80°C until analysis. Blood samples from healthy volunteers, used as blank samples, were treated in the same way.

Sample pre-treatment

For the analysis of GHB in plasma samples, 100 µL of plasma were added to aliquots of 10 µL of GHB and SI (or IS only) taken from the stock solutions. MeOH was then added to this solution to obtain a final volume of 400 µL. The solution thus obtained was stirred for 3 minutes and then centrifuged (4000 rpm, 5° C, 10 minutes). The supernatant was collected and filtered through a cellulose filter 0.20 µm. The final solution was injected into the CE system.

Equipment

The instrument used for the analysis of GHB in plasma is a capillary electrophoresis system (CE-3D, Agilent, Palo Alto - CA, USA) equipped with a photodiode array detector (DAD).

The separation was conducted using a fused silica capillary (Composite Metal Services, Hallow

UK) with the following characteristics: total length (TL) of 48.5 cm, effective length (EL) of 40.0 cm and internal diameter (ID) of 50 microns.

The samples were injected from the anode side of the capillary by means of a pressure of 50 mbar for 25s.

The applied voltage was -30 kV. The wavelength of 210 nm was set for detection.

At the beginning of each day, the capillary was conditioned with ultrapure water, 1N NaOH, ultrapure water and BGE, for ten minutes each.

A Crison (Barcelona, Spain) MicropH 2000 pHmeter, a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge and an IKA (Staufen, Germany) RV 10 rotary evaporator were also used.

Data were handled by means of ChemStation software from Agilent (Santa Clara, CA, United States).

Method validation

The method was validated according to USP XXXVIII [26] and "Crystal City"[27],[28] guidelines.

- *Linearity, limit of quantitation, limit of detection*

Aliquots of 10 μL of analyte standard solutions at six different concentrations, containing the IS at a constant concentration, were added to 100 μL of blank plasma. The resulting calibration standards were subjected to the previously described pretreatment procedure and then injected into the CE system. Calibrators were prepared in triplicate for each point and covered the following plasma range: 2.0-320 μg mL^{-1}. The concentration of the IS was 20 μg mL^{-1}. The analyte/IS peak-area ratios obtained were plotted against the corresponding concentrations of the analytes (expressed as μg mL^{-1}) and the calibration curves were constructed by means of the least-square method.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the analyte concentrations which give rise to peaks whose heights are 3 and 10 times the baseline noise, respectively.

- *Extraction yield and precision*

For these assays plasma samples at three different concentrations of the analytes, corresponding to the lowest, the intermediate and the highest point of the calibration curve, were prepared. The samples, containing 2.0, 160.0 and 310.0 µg mL^{-1} of GHB and were subjected to the previously described pre-treatment procedure and injected into the CE. The analyte peak areas of the spiked samples were compared to those obtained injecting standard solutions at the same theoretical concentrations and the extraction yield (expressed as percentage value) was calculated.
The assays described above were repeated six times within the same day to obtain repeatability (intraday precision) and six times over six different days to obtain intermediate precision (interday precision), both expressed as percentage relative standard deviation values (RSD%).

- *Selectivity*

The selectivity of the method was evaluated with respect to endogenous and exogenous compounds. Blank plasma samples from six different healthy volunteers were processed in the absence of the IS and the resulting electropherograms were checked for possible interference at the retention time of the analytes and the IS. Selectivity towards exogenous substances was investigated by injecting into the CE system standard solutions of several compounds that could be co-administered in clinical practice. A substance was classified as interfering if it gave rise to a peak that was not baseline separated from those of the analytes or the IS.

- *Stability*

Stock solution stability was tested by comparing the analyte/IS peak-area ratios of a standard solution (containing 10 µg mL^{-1} of GHB) prepared from stock solutions stored for 1 month at -20°C with those of a standard solution at the same theoretical concentrations obtained from fresh stock solutions (n = 3). Stability assays were also carried out in blank plasma samples fortified with 10 µg mL-1 of GHB. Sample stability was evaluated over five hours at room temperature, after three freeze- thaw cycles and after 1 month of storage at -80°C (n = 3 per storage condition).

- *Analysis of plasma samples from patients in treatment with GHB*

For the analysis of GHB in real samples, 10 µL of SI were added to 100 µL of plasma samples collected from patients in therapy with Alcover®. The mixtures thus obtained were subjected to the procedure of precipitation with MeOH. The solution is then injected in the CE.
The values of the ratios of the peak areas electrophoretic analyte / SI were interpolated on the calibration curve built on plasma samples to obtain plasma concentrations of the analyte.

- *Accuracy*

Analyte standard solutions at three different concentrations (in order to obtain analyte additions of 5.0, 20.0 and 50.0 µg mL^{-1} of GHB) were added to plasma samples collected from patients under Alcover® treatment whose analyte concentrations had been previously determined (n = 3 for each level); then, the mixtures were subjected to the pretreatment procedure. Accuracy was expressed as percentage recovery and was calculated according to the following formula: 100 ([after spiking] – [before spiking]) / [added], where [after spiking] is the sum of the concentrations of the analyte in the real plasma and in the analyte standard solution added; [before spiking] is the concentration of the analyte in the real sample; and [added] is the concentration of the analyte standard solution added to the real sample.

3.3. **Results and discussion**

Development of indirect spectrophotometric determination

GHB is a molecule that does not have unsaturation or other chromophores that allow direct detection by UV-visible spectrophotometry. To overcome this problem it can be derivatized, but this strategy sometimes is not reliable for analytical purposes.

An indirect spectrophotometric determination of the analyte can be done in CE, using, as a component of BGE, an ionic compound with advantageous absorption properties called probe.

In this case, for the determination of GHB has been used a BGE consisting of benzoic acid (Figure 2). Benzoic acid contains a chromophoric group, an aromatic carboxylic acid, that shows a an absorption maximum at 210 nm.

Each time that the analyte, migrating in the capillary, is located in front of the optical window, it is observed a negative peak on the absorption line produced by BGE: this peak is negative because the analyte considered shows no absorption.

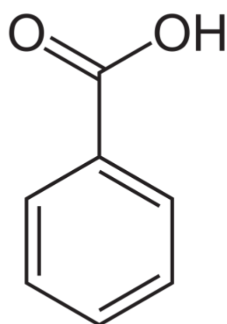


Figure 2. Chemical structure of benzoic acid

BGE optimization

In capillary electrophoresis with indirect spectrophotometric detection the separation efficiency and sensitivity of the method is influenced by many factors: the concentration of the probe, the running buffer, the pH value, the concentration of the Electroosmotic Flow (EOF) modifier.

A good Background Electrolyte (BGE) for the indirect detection must provide a high molar extinction coefficient. The para-hydroxybenzoic acid and benzoic acid, both compounds which contain a chromophore group that allows indirect UV detection were evaluated as potential probes. It was chosen benzoic acid which allows a better detection of the analyte.

Very important is the mobility of the analytes within the BGE that can be controlled by changing the concentration of benzoate or the pH.

The effect of the concentration of benzoate, on the mobility of analytes, was studied using a Cetyl-Trimethyl-Ammonium Bromide (CTAB) concentration of 0.5 mM at pH 5.5, in the range between 10 mM and 25 mM. It is observed that the effective mobility of the analytes is reduced by increasing the concentration of benzoate, this reduction is due to an increase in total ionic strength of the electrolyte. The decrease of the mobility of the analytes is accompanied by a parallel increase in the migration time, which improves the resolution of GHB, especially in plasma samples of healthy volunteers, increasing the migration times of the analyte with respect to the interferences of the plasma.

However with increasing concentration of benzoate is a decrease of sensitivity in the detection, due to the decrease of the ratio of UV absorbance the analyte.

For this reason, a good compromise, in terms of sensitivity and resolution was obtained by choosing a concentration of benzoate equal to 15 mM.

The electrophoretic mobility of the analytes is pH dependent, because the ionization is directly related to the pH of BGE.

The pH of the BGE was studied in a range from 5 to 6.5, taking into account that the pKa of the analyte is ~ 4.5. It was chosen value of 5.5 because it was observed that in samples of healthy volunteer this value allowed to have migration times more similar to those of the standard solutions. This is probably attributable to an increase of the buffering power of BGE at effective pH of 5.5.

The EOF is considerably reduced, or even eliminated, if the BGE is added a cationic surfactant, so it was chosen to achieve this purpose the Cetyl-Trimethyl-Ammonium Bromide (CTAB). Tests were carried out also using, as surfactant, the Tetrabutylammonium Bromide (TBAB), in a concentration range between 0.25 mM and 0.5 mM, which, however, has led to a considerable increase of the migration times of the analyte, for this reason it was discarded.

Even the concentration of CTAB in the BGE was studied, in a range between 0.3 and 0.7 mM, and was observed as an increase in the mobility of the analytes proportional to the increase of the concentration of CTAB. However, increasing the concentration of CTAB is a reduction of sensitivity because it adds an anion which competes with the ions of the solute, while, for concentrations of CTAB less than 0.5 mM is observed a lack of reproducibility of migration times. A good compromise between resolution and sensitivity was achieved with a concentration of 0.5 mM CTAB.

The addition of an organic modifier which varies the mobility of the analytes in a selective manner can significantly reduce the interference. Since methanol is required by pretreatment of biological samples, which occurs through precipitation of the protein, this was added to the BGE.

The percentage of methanol is added has been studied in a range between 10% and 40%. From the analysis it was evident that the best results, in terms of stability and reproducibility of the electrophoretic system, were obtained with 30% methanol.

The optimal separation was therefore obtained using a BGE with the following composition:

- Benzoate buffer 15 mM pH 5.5
- 0.5 mM CTAB
- MeOH 30%

Internal standard choice

As regards the selection of a suitable internal standard some compounds with no chromophore groups, as carboxylic acids, were tested. Among these, the most appropriate was found to be hexanoic acid (Figure 1) which, as GHB, does not absorb UV radiation: when it passes in front of the optical window it produces a negative peak. Furthermore it showed an electrophoretic behavior similar to that of the analyte. In fact his log P is comparable to GHB.

In this the experimental conditions, the migration time of the internal standard is slightly higher than GHB, but still allow short analysis times, less than 4 minutes. The hexanoic acid was used as IS at the concentration of 20 μg / mL.

Optimization of injection conditions

Studies have been performed to optimize the injection conditions to increase the sensitivity of the method and avoid a reduction in efficiency. Several tests were carried out while maintaining the pressure constant at a value of 50 mbar and gradually increasing the injection time from 5 seconds to 35 seconds. There is increased sensitivity of up to 25 seconds, follow it is observed a peak broadening with consequent loss of efficiency.

The optimum condition is reached by injecting pressure from the anodic side at 50 mbar for 25 seconds.

Solvent used for dilution of the analytes

The solvent used to perform dilutions is made from a mixture 75:25 MeOH / 25 mM phosphate buffer at pH 7.4.

This choice has been very important as it has influenced both the height and the efficiency of both peaks.

During an initial screening of solvents to make the dilutions of the standard solutions, the use of methanol was observed to produce a marked increase in sensitivity, compared to the use of ultrapure water as shown in Figure 5 and Figure 6.

Subsequently, performing the tests on healthy volunteers plasma fortified a reduction of

reproducibility of the times and areas was observed between the standard solutions and the plasma solutions. This phenomenon has been attributed to excessive ionic strength of the matrix compared to standard solutions and to the presence of a percentage of organic solvent resulting from the pretreatment procedure of the sample. To be able to compare the analysis of standard solutions the analysis of plasma samples, the conditions more similar to those of the matrix was reproduced using a 25 mM phosphate buffer pH 7.4 that mimics the characteristics of the plasma and the methanol which is used in pretreatment mixture in the same ratio 25:75. As can be seen, in the optimized electrophoretic conditions the analytes are well resolved within a short total run time and GHB is reasonably separated from EOF(Figure 7).

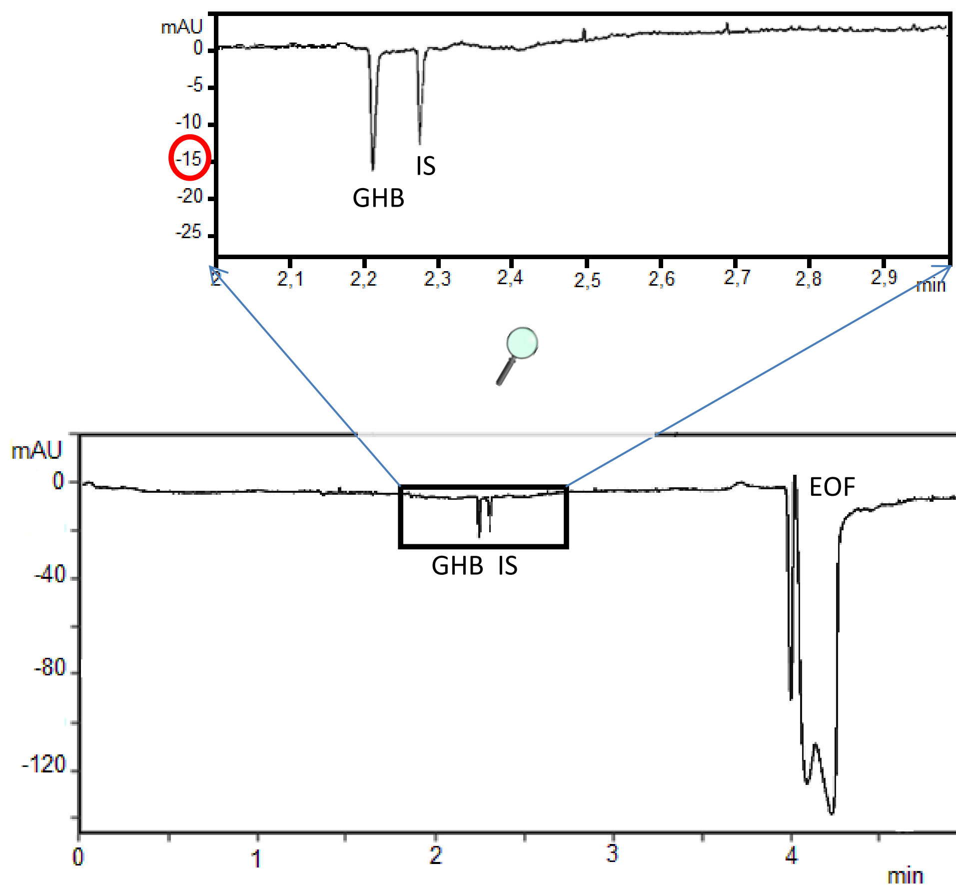


Figure 3. Electropherogram of standard water solution containing GHB (20 μg/mL) and hexanoic acid (IS) (20 μg/mL)

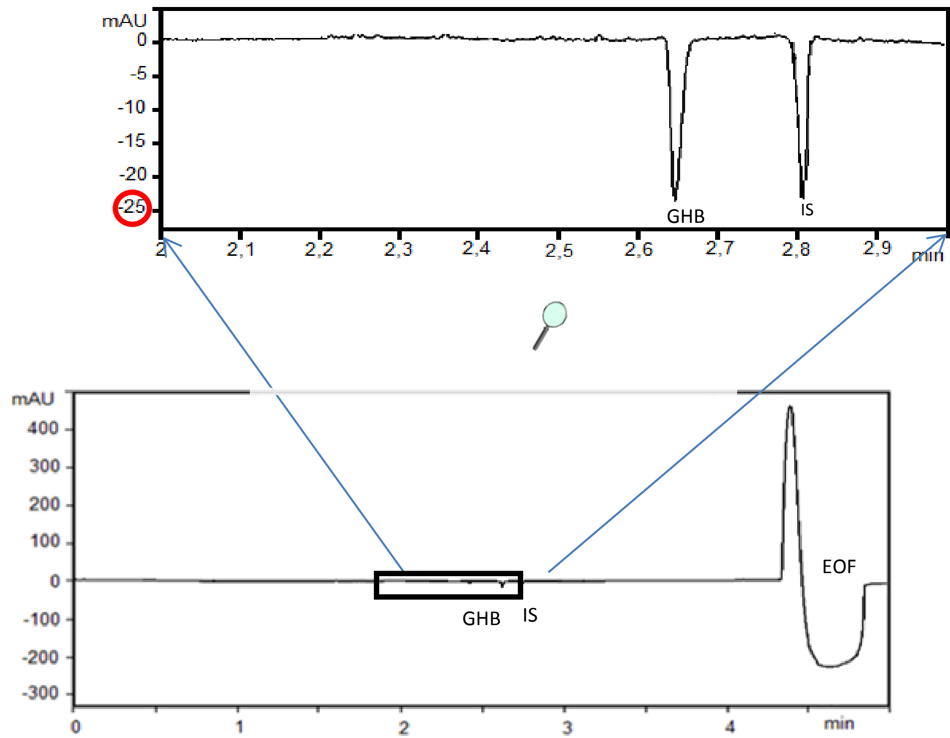


Figure 4. Electropherogram of standard methanolic solution containing GHB (20 µg/mL) and hexanoic acid (IS) (20 µg/Ml)

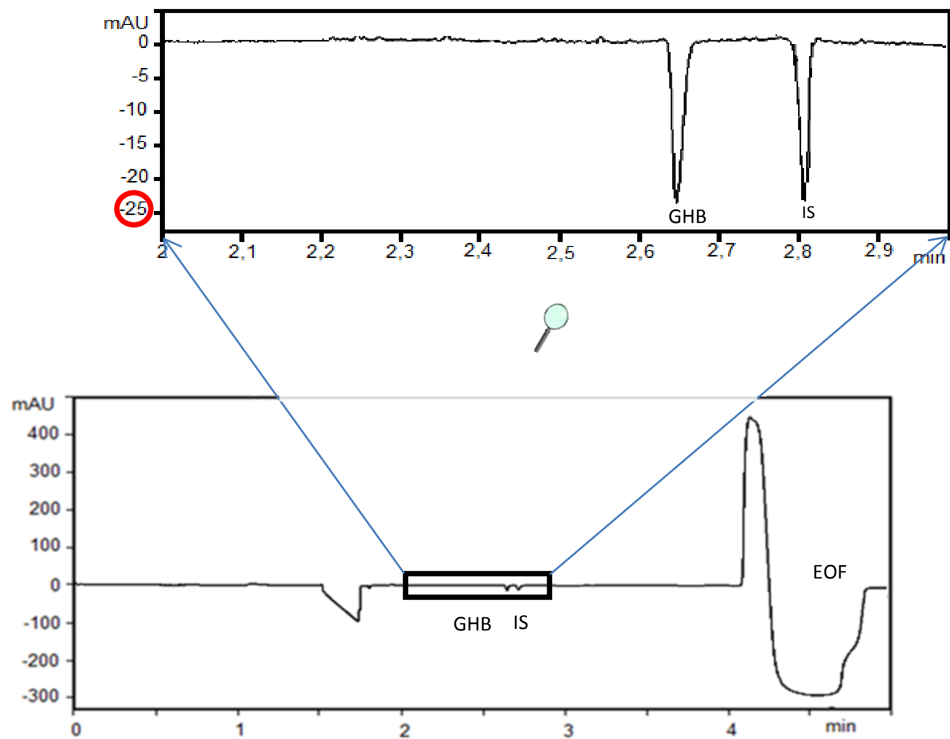


Figure 5. Electropherogram of standard solution methanol : water 75:25 containing GHB (20 µg/mL) and hexanoic acid (20 µg/mL)

Method Validation

- *Calibration curve*

A calibration curve for the standard solutions was built. The concentration range covered was between 0.5 and 80 µg / mL. The concentration of the IS was 20 µg mL^{-1}.

The analyte/IS peak-area ratios obtained were plotted against the corresponding concentrations of the analytes (expressed as µg mL^{-1}) and the calibration curves were constructed by means of the least-square method. The equation of the calibration curve is:

$$y = 0,0592x - 0,002$$

while the corresponding correlation coefficient is:

$$R^2 = 0.9995$$

demonstrating a good linearity in this concentration range of the calibration curve is shown in Figure 6.

The limit of detection (LOD) and the limit of quantitation (LOQ) were found to be, respectively: LOD = 0.15 µg / mL LOQ = 0.5 µg / mL

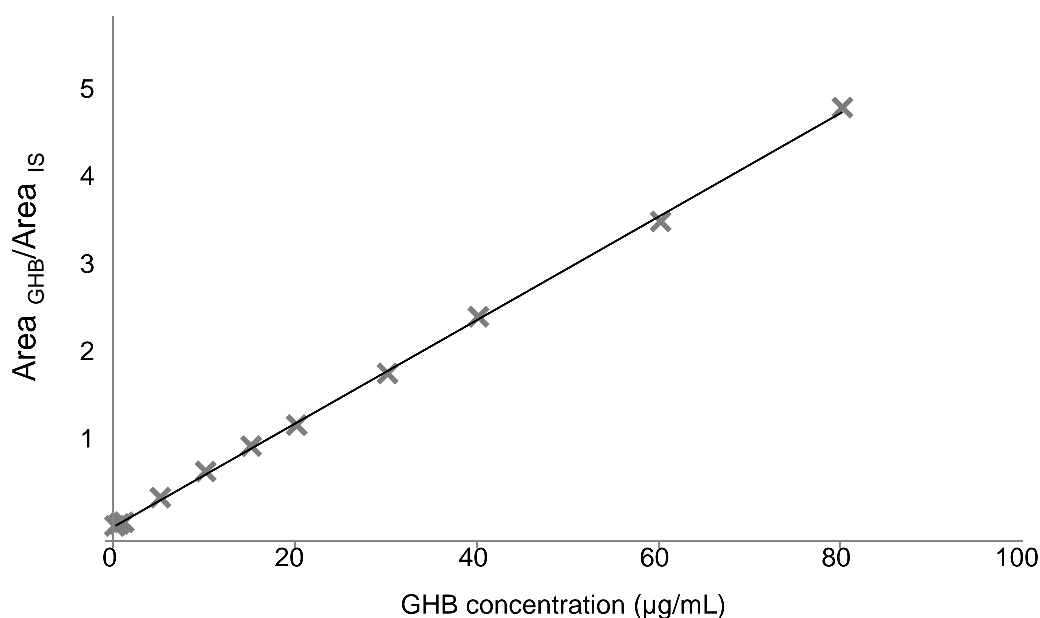


Figure 6. Calibration curve of GHB on standard solutions

- *Repeatability and intermediate precision*

The repeatability of the method was verified by running tests on standard solutions of GHB at 3 different concentrations in the same day. The concentrations of GHB analyzed were 0.5, 40 and 80 µg / mL, while the concentration of the SI is always remained constant at 20 µg / mL.

The data relating to the precision of the analysis results are good: the repeatability, expressed as intraday RSD% (relative standard deviation percentage) was found to be between 0.45% and 1.32% (Table 1). Even the intermediate precision, calculated as RSD% interday, was found to be good, with values between 0.71% and 1.91% (Table 2).

	GHB 0,5 µg/mL	*GHB 40 µg/mL*	*GHB 80 µg/mL*
	$Area_{GHB}$ / $Area_{IS}$	$Area_{GHB}$ / $Area_{IS}$	$Area_{GHB}$ / $Area_{IS}$
1	0,0218	2,090	4,154
2	0,0213	2,070	4,106
3	0,0221	2,052	4,124
4	0,0220	2,076	4,116
5	0,0216	2,109	4,148
6	0,0217	2,082	4,122
RSD%	***1,32%***	***0,92%***	***0,45%***

Table 1. Repeatability in standard solutions

	GHB 0,5 µg/mL	GHB 40 µg/mL	GHB 80 µg/mL
	Area $_{GHB}$ / **Area** $_{IS}$	**Area** $_{GHB}$ / **Area** $_{IS}$	**Area** $_{GHB}$ / **Area** $_{IS}$
1	0,0224	2,038	4,152
2	0,0216	2,088	4,090
3	0,0221	2,118	4,116
4	0,0225	2,076	4,126
5	0,0215	2,098	4,068
6	0,0222	2,086	4,108
RSD%	***1,91%***	***1,28%***	***0,71%***

Table 2. Intermediate precision in standard solutions

- *Development of pretreatment procedure*

The analysis of pharmacologically active compounds present in a biological matrix involves a preliminary treatment of the clean-up of the biological sample that may be made by various techniques: liquid-liquid extraction, protein precipitation (with organic solvents, with suitable saline solutions or with acids), procedures for solid phase extraction (SPE) or micro extraction (SPME, MEPS).

For the extraction of GHB from biological samples of plasma various pre-treatment procedures were tried including various SPE methods.

Considering the chemical and physical characteristics of GHB cartridges with different characteristics have been tested:

- SAX, strong anion exchange cartridges, and MAX cartridge mixed mode: d hydrophilic / lipophilic balance and anion exchange. Both were discarded due to the incompatibility of the eluent used with electrophoresis system.
- C8: do not recover the IS and the recovery of GHB is less than 70%.
- HLB cartridges hydrophilic / lipophilic balance, discarded because it restrains the GHB.

In light of the tests performed has abandoned the way of treatment by SPE for the clean-up of biological samples.

The precipitation by addition of organic solvents was performed using acetonitrile and methanol. The electropherograms obtained, Figure 8 and Figure 9, using acetonitrile as the precipitating agent, there is a yield lower than the extraction of methanol and also a minor purification.

The methanol was chosen as the precipitating agent and experiments have been made by adding to 100 µL of plasma, different volumes of methanol (from 200 µL to 600 µL). The best results in terms of yield of extraction were obtained by adding 300 µL of methanol, and then this volume was used to precipitate the plasma proteins.

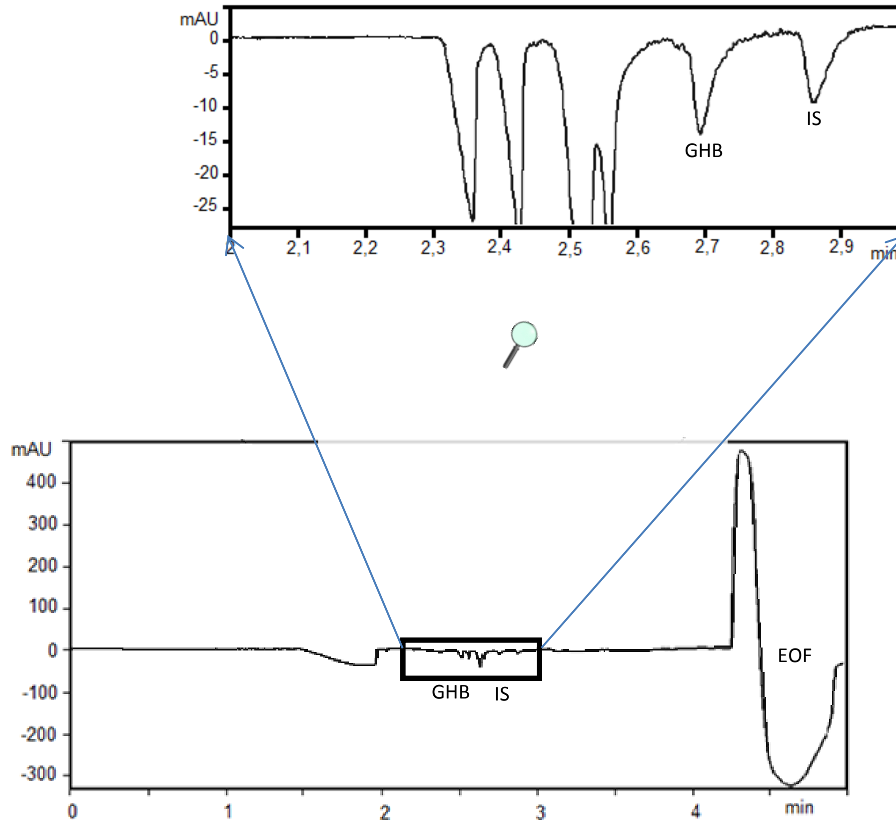


Figure 7 Electropherogram of fortified plasma sample spiked with GHB (20 µg/mL) and hexanoic acid (20 µg/mL) after acetonitrile protein precipitation pretreatment

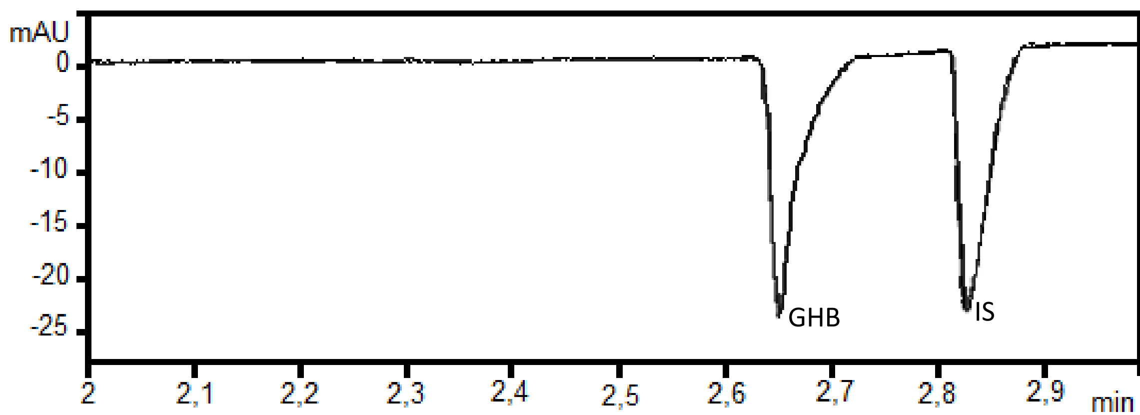


Figure 8. Electropherogram of fortified plasma sample spiked with GHB (20 µg/mL) and hexanoic acid (20 µg/mL) after methanol protein precipitation pretretment (zoom of the peak area of interest)

- *CE analysis of GHB in fortified plasma*

After the setup of pretreatment procedure, the method has been applied to plasma samples. The electropherogram of a blank plasma sample collected from an healthy volunteer, after methanolic protein precipitation, is showed in Figure 9. It can be observed that the extraction procedure is able to remove the interferences from the matrix.

The electropherogram of a plasma fortified with 80 µg / mL GHB (plasma concentration), corresponding to 20 µg/mL(effective concentration), and 20 µg / mL of IS is shown In Figure 10. The discrepancy between the concentration in plasma and the concentration in the injected solution is due to the 1:4 dilution of the sample introduced by the process of precipitation of the proteins.

The two peaks have good symmetry, they appear to be well separated and they have the same migration times observed in the analysis of standard solutions.

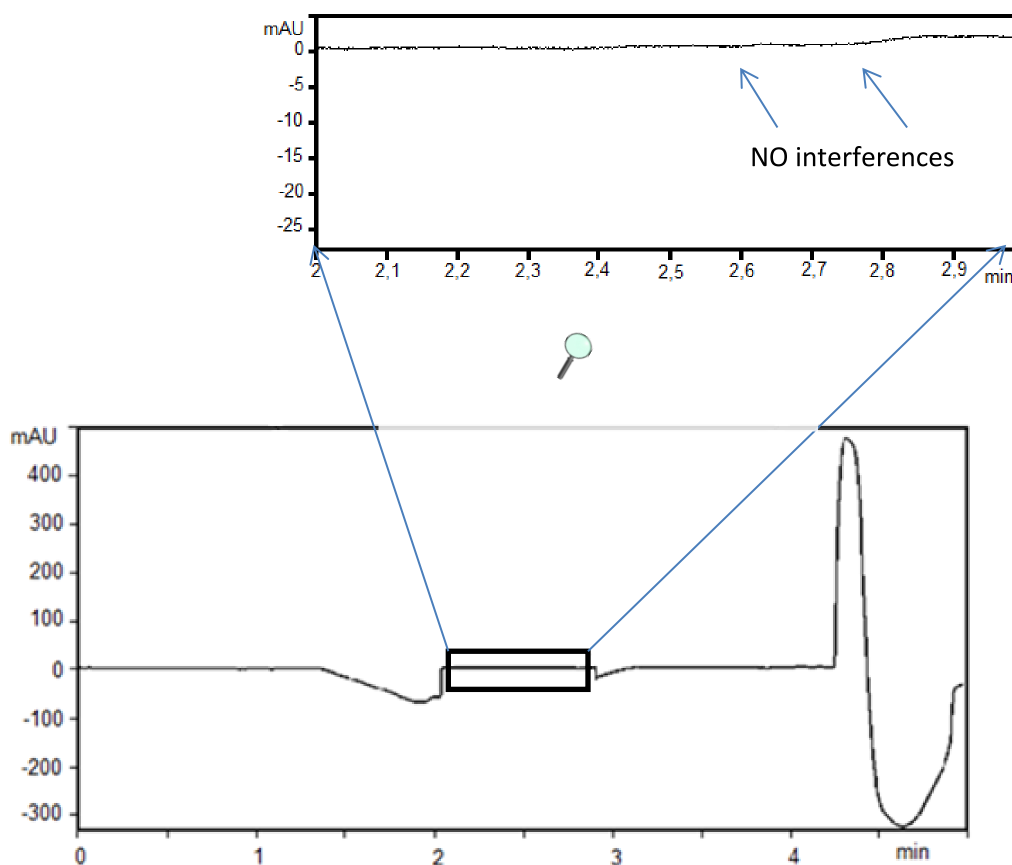


Figure 9. Electropherogram of blank plasma after sample pretreatment procedure

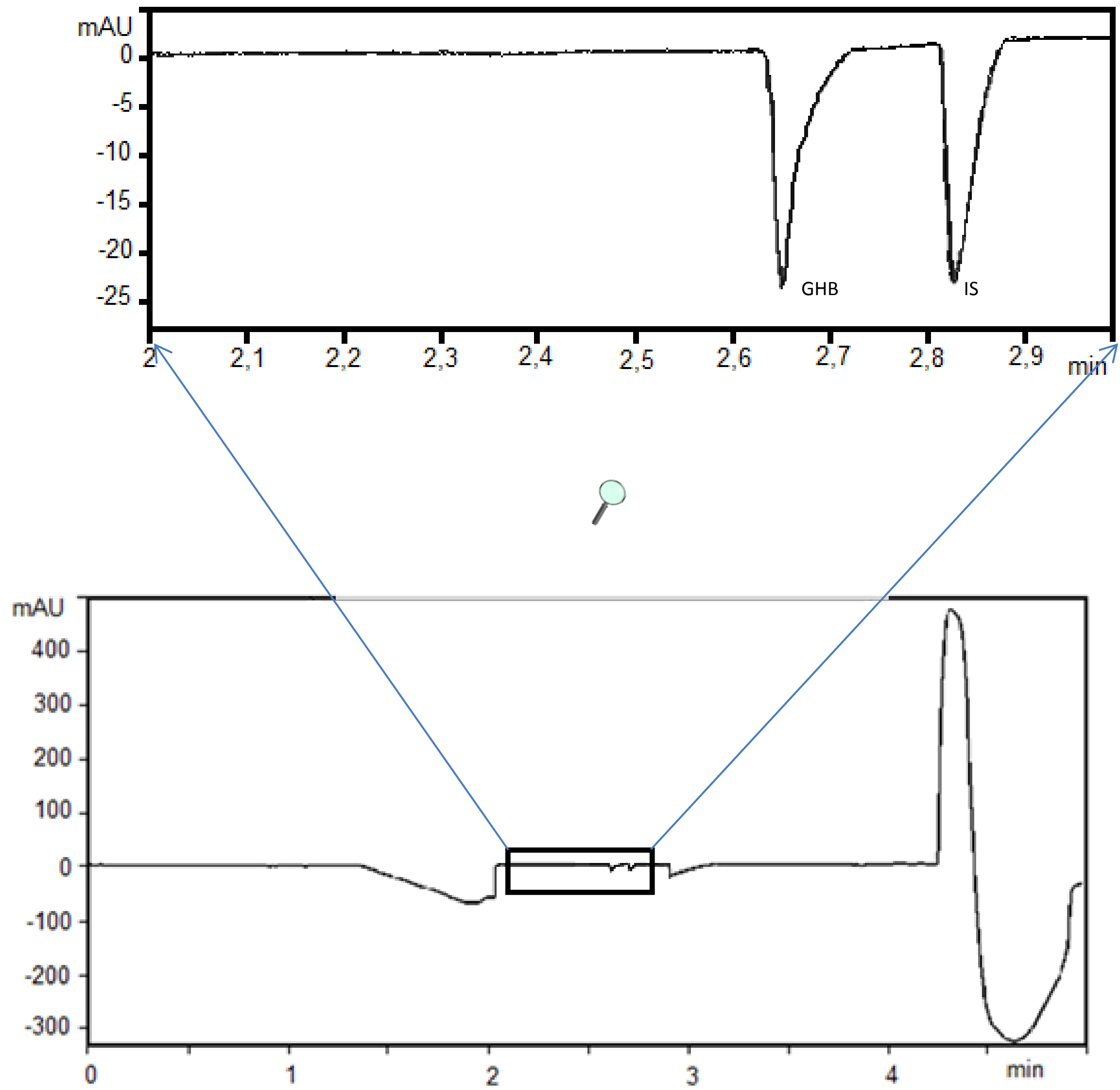


Figure 10. Electropherogram of plasma fortified with 80 µg/mL di GHB e 80 µg/mL di SI (plasmatic concentrations)

- *Calibration curve*

Calibration curve was set up for the analyte in fortified plasma and good linearity (r^2 > 0.9993) was found over the concentration range from 2 to 320 µg / mL plasma concentration, (corresponding to 0.5 to 80 µg / mL in the injected solution).

The equation of the curve obtained by the method of least squares is found to be:

$$y = 0{,}0127x - 0{,}227$$

while the regression coefficient is:

$$R^2 = 0.9993$$

demonstrating a good linearity in this concentration range of the calibration curve shown in Figure 11.

The limit of detection (LOD) and the limit of quantitation (LOQ) were respectively:

LOD = 0.6 g / mL; LOQ = 2 g / mL showing a good sensitivity of the method.

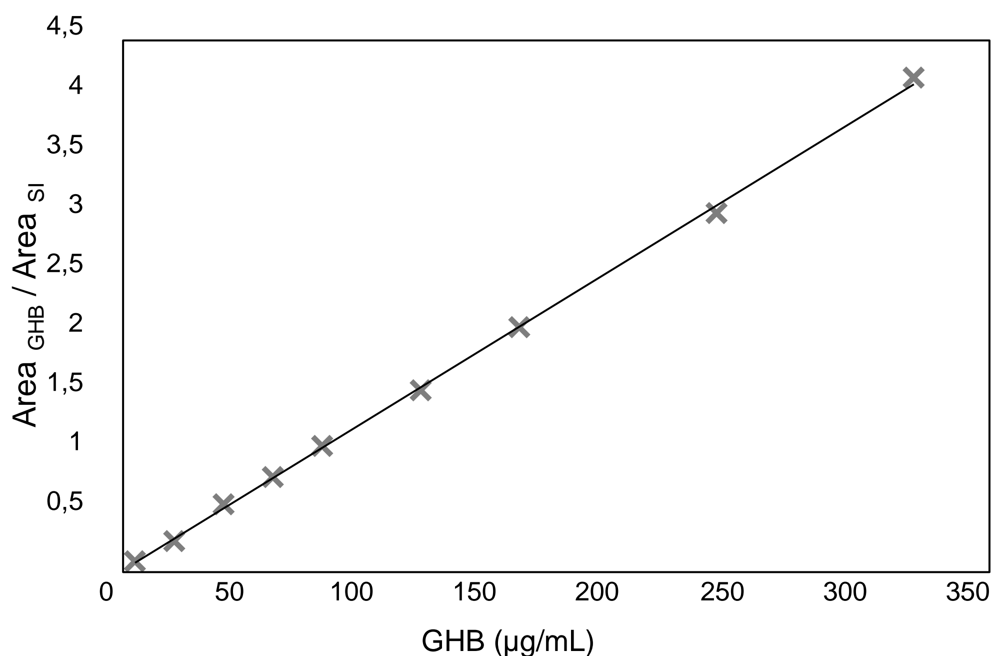


Figure 11. Linearity in fortified plasma

- *Extraction yield and precision*

The tests of repeatability, or intraday precision, and intermediate precision, or interday precision, have been performed on fortified plasma samples subjected to protein precipitation procedure with methanol. Both are expressed as percentage relative standard deviation values (RSD%)

Extraction yield and precision assays were carried out at three different concentrations, corresponding to the lowest, the intermediate and the highest point of each calibration curve. Three different concentrations of GHB: 2, 160 and 320 µg / mL were added to 100 µL of blank plasma, while the IS added is maintained at a constant concentration of 20 µg / mL.

The results of these assays are reported in Table 3. As can be seen, good extraction yield was obtained with values higher than 88%; the mean extraction yield of the IS was 95%. Precision was also satisfactory, with RSD values always lower than 4.9%.

The repeatability, expressed as RSD% intraday was found to be less than 2.36% (Table 4). Even the intermediate precision, calculated as RSD% interday, was found to be very good with values less than 4.91% (Table 5).

Analyte	Concentration (µg/mL)[a]	Extraction yeld (%)	Repetability (RSD %)	Interday Precision (RSD %)
GHB	2	88,03	2,36	4,91
	160	89,30	1,61	2,13
	320	90,90	1,08	1,49

a = plasmatic concentration

Table 3. Main validation parameters on plasma

	GHB 2 µg/mL	GHB 160 µg/mL	GHB 320 µg/mL
	Area *$_{GHB}$* / ***Area*** *$_{IS}$*	***Area*** *$_{GHB}$* / ***Area*** *$_{IS}$*	***Area*** *$_{GHB}$* / ***Area*** *$_{IS}$*
1	0,0204	1,996	4,022
2	0208	1,976	3,996
3	0,0200	1,936	4,090
4	0,0214	1,916	4,114
5	0,0203	1,974	4,068
6	0,0205	1,990	4,042
RSD%	***2,36%***	***1,61%***	***1,08%***

Table 4.intraday precision in fortified plasma.

	GHB 2 µg/mL	GHB 160 µg/mL	GHB 320 µg/mL
	Area *$_{GHB}$* / ***Area*** *$_{IS}$*	***Area*** *$_{GHB}$* / ***Area*** *$_{IS}$*	***Area*** *$_{GHB}$* / ***Area*** *$_{IS}$*
1	0,0206	1,912	4,146
2	0,0197	1,990	4,134
3	0,0208	1,968	4,050
4	0,0214	2,024	4,236
5	0,0193	1,926	4,114
6	0,0220	1,984	4,172
RSD%	***4,91%***	***2,13%***	***1,49%***

Table 5.Interday precision in fortified plasma

- *Selectivity*

The analysis of plasma samples from six different healthy volunteers showed no evidence of unacceptable interference from endogenous compounds at the retention time of the analytes and the IS. Several compounds that could be co-administered with GHB during clinical practice, such as antipsychotics, antidepressants, nonsteroidal anti-inflammatory and sedative-hypnotics, were tested for possible interference; none of them gave rise to peaks that could interfere with the determination of the analytes. including, all reported in Table 6.

Most of the substances have not been revealed in a normal electrophoretic run; other have different migration times with respect to the analyte, thus not interfere with the indirect revelation of GBH by CE- DAD, ensuring a high selectivity of the method.

ANALYTE	***Migration time (min)***	***ANALYTE***	***Migration time (min)***
GHB	**2,64**	**HEXANOIC ACID (SI)**	**2,83**
VALPROIC ACID	3,11	ISOBUTYRIC ACID	2,07
TOPIRAMATE	n.d.	GABAPENTIN	n.d.
SALICILIC ACID	4,17 (positive peak)	ACETILSALICILIC ACID	4,5 (positive peak)
INDOMETACIN	n.d.	QUETIAPINE	n.d.
GABA	n.d.	VENLAFAXINE	n.d.
LEVOMEPROMAZINE	n.d.	CARBAMAZEPINE	n.d.

Table 6. Selectivity

- *Stability*

The mean difference in the analyte/IS peak-area ratios between a standard solution of the analytes prepared from stored stock solutions and a standard solution at the same nominal concentration obtained from fresh stock solutions was -0.1%, indicating that all the analytes were stable in methanol when stored at -20°C for 1 month.

Stability was also assessed in plasma samples fortified with known amount of the analytes and stored under various conditions. The concentrations of the analytes found after storage were compared to the respective theoretical concentrations. The mean differences observed were -0.5% for plasma sample kept at room temperature for 6 hours, -1.1% for samples subjected to three freeze-thaw cycles and -1.3% for samples stored at -80°C for 1 month. The loss of the analytes was not significant, indicating that the compounds are stable under the tested storage conditions.

Analysis of plasma samples from patients. Analysis of GHB in the plasma of patients under treatment with Alcover®

The validated method was then applied to the analysis of plasma specimens collected from patients treated with Alcover®. A 2 μL aliquot of IS were added to a volume of 100 μL of plasma sample. This mixture was subjected to the pretreatment procedure than injected. The electropherogram shown in Figure 12 is an example of a plasma sample from a patient treated with 50 mL / day of Alcover® corresponding to 8.75 g / day. The usual dosage for alcohol withdrawal is 50 mg/kg/die, for 3 times in a day corresponding to 10.5 g/day for a 70 kg adult male and could be augmented till 21g /die after 60 days of treatment.[29]

The value of the concentration of GHB obtained by interpolation on the plasma calibration curve is 35 μg /mL. The blood sampling was carried out 35 minutes after administration of the drug. The maximum plasma concentration is observed, in fact, after 30 minutes of administration. This low plasma value found, which is below the therapeutic range that can vary between 50 μg / mL and 120 μg / mL[30], is consistent to clinical responses registered by the medical staff. In fact the patient was considered “non- responder” to the therapy.

A notable interindividual variability was found among the analysed patients in the plasmatic concentrations of GHB (Fig. 13). In some case plasma levels were below therapeutic range. These results support the importance of an appropriate monitoring to optimize administration, especially during the beginning of the therapy. It’s clear that these analytical data could be powerful tools supporting physicians in the pharmacological management of alcohol withdrawal.

Figure 12. Electropherogram of a plasma sample from a patient treated with 50 mL/day of Alcover® (8.75 g / day)

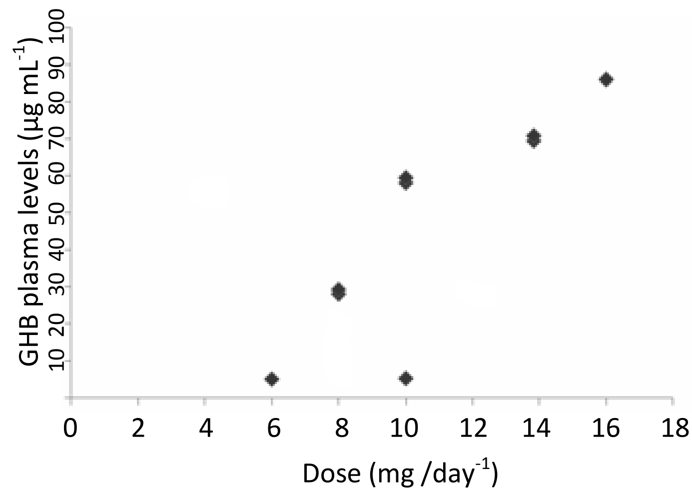


Figure 13. Plasma levels of GHB found in patients under Alcover® treatment plotted against the daily administered dose.

Accuracy

Method accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at three different concentrations were added to plasma samples from patients under GHB treatment (n = 3 for each level) previously analysed and the percentage recovery was calculated. Results were satisfactory, as recovery values were always higher than 88.0%.

Analyte	Added concentration (µg/mL)[a]	Recovery (%)
GHB	20	88,76
	80	92,97
	200	93,84

Table 7. Accuracy expressed as recovery %

3.4. **Conclusions**

An analytical method based on the use of a CE-DAD system, with indirect revelation has been developed for the analysis of GHB in plasma of alcoholic patients in therapy with Alcover®.A simple technique has been used to perform the clean-up of the matrix, based on the precipitation of the protein that allows to obtain high extraction yields (> 88%). Good results in terms of precision (RSD% always less than 4.91%) and accuracy (recoveries always above 89.0%). were obtained. The indirect revelation method gives a remarkable selectivity. Moreover the use of this electrophoretic technique implicates some other advantages as a limited use of organic solvents and very short time of analysis, always less than 5 minutes.

One of the novelties of this work is represented by the analysis of GHB based on an indirect DAD detection, taking advantage of GHB physical and chemical properties. When compared to other methods reported in the literature which make use of mass spectrometry [17,18,20-23,25,27-34,36], CE with indirect DAD detection represents a good alternative, as it offers satisfactory sensitivity and high selectivity, it requires less maintenance and it is less expensive. Moreover, it is less prone to suffer from matrix effect, which could be a drawback in mass spectrometry detection.

In conclusion the present work describes an innovative method that has proven to be accurate, fast and therefore suitable for therapeutic monitoring of alcoholic patients in therapy with Alcover®.

Acknowledgments

We would like to thank Dr. Maria Chiara Pieri (Ser.T. East, AUSL Bologna) for providing the blood samples of patients receiving Alcover®.

4. DETERMINATION OF GHB IN DRIED BLOOD SPOTS FOR THERAPEUTIC DRUG MONITORING

4.1. Introduction

The purpose of the present work was the development of a rapid analytical method for the analysis of GHB in innovative biological matrix, dried blood spots (DBSs). The technique is based on capillary zone electrophoresis with indirect UV absorption detection at 210 nm.

One of the main difficulties physicians have to face in the management of GHB treatment is represented by an heterogeneous response pattern which is partly due to GHB large interindividual pharmacokinetic variability[31]. In fact, different studies have evidenced that GHB plasma levels vary for a given dose, so contributing to variability in clinical response In addition, during therapy, many patients take concomitant medications that might induce or inhibit some of the CYP isoforms involved in GHB metabolism, thus affecting its pharmacokinetics. This accentuates interindividual response variability and consequentially influences the clinical effects and the safety profile of the drug.

The measurement of hematic concentrations of GHB can help assess patient compliance, tailor the dose to the patient's needs, minimize the risk of dose-related toxicity and can be useful for clinical decision making. Thus, an accurate therapeutic drug monitoring (TDM) of GHB should be carried out.

As blood (plasma, serum or whole blood) is the optimal matrix for TDM, the use of dried blood spot (DBS) sampling technique can be an attractive approach. In fact, DBSs are easily obtained by a finger prick. The resulting drops of blood are collected onto filter paper and allowed to dry. The use of DBSs offers several advantages: it avoids venous blood withdrawal, thus reducing patient worry, it simplifies storage and transport as no refrigeration is required and it decreases the risk of infection with blood-borne pathogens[32] .

To the best of knowledge, no analytical method is currently available for the determination of GHB in DBSs by means of capillary electrophoresis from patients under alcohol withdrawal

treatment. In fact, papers from the literature deal with the determination of GHB in human plasma/serum[33], urine, whole blood[34] samples by means of HPLC methods with fluorecence detection[35], Raman spectroscopy[36] or GC coupled with mass spectrometry (MS)[37].The proposed method is faster and simpler than the others present in literature, and did not require derivatisation step.

Therefore, given the importance of TDM for GHB and the advantages offered by DBS sampling technique, the aim of the present study was to optimize the method previously developed for the indirect determination of the GHB in human plasma (see page 4) for his analysis in DBS.

Chemicals

Methanolic stock solution of GHB (1 mg mL^{-1}) was purchased from LGC Standards (Teddington, UK). Hexanoic acid, used as the internal standard (IS, Figure 1), was from Janssen Chemicals (Beerse, Belgium). Acetone and methanol, 85 % (w/w) phosphoric acid and disodium hydrogen phosphate were obtained from Sigma Aldrich.

TRIS (Tris(hydroxymethyl)aminomethane), TBAB Tetrabutylammonium Bromide, isovanillic acid, didhydroxybenzoic acid, acetilsalicilic acid, phthalic acid were provided by Sigma Aldrich (Steinheim, Germania);

CTAB (Cetyl-Trimethylammonium Bromide), used for the preparation of BGE, and sodium hydroxide, were supplied by Carlo Erba (Milan, Italy).

Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA).

Preparation of stock and working solutions

The stock solution (1 mg/mL) of GHB was purchased from the pharmaceutical manufacturer. The stock solutions (1 mg/mL) of hexanoic acid (SI) were prepared by diluting the solution of hexanoic acid with a density of 0.927 g / mL. The exact volume of 10.8 μL of this solution were diluted with MeOH in a 10 mL volumetric flask.

Working standard solutions were prepared, daily, by diluting primary stock solutions to desired concentrations, in ultrapure water. All solutions were stored in the freezer at a temperature of -20 ° C. Stock solutions were stable for at least 3 months when stored at -

20°C (as assessed by CE assays).

Sample collection

DBS samples were obtained by puncturing the subjects (healthy volunteers and patients under GHB) on a finger with single use lancing device. The first drop of blood was wiped away using dry sterile gauze, then the blood drops were collected on a FTA® card Whatman® International Ltd (Maidstone, Kent, UK), paying attention not to go outside the pre-marked circles. The blood spots thus obtained were left to dry for 3 h in the dark at room temperature and then stored in a sealed paper bag (if necessary with a suitable desiccant, i.e. silica gel).

All the blood samples were collected between 8.00 a.m. and 10.00 a.m. from fasting patients under GHB at local Drug Addiction Treatment Centres (Ser.T.); this use for TDM was already authorised at the time of sampling.

Equipment

The instrument used for the analysis of GHB in plasma is a capillary electrophoresis system (CE-3D, Agilent, Palo Alto - CA, USA) equipped with a photodiode array detector (DAD).

The separation was conducted using a fused silica capillary (Composite Metal Services, Hallow UK) with the following characteristics: total length (TL) of 48.5 cm, effective length (EL) of 40.0 cm and internal diameter (ID) of 75 microns.

The samples were injected from the anode side of the capillary by means of a pressure of 50 mbar for 10s.

The applied voltage was -30 kV. The wavelength of 210 nm was set for detection.

At the beginning of each day, the capillary was conditioned with ultrapure water, 1N NaOH, ultrapure water and BGE, for ten minutes each.

A Crison (Barcelona, Spain) MicropH 2000 pHmeter, a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge, an IKA (Staufen, Germany) RV 10 rotary evaporator and a microwave oven were also used. Whatman (Maidstone, UK) FTA® classic cards (WB120205) were used for DBS sample collection.

Data were handled by means of ChemStation software from Agilent (Santa Clara, CA, United States).

BGE preparation

Prior to use, the mobile phase was filtered through Varian nylon filters (47 mm diameter, 0.2 µm pore size) and degassed by sonication.

The backgound electrolyte (BGE) is constituted by a TRIS buffer 50 mM and benzoate buffer 7mM obtaining pH 8.9.

This buffer is prepared as follows: 50.4 mg of benzoic acid is weighted ad dissolved with approximately 25 mL of water, agitated on a plate heated to 37 ° C. The solution is cold avoiding the formation of benzoate cristals and 302,8 mg of TRIS are added to the clear solution obtained. The solution is adjusted to pH 8.9 with 1 M NaOH, appropriately diluted, and then transferred to a 50 cc flask and filled with ultrapure water. Prior to use, the BGE was filtered through Varian cellulose filters (47 mm diameter, 0.45 µm pore size) and degassed by sonication.

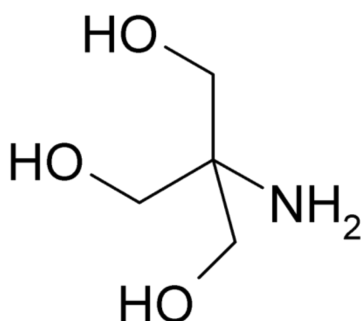


Figure 14.TRIS molecular structure

Extraction from DBS and sample pre-treatment

One or more DBS disks were cut out of the card after the addition of 10 µL of IS solution (on CE concentration 10 µg mL^{-1}) and were placed into a vial with 300 µL of methanol. The vial was put in a votex for 60 s, then it was sonicated for 5 minutes and finally centrifuged for 10 min at 4000 rpm. Then the extract was brought to dryness under vacuum using a rotary evaporator, dissolved again with 50 µL of methanol and directly injected to CE.

The volume of blood contained in the blood spot disks was determined by pipetting known volumes of blood (from 5 to 50 µL) onto the FTA® paper cards with a micropipette: the diameter of the regular-shaped blood spots was then measured and a calibration curve constructed. A power equation was fitted and the equation was used to determine the volume of blood contained in the DBS disks from patients:

$$y = 0.931\ x^{0.352}$$

where y and x were the diameter and the volume of blood spot, respectively. The volume of blood determined in this way was used for calculating the concentration of GHB in all samples analysed.

Method validation

The method was validated following USP XXXVIII[26] and “Crystal City”[27] guidelines.

- *Linearity, limit of quantitation, limit of detection*

Aliquots of 6,25 μL of GHB standard solutions at six different concentrations (in order to obtain on-capillary concentrations over the 1-200 μg mL^{-1} range), containing the IS at a constant concentration (in order to obtain on-capillary concentration of 25 μg mL^{-1}), were added to DBS disks (after the addition DBS samples were left to dry for at least 1 h). The resulting fortified DBS were subjected to the previously described sample preparation and injected into the CE system. This procedure was done in triplicate for each point. The analyte/IS peak-area ratios were plotted against the corresponding concentrations of the analyte (expressed as ng mL^{-1}) and the calibration curve was constructed by means of the least-square method.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the analyte concentrations which give rise to peaks whose heights are 3 and 10 times the baseline noise, respectively.

- *Extraction yield and precision*

Aliquots of 6,25 μL of GHB standard solutions at three different concentrations (in order to obtain on-capillary concentrations of 4, 50 and 100 μg mL-1), containing the IS at a constant concentration (in order to obtain on-capillary concentration of 25 μg mL^{-1}) were added to DBS disks. The resulting spiked DBS were subjected to the previously described pre-treatment procedures and finally injected into the CE system. The analyte peak areas were compared to those obtained injecting standard solutions at the same theoretical concentrations and the absolute recovery was calculated.

The assays described above were repeated six times within the same day to obtain

repeatability (intraday precision) and six times over six different days to obtain intermediate precision (interday precision), both expressed as percentage relative standard deviation values (RSD%).

- *Selectivity*

Blank DBS samples from six different healthy volunteers were subjected to the sample pre-treatment procedure and injected into the CE system. The resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was no interfering peak higher than an analyte peak corresponding to its LOD. Furthermore, standard solutions of several different compounds that could be co-administered in clinical practice were injected into the CE system. A substance was classified as interfering if it gave rise to a peak that was not baseline separated from those of the analytes or the IS.

- *Stability*

Stability assays were carried out in DBS samples from three patients stored at room temperature (25 ± 3°C) over a period of 1 month (n = 3). DBSs were kept in the dark and in paper bags with a suitable desiccant (i.e. silica gel). The concentrations of GHB found in the stored DBSs were compared to those obtained from the corresponding samples extracted and analysed immediately after initial spotting and drying.

- *Accuracy*

Accuracy was evaluated by means of recovery assays. Aliquots of 6,25 μL of GHB standard solutions at three different concentrations (i.e. 10, 50 and 100 μg mL^{-1} of GHB on-capillary concentrations) containing the IS at a constant concentration (in order to obtain on-capillary concentration of 25 μg mL^{-1}) were added to real DBS or plasma samples from subjects under

Alcover® therapy whose content of GHB was previously determined.

Recovery values were calculated according to the following formula: 100 ([after spiking] – [before spiking]) / [added].

4.2. **Results and discussion**

Development of electrophoretic conditions

Initially, the same electrophoretic conditions used for the analysis of GHB in human plasma (i.e. small inner diameter capillary, 50 µm, combined with a mixture of benzoic acid CTAB and methanol as BGE) were applied (see page 10). However, for cheapness and simplicity the previous capillary was replaced by another one with grater inner diameter the same functional group but with a conventional inner diameter (4.6 mm instead of 3.0 mm). Consequently, the mobile phase was adapted to suit the chromatographic behaviour of the analyte and the IS on the new column: a mixture of phosphate buffer (25 mM, pH 6.4) and acetonitrile (45:55, v/v) gave satisfactory results.

Some trials were carried out to improve the performance of the CE system in terms of selectivity and sensitivity.

The electropherogram of a standard solution containing 50 µg mL^{-1} of MTD and 25 µg mL^{-1} of IS is shown in figure. Migration times (mr) are: GHB, tm = 3.0 min; IS, tm = 5.6 min.

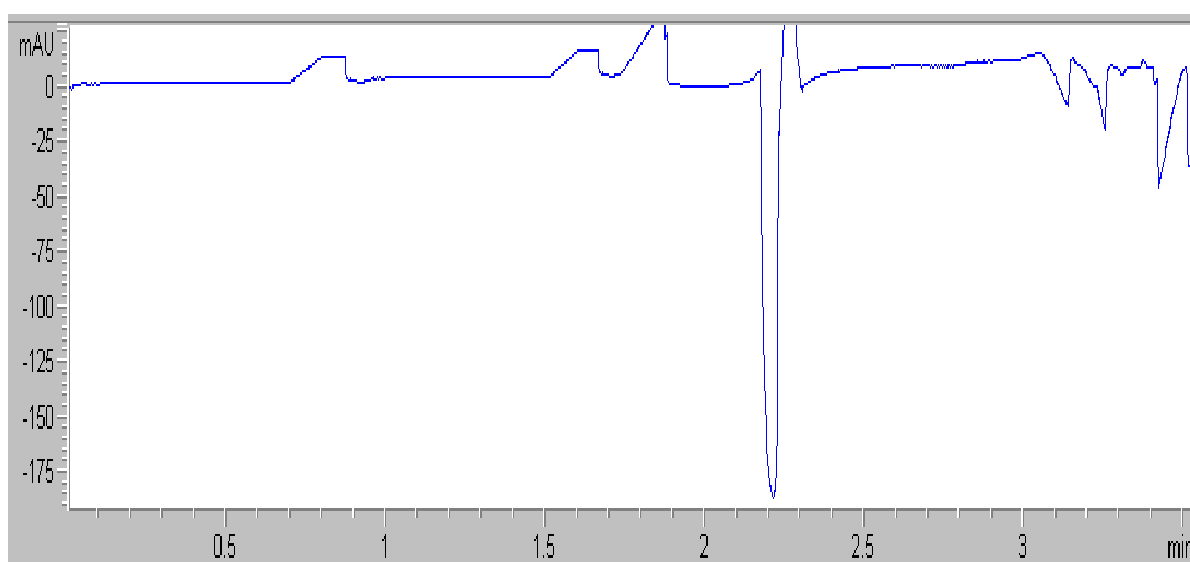


Figure 15 electropherogram of a standard solution containing 50 µg mL^{-1} of MTD and 25 µg mL^{-1} Migration times (mr) are: GHB, tm = 3.0 min; IS, tm = 5.6 min

Development of the extraction from DBS

Several assays were carried out to obtain reliable results and to optimize extraction efficiency of molecules from DBSs. It may vary depending on the best combination of some operating parameters, such as the duration of the extraction procedure, the nature of the solvent employed and the technique used to aid the removal of the compounds from the filter paper.

Spiked DBSs were treated with different solvents or mixtures (acidic or basic buffers, methanol, acetonitrile, buffer/organic solvent mixtures): aprotic organic solvents proved to be unsuitable to remove GHB from the paper (extraction yields < 70%), while aqueous buffers eluted red blood cells which interfered with the assay. Only methanol gave promising results in terms of sample cleaning and extraction yields (> 90 %).

Extraction time and mixing technique were also tested. In particular, ultrasound and vortex agitation were investigated. The extraction efficiency for vortex and ultrasound agitation increased while increasing the duration of the extraction time from 1 to 5 minutes giving satisfactory extraction yield, hence, the combination of these techniques was adopted.

The methanol was then chosen as the precipitating agent; different volumes of methanol, from 200 μL to 600 μL were added to a dried blood spot of 25 μL (finely cut to increase the surface area). The best results in terms of yield of extraction were obtained by adding 300 μL of methanol, and then this volume was used to extract the analyte in solution and at the same time, precipitate the proteins.

The subsequent centrifugation step (4000 rpm for 10 min) allowed to separate the solid residue consisting of fragments of paper and denatured proteins by metabolic solution containing the analyte.

The use of pure methanol allowed to dry out the solution obtained by rotary evaporation and concentrate the analyte six times (from 300 μL to 50 μL).

Method Validation

Calibration curves were set up in blank matrices fortified with different concentrations of GHB and a constant concentration of the IS. Good linearity was found in the 1-200 µg mL^{-1} on-capillary concentration range . The LOD and LOQ values were 0.3 µg mL^{-1} and 1 µg mL^{-1}, respectively.

Extraction yield and precision assays were carried out at three different concentration levels of GHB, corresponding to the lowest, the intermediate and the highest point of the calibration curve The results were satisfactory, being the extraction yield values higher than 90%; the mean extraction yield of the IS was 92%. Precision was also good, with RSD values always lower than 4.6%.

Analita	Concentration (µg/mL)[a]	extraction yeld (%)	Repeatabilit y (RSD %)	intraday precision (RSD %)
GHB	1	90,10	2,41	4,62
	50	92,30	1,78	2,17
	200	93,90	1,17	1,27

Table 8 validation parameters

- *Selectivity*

The analysis of blank DBS and plasma samples from six healthy volunteers showed no evidence of unacceptable interference from endogenous compounds at the retention times of MTD and the IS. Standard solutions of some opioids and some antipsychotic, antidepressant and sedative-hypnotic drugs were injected into the CE system at concentrations higher than the upper limit of the calibration curve. None of them gave rise to peaks that could interfere with the determination of GHB.

- *Stability*

Stability was evaluated in DBS samples from 3 patients stored for 1 month at room temperature. The concentrations of GHB found in the specimens after storage were compared with those determined in the fresh samples, revealing a mean difference of -2.3%. This percentage of loss is not significant, especially if the relative long time of storage (1 month) is considered. Thus, GHB can be considered stable in DBS under the tested condition of storage.

Analysis of samples from patients

The method was applied to the analysis of DBS and plasma samples collected from 6 former alcohol addicted subjects under Alcover. As an example, the electropherogram of a DBS sample from one of these patients treated with 50 mg day-1 of GHB is reported in figure 16 , , a good correlation ($r^2 = 0.998$) was obtained between the concentrations of GHB found in DBS and those found in the corresponding plasma samples, taking into account the presence of haematocrit in DBS and its absence in plasma. In fact, since the haematocrit is normally about 40–54% for men and 36-46% for women (mean 45%), the concentrations found in DBS samples were multiplied by a correction factor of 1.79 to obtain the corresponding plasma concentrations.

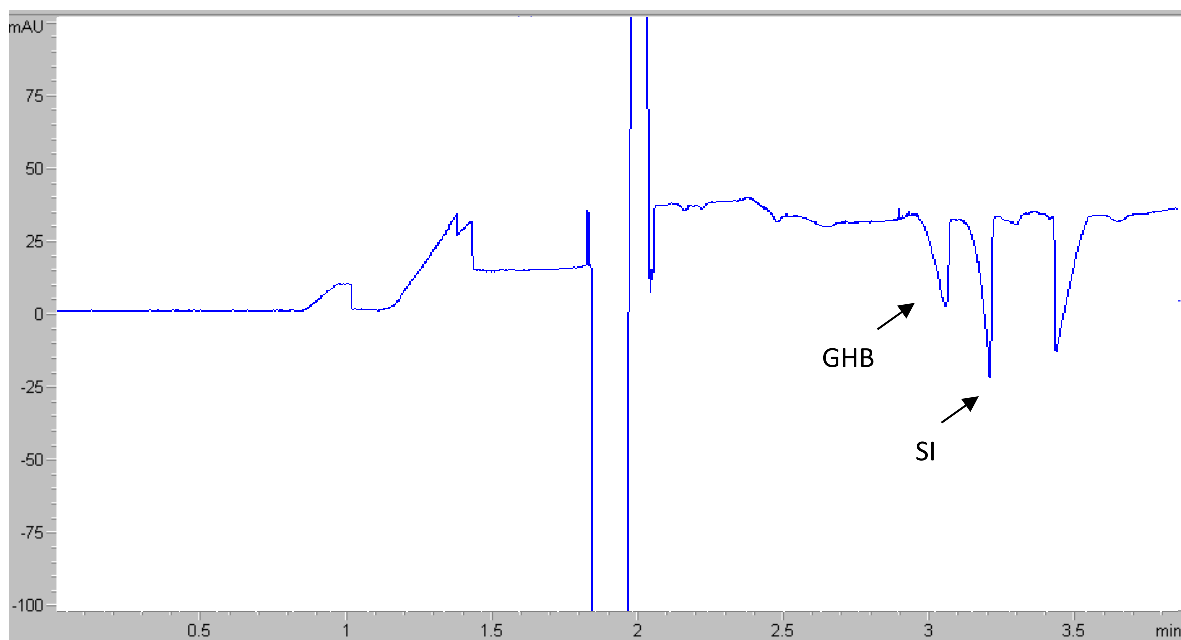


Figure 16 electropherogram of a DBS sample from one of these patients treated with 50 mg day-1 of GHB

Accuracy

Method accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at three different concentrations were added to DBS or plasma samples from patients previously analysed and the percentage recovery was calculated. Accuracy was satisfactory, being recovery values always higher than 90.0%.

Analita	***Concentration added (µg/mL)[a]***	***Recovery (%)***
	1	*90,16*
	5	*90,66*
	50	*92,84*

Table 9 Accuracy

4.3. Conclusions

A reliable CE method with indirect DAD detection has been optimized for the determination of GHB in DBS and human plasma samples. GHB removal from DBSs was performed by means of ultrasound-assisted extraction, clean up and precipitation with methanol.. The reliability of DBS specimens in the determination of GHB blood levels was evaluated: the analyte proved to be stable in the DBS matrix for at least one month and the concentrations of GHB obtained from the analysis of DBS samples were perfectly comparable with those found in the corresponding plasma specimens. No interferences were found neither from the most common exogenous nor from endogenous compounds. Our procedure can offer a rapid, precise and accurate method for GHB determination in innovative biological fluids, which could be important for screening purposes in routine clinical analysis.

Since the outcome of alcoholic withdrawal therapy is highly variable depending on different factors, such as individual metabolism, it is of principal importance to accurately determine GHB blood levels in order to optimize the dosage in each subject. The developed method allows to perform TDM of patients under Alcover® treatment with all the advantages offered by DBS technique, including low invasiveness of the sampling procedure and low cost of sample collection, transport and storage.

5. ANALYSIS OF ADULTERANTS PRESENT IN STREET DRUGS BY CE-DAD

5.1. Introduction

The presence of pharmacologically active adulterants in seized illicit drugs is common, and many of these substances can cause unwanted local and systemic reactions, therefore contributing to the overall toxicity of the "street drug".

Due to this situation, the analytical information derived from the analysis of street drugs is very important for legal and crime investigation purposes. Capillary electrophoresis (CE) is well suited for the analysis of illicit drugs: it can separate a wide variety of solutes with high efficiency and selectivity, including highly polar, thermally labile and/or non-volatile compounds which could prove difficult to analyse via gas chromatography (GC) and high performance liquid chromatography (HPLC)[38].

The aim of this study is the development of a micellar electrokinetic chromatography (MEKC) method suitable for the analysis of several different basic, acidic and neutral solutes, for the purpose of screening adulterants in seized substances.

The proposed method uses a BGE containing SDS in phosphate buffer, a 48.5 cm uncoated fused silica capillary and a photodiode array (PDA) detector set at 233 nm. It is capable of discriminating atropine, lidocaine, quinidine, acetaminophen, scopolamine, caffeine, theophylline, salicylic acid and tetramisole in their mixtures.

The illicit market of drugs of abuse is constantly evolving and the active ingredients, especially when they reach a status relevant business, may be subject to adulteration and sophistication.[39]

The main reason that pushes to dilute drugs is economic. In fact the so-called "cut" allows cost reduction in production that has as a direct result of the increase in profits of drug traffickers[40].

The knowledge of the impurities present in street drugs, "street drugs", it is important for the

understanding of the phenomena of "accidental overdose" of unexpected side effects and routes of distribution to various countries[41]

Among these are harmless diluents such as sugars, but also substances that induce a physiological response defined adulterants and processing residues, contaminants.

Substances that have a pharmacological, that mimic or enhance the effects of the drug are considered adulterants.

Adulterants can be in any illegal substance, at any concentration. It is important to recognize the presence of these substances when a person has a "mixed picture", a manifestation of unusual side effects, after taking an illegal substance. The synergistic effect produced by the presence of adulterants in the composition of "street drugs" can be a determining factor in the pathogenesis of acute intoxication by drugs as well as a previous or concomitant use of other substances such as alcohol or drugs may amplify the toxicity .Adulterants used are countless variations and ever new, difficult to categorize in analysis laboratories. For the Police Forces, tracking drugs requires constant updates and high costs: the lists of prohibited substances, despite efforts in the media and in research, are not always complete and still differ from state to state[42]. The content of adulterants increases in the various steps of the distribution chain[43].

The main adulterants properties are reported in table 8.

Adulterant	Street Drugs	legal use	Potential as adulterant	toxicity
Caffeine	Heroine, Cocaine, Amphetamine	Stimulant	psychoactive effect, essentially legal, economic, easily available, facilitates vaporization heroin;	Anxiety, mood disorders and sleep disorders; Malignant syndrome MDMA
Paracetamol	Heroine	Anti inflammatory	substance readily available, inexpensive analgesic properties similar to heroin	Gastro-intestinal disorders, damage to the liver Adri, interaction with alcohol
Quinine, quinidine	Heroine, cocaine	Antimalarial, anti-arrhythmic	Bitter taste similar to heroin, mimics the rush respiratory heroin injection	Kidney damage, cinconism, gastric disorders, thrombosis,
Scopolamine	Heroine	Anticholinergic alkaloid, spasmolytic mydriatic,	Colorless, tasteless, odorless, and not easily detectable, amnesia, impaired vision, relaxing	Low doses cause drowsiness; high doses cause euphoria Hallucinations, dry mouth, tachycardia
Atropine	Heroine	mydriatic,	impaired vision, relaxing	Hallucinations, dry mouth, tachycardia
Lidocaine, procaine	Cocaine	Local anesthetic, antiarrhythmic	Anesthetic effects similar to those produced by cocaine	Cardiovascular and CNS; Increases toxicity of cocaine. Nausea, vomiting, dizziness, tremors, convulsions
Tetramisole	Cocaine	anthelmintic	active metabolite aminorex CNS stimulant, increasing dopaminergic trasmmission	Highly toxic. Fever, agranulocytosis
diphenhydramine	Cocaine, heroine	antihistamine	perception of better quality of drugs	euphoria, disorientation, drowsiness appetite effect gastrolesivo
Acetylsalicylic Acid	MDMA, heroine	anti-inflammatory, antipyretic, anticoagulant	perception of better quality of drugs, mitigates the negative effects (headache)	ulcer

Table 10. Main adulterants properties

The opportunity to determine the content of adulterants in "street drugs" has always aroused great interest especially the implications in terms of legal and investigation and has seen the use of different analytical methods[44].

The first analyzes that are performed on samples of drugs usually using colorimetric techniques. These tests are often performed on the raw sample before going to isolate individual compounds. For the identification of heroin, morphine, amphetamine, methamphetamine and ecstasy using the Marquis reagent while for cocaine using the reagent of Scott. These substances can also react with adulterants for which they are requested analytical further confirmations[45].

The direct identification and screening of mixtures of many substances of abuse and adulterants was carried out using techniques of infrared spectroscopy (IR)[46]. The IR spectrum produces a pattern that is unique in that it bound to the chemical structure of the drug and can be compared with a reference. Some authors consider the fact IR as a method of identifying "fingerprint". This method, however, has limitations: to obtain a spectrum acceptable sample must be very clean and free of debris watery. This means that to use this method at the legal level is often necessary to resort to extraction processes to remove impurities, consequently extending the analysis time.

Numerous methods developed for the analysis of adulterants in the literature involve the use of gas chromatography, usually coupled to a flame ionization detector (GC-FID)[47]. This is a standard technique for forensic investigations in the last three decades[43]. In 2003 was published a research which used this method for the identification and quantification of cocaine and adulterants detected in powder seized in Brazil[44]. A subsequent publication has shown the ability to optimize and harmonize the analytical data obtained from two different GC-FID in two different laboratories, in France and in Switzerland in order to draw a profile of the samples of cocaine[41]. This method, however, does not allow the analysis of non-volatile compounds that you might be present in dust from the road, certain thermolabile substances can undergo thermal degradation due to phenomena of catalytic pyrolysis, some substances requiring chemical derivatization and the analysis involves high costs. In addition, the FID detector has limits because it allows to detect only the compounds that can burn. Other components can be ionized by passing along the flame, but could not produce a signal strong enough to be detected by the instrument[48].

The GC method is used mainly coupled to the mass spectrometer (GC-MS) for the analysis of

samples of heroin and cocaine. A recent study reported the purity, the concentration range and frequency of use of adulterants in 471 samples of 962 cocaine and heroin seized in Luxembourg[40]. This allowed us to estimate the variation of these substances in the years and speculate about their origin.

New analytical methods have also been developed in high-performance liquid chromatography (HPLC) coupled with various detectors; Therefore, it has been possible to perform new analysis more reliable for the determination of the concentration of adulterants in various powders sold in the street. It is often used a reverse-phase chromatography in which the separation takes place in a adulterants C8 or C18 column with a mobile phase polar such as methanol, acetonitrile, THF or acid solutions and the elution is carried out according to gradient[49]. In 2009 different samples of cocaine were analyzed by reverse phase HPLC with electrochemical detector with accurate results. This proposed method has proved particularly convenient and suitable for routine analysis of samples of substances of abuse seized. The HPLC method-DAD[49] uses multiple wavelengths and allows to highlight the potential presence of adulterants in all samples examined. In this way it is possible to perform a monitoring of these with savings in terms of analysis time and cost.

Although some HPLC methods are advantageous because they can simultaneously analyze abuse substances, impurities and adulterants, also have some disadvantages including the resolution of peaks limited. Furthermore, the basic compounds, acids and some neutral substances are separated in the same region as the chromatographic lipophilicity of the solute (log P) plays a predominant role in the mechanism of separation.Other methods developed for the analysis of adulterants in the literature involve the use a liquid chromatography coupled to the mass spectrometer (LC-MS).

The capillary electrophoresis (CE), in offers important advantages over HPLC and GC including excellent separative efficiency of complex samples, rapid analysis, a reduced consumption of samples and of organic solvents, an instrumentation simple and more resistant, and various modes of separation suitable for the determination of a large number of analytes: very polar substances, heat-labile, and non-volatile compounds. As a result, the EC has been widely used in quality control and monitoring therapeutic drug but still little used in the legal and forensic.

Since adulterants are substances of various nature (different charge, different different lipophilicity and pKa) it was considered appropriate to develop an electrophoretic technique that

exploits these characteristics to improve separation. The method MEKC is the most appropriate because it takes the advantages of the EC and those of HPLC in a single system.

5.2. Experimental

Chemicals

Paracetamol, acetylsalicylic acid, the tetramisole, quinidine, diphenhydramine, atropine, scopolamine, lidocaine and caffeine were supplied by Sigma Aldrich (St. Louis, Missouri, USA), sodium dodecyl sulfate (SDS), lactose, starch and the solutions of methanol, and ethanol were purchased from the same company, the disodium hydrogen phosphate and phosphoric acid were produced by Carlo Erba (Milan, Italy). The L-sulpride (N - [[(2S) -1-etilpirrolidin-2-yl] methyl] -2-methoxy-5-sulfamoilbenzammide) 4 used as the internal standard (IS, Figure) was supplied by Sigma Aldrich (St. Louis, Missouri, USA).

The ultrapure water (18.2 MΩ cm) was obtained using a Milli-Q system supplied by Millipore (Milford, Massachusetts, USA)

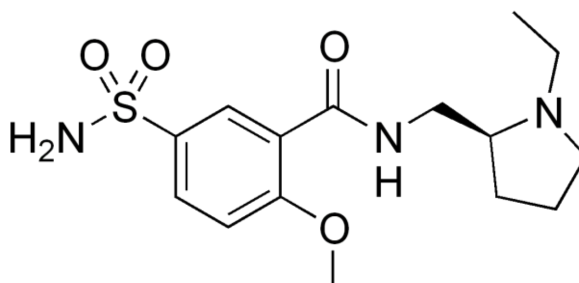


Figure 17. L Sulpiride structure

Preparation of stock and working solutions

The stock solutions of adulterants were prepared by weighing 1.00 mg of the substance and solubilizzandola in 1,000 mL of methanol. The stock solution of sulpiride was prepared by weighing 1.00 mg of the substance and solubilizing in 1,000 mL of methanol.

Both stock solutions were diluted to the desired concentrations using the BGE (Back Ground Electrolyte) to obtain working solutions. All solutions were stored in a freezer at -18 ° C. The backgound electrolyte (BGE) is constituted by a benzoate buffer 15 mM at pH 5.5 containing 0.5 mM CTAB and MeOH to 30% (v / v).

The BGE consists of 12.5 mM phosphate buffer at pH = 8.5 + 25 mM SDS. The phosphate buffer was prepared in the following way: were weighed 88.72 mg of dibasic sodium phosphate and were dissolved in about 15 mL of ultrapure water; the solution was then brought to the desired pH with a solution of concentrated phosphoric acid freshly prepared and finally the volume was brought to 50.0 mL with distilled water. Subsequently, 5.0 mL of buffer were used to solubilize 36,05 mg of SDS.

The solution was then filtered with filters of regenerated cellulose with a pore diameter of 0.20 µm.

Simulated sample preparation

The powder samples were prepared by reproducing the composition of street drugs reported in literature due to seizure of the police. The percentages of diluents and adulterants were reproduced exactly. The drug was not present instead (simulated sample). The "cut" occurred by means of geometric dilutions

Sample pre-treatment

The pretreatment of the powders from simulated strret drugs is carried out by extraction with methanol. Are weighed 50.0 mg of powder to which is added 1.00 mL of methanol. The mixture is left under stirring at room temperature for a total time of 30 min. After 10 min, the heterogeneous mixture is centrifuged for ten minutes at 4000 rpm, the supernatant is recovered and the residue is done two more extractions with 1.00 mL of methanol. The three solutions are then combined in a test tube. They are then taken 100 L of methanol solution and diluted 1:10 in BGE. Before injection into the EC solution is filtered with filter regenerated cellulose by 0.20 m.

Equipment

The instrument used for the analysis is a capillary electrophoresis system (CE-3D, Agilent, Palo Alto - CA, USA) equipped with a photodiode array detector (DAD)set to 218 nm, 233nm, 274nm.

The separation was conducted using a fused silica capillary (Composite Metal Services, Hallow UK) with the following characteristics: total length (TL) of 48.5 cm, effective length (EL) of 40.0 cm and internal diameter (ID) of 50 microns.

The samples were injected from the anode side of the capillary by means of a pressure of 50 mbar for 10s.

The applied voltage was 20 kV. The wavelength of 210 nm was set for detection.

At the beginning of each day, the capillary was conditioned with ultrapure water, 1N NaOH, ultrapure water and BGE, for ten minutes each.

A Crison (Barcelona, Spain) MicropH 2000 pHmeter, a Hettich (Tuttlingen, Germany) Universal 32 R centrifuge and an IKA (Staufen, Germany) RV 10 rotary evaporator were also used.

Data were handled by means of ChemStation software from Agilent (Santa Clara, CA, United States).

Method validation

The method was validated according to USP XXXVIII26[26] and “Crystal City”[27] guidelines.

- *Linearity, limit of quantitation, limit of detection*

Aliquots of 10 μL of analyte standard solutions at six different concentrations, containing the IS at a constant concentration, were added to 100 μL of BGE. The resulting calibration standards were subjected to the previously described pretreatment procedure and then injected into the CE system. Calibrators were prepared in triplicate for each point and covered the following plasma range: 2.0-320 μg mL^{-1}. The concentration of the IS was 10 μg mL^{-1}. The analyte/IS peak-area ratios obtained were plotted against the corresponding concentrations of the analytes (expressed as μg mL^{-1}) and the calibration curves were constructed by means of the least-square method.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the analyte

concentrations which give rise to peaks whose heights are 3 and 10 times the baseline noise, respectively.

Extraction yield and precision

For these assays simulated samples at three different concentrations of the analytes, corresponding to the lowest, the intermediate and the highest point of the calibration curve, were prepared. The samples, containing 2.0, 50.0 and 100.0 μg mL^{-1} of adulterants were subjected to the previously described pre-treatment procedure and injected into the CE. The analyte peak areas of the spiked samples were compared to those obtained injecting standard solutions at the same theoretical concentrations and the extraction yield (expressed as percentage value) was calculated. The assays described above were repeated six times within the same day to obtain repeatability (intraday precision) and six times over six different days to obtain intermediate precision (interday precision), both expressed as percentage relative standard deviation values (RSD%).

- *Selectivity*

The selectivity of the method was evaluated with respect to exogenous compounds. Selectivity towards exogenous substances was investigated by injecting into the CE system standard solutions of several compounds that could be co-administered in clinical practice. A substance was classified as interfering if it gave rise to a peak that was not baseline separated from those of the analytes or the IS.

- *Stability*

Stock solution stability was tested by comparing the analyte/IS peak-area ratios of a standard solution (containing 10 μg mL^{-1} of adulterants) prepared from stock solutions stored for 1 month at -20°C with those of a standard solution at the same theoretical concentrations obtained from fresh stock solutions (n = 3). Stability assays were also carried out in blank plasma samples fortified with 10 μg mL-1 of adulterants. Sample stability was evaluated over five hours at room temperature, after three freeze- thaw cycles and after 1 month of storage at -80°C (n = 3 per storage condition).

- *Analysis of street drugs simulated samples*

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The validated method was applied to quantify the content of adulterants in dust samples from simulated street drugs to the solutions was added the internal standard to the constant concentration of 10 g / mL.

- *Accuracy*

The accuracy was evaluated by performing recovery tests, adding known amounts of standard solutions of the analytes methanol extract diluted in BGE. The concentrations were added to 5 µg / mL, 10 µg / mL and 15 µg / mL whose analyte concentrations had been previously determined (n = 3 for each level); then, the mixtures were subjected to the pretreatment procedure. Accuracy was expressed as percentage recovery and was calculated according to the following formula: 100 ([after spiking] – [before spiking]) / [added], where [after spiking] is the sum of the concentrations of the analyte in the real plasma and in the analyte standard solution added; [before spiking] is the concentration of the analyte in the real sample; and [added] is the concentration of the analyte standard solution added to the real sample.

5.3. Results and discussion

Development of electrophoretic conditions

Normally the electrophoretic analyzes are conducted in an aqueous environment. In the electrophoresis capillary zone (CZE) separation of the analytes takes place by differential migration of charged species (ions) in a buffered solution, for the effect of the application of an electric field inside the capillary.Some of adulterants considered, however, are quite lipophilic molecules, without fillers and with a high degree of unsaturation.So the CZE does not lend itself to the analysis of this type of molecules as the relative mobility is equal to zero. The development of strategies that allow the use of capillary electrophoresis for the determination of substances of this type has been a critical step in the development of the method.The approach was systematic.

• First you are looking for the ideal pH conditions to favor the formation of a strong electroosmotic flow such as to allow the migration of the analyte towards the detector.

• Then it is tried to improve the mobility of some analytes in BGE with the addition of additives loads: β-cyclodextrin sulfate and SDS.

• Finally, there was the effect of additives on the mobility of all analytes.Considering the structures of the compounds it was considered appropriate to operate at a basic pH, in order to exploit the establishment of a strong electroosmotic flow (EOF). The choice of the BGE and then was carried out by evaluating the pKa of the acids used in the preparation of the pad.The various tests were carried out using phosphate buffer (pKa = 7.20), TRIS buffer (pKa = 8.10), carbonate buffer (pKa = 9.70) and borate buffer (pKa = 9.23). The carbonate buffer was discarded because it has not produced a stable current and reproducible over time.The best result was obtained with phosphate buffer, which was tested at various concentrations (from 10 mM to 30 mM) and at various pH (7.00 to 9.00), leaving unchanged the percentage of additive in order to obtain the better mobility of the analyte compared to that of 'EOF. The optimal concentration of the buffer is 12.5 mM and was prepared using dibasic sodium phosphate, ultrapure water and a solution of phosphoric acid to lower thestarting pH .Subsequently were tested increasing concentrations of sodium dodecyl sulfate (SDS from 10 mM to 50 mM) to favor the formation of micelles and assess the interaction of the analyte with them. For low quantities was observed a low, if any, interaction, while an excessively high percentage the base line was not stable due to the increase of the charges

present in the BGE. The optimal concentration of SDS was found to be 25 mM. In place of the sodium dodecyl sulphate was also tested β-CD sulphate in percentage ranging from 0.5% to 1.5% (w / v) without obtaining meaningful results. The BGE chosen for analysis, then, consists of a 12.5 mM phosphate buffer at pH 8.5 + 25 mM SDS. Even the voltage has been the object of numerous tests based on the percentage of surfactant to obtain the analysis in the shortest possible time. The chosen final voltage is 20 kV, which guarantees a stable and consistent current around 70mA.The temperature of the capillary was maintained constant at 25 ° C and, despite this, it was revealed that the total travel time tended to increase in the course of the day, probably due to the aging of the BGE.The injection of samples was performed by the anode side with a pressure of 50 mBar for ten seconds. Subsequently, the injection time was increased up to twenty seconds, to improve the sensitivity in the quantification of the analyte. For injection times greater were detected deterioration in the selectivity, as the peak appeared scodato.The procedure of pre-conditioning and post-conditioning consists in flushing the capillary with BGE for two minutes. Although the solvent for dilutions of the solutions to be injected in the EC has been the subject of study; dilutions prepared using solvents such as water and phosphate buffer gave peaks with areas and little reproducible migration times. The best results were obtained by diluting the substances with the BGE.

Internal standard selection

The internal standard is a substance that from the chemical point of view must be sufficiently different from the analyte, so as to be able to be determined, in the same experiment, without interfering in the measurement. Also his recovery should reflect that of the test species. So the selected molecule should be quite lipophilic and at the same time must not be used as an adulterant in the street drug to avoid interference.

Initially were analyzed propranolol, which has a migration time very close to that of atropine, amiloride and benserazide, which have migration times are too long; subsequently tiapride, L-sulpiride and amisulpride.

Among these was chosen the L-sulpiride that has a migration time and the intermediate is not present in the samples of street drugs

Linearity

Having developed the electrophoretic conditions, we moved to the validation of the method of standard solutions. The area under the curve of the peak electrophoretic each analyte is directly proportional to its concentration. To assess the linearity range of the method, mixtures were prepared at different concentrations of analytes, keeping constant the internal standard L sulpiride to (tm = 6.3 min) to 10 µg / mL and were injected into the CE (Figure 16)

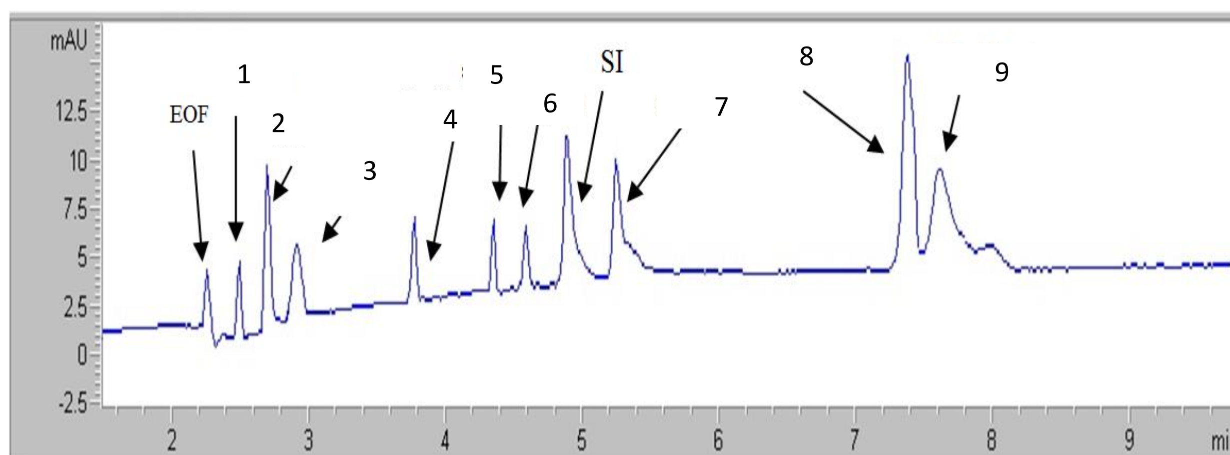

Figure 18. mix standard of adulterants: 1Caffeine, 2 Lidocaine,3 Paracetamol, 4Acetylsalicylic Acid,5 Scopolamine,6 Atropine, 7 Tetramisole, 8 Quinidine, 9 Diphenhydramine 10 ug mL

Then the calibration curve of the analyte concentration range were buit between 5 and 150.0 g / mL (Table 9), plotting the relationship between the peak areas of the analyte electrophoretic and those of the internal standard peak, in function of the concentration of the analyte. The equation of the line obtained by interpolation was calculated by the method of least squares.

The repeatability of the method was verified by running six tests on standard solutions at different concentrations of adulterants in the same day. These solutions correspond to the lower limit, to an intermediate point and a high point of the calibration curve. To simplify the work, were injected mix of all analytes for each test (Table 10)

To evaluate the interday precision of the method, the same concentrations of standard solutions were injected in 6 different days (Table 11)

Analytei	Tm (min)	Range (µg/mL)	$y = ax + b$		r^2	LOD (µg/mL)
			a	b		
Caffeine	2.3	5-150	1.473	-0.032	0.9997	1.5
Lidocain	2.7	5-150	1.376	0.012	0.9995	1.5
Paracetamol	2.9	5-100	2.838	0.004	0.9996	1.5
Acetylsalicylic Acid	3.8	10-150	0.801	0.003	0.9991	3.0
Scopolamine	4.4	5-100	1.239	0.014	0.9993	1.5
Atropine	4.6	15-150	0.539	-0.003	0.9992	5.0
Tetramisole	5.3	10-150	0.940	0.003	0.9992	3.0
Quinidine	7.4	5-100	2.400	0.005	0.9995	1.5
Diphenhydramine	7.9	5-100	1.222	0.005	0.9994	1.5

Table 11 Linearity parameters

Analyte	Migration time (min)	Concentration (µg mL^{-1})	Intraday precision,	
			Area Ratio analyte/I.S. (R.S.D.%)	Migration Time (R.S.D.%)[a]
Caffeine	2.3	5	3.80	2.0
		50	2.10	
		150	1.49	
Lidocaine	2.7	5	3.20	1,7
		50	2.50	
		150	1.75	
Paracetamol	2.9	5	3.17	1.8
		10	2.06	
		50	1.08	
Acetylsalicylic ac	3.8	10	4.07	1.1
		50	3.00	
		100	2.73	
Scopolamine	4.4	5	3.15	2.3
		50	3.00	
		100	2.78	
Atropine	4.6	15	3.13	2.0
		50	3.09	
		100	2.85	
Tetramisole	5.3	10	3.10	1.8
		50	2.04	
		100	1.14	
Quinidine	7.4	5	3.09	0.9
		10	3.00	
		100	1.47	
Diphenhydramine	7.9	5	3.04	2.02
		10	3.03	
		50	2.00	

Table 12 Intraday precision

Analyte	migration time	Concentration	Interday Precision	
	(min)	*(μg·mL^{-1})*	*Area Ratio analyte/I.S. (R.S.D.%)*	*Migration Time (RSD%)^{b}*
Caffeine	2.3	5	5.08	3.2
		50	5.00	
		150	3.90	
Lidocaine	2.7	5	4.90	2.7
		50	3.89	
		150	4.01	
Paracetamol	2.9	5	5.07	3.1
		10	4.06	
		50	3.00	
Acetylsalicylic ac	3.8	10	5.07	3.1
		50	4.00	
		100	4.02	
Scopolamine	4.4	5	5.02	2.9
		50	4.00	
		100	3.87	
Atropine	4.6	15	6.03	3.0
		50	5.09	
		100	4.05	
Tetramisole	5.3	10	5.00	3.4
		50	4.04	
		100	3.04	
Quinidine	7.4	5	5.09	2.3
		10	4.00	
		100	3.08	
Diphenhydramine	7.9	5	6.03	3.0
		10	5.03	
		50	3.00	

Table 13 Interday precision

- *Application to simulated street drugs*

The validated method was applied for the control of dust samples from simulated road or prepared in the laboratory trying to reproduce the composition of the major national and international drug seizures reported in the literature. The results are expressed in mg of adulterants of grams of powder from the road. Note that the diluents (such as starch and lactose) precipitated during the step of pretreatment of the sample and do not interfere with the electrophoretic system.

The results of analyzes carried out on samples of dust from the road simulated are summarized in Table .

Sample A reproduces the content of adulterants present in seizures of cocaine and heroin made in Luxembourg from 2005 to 2010.Figure 17

The sample B simulates the content of adulterants in cocaine seizures made in France in 2006.Figure 18

The sample C follows the composition of adulterants in cocaine seizures made in Brazil in 2003.26

In the sample D are the same as adulterants of seizures made in Switzerland and France from 2004 to 2005.6

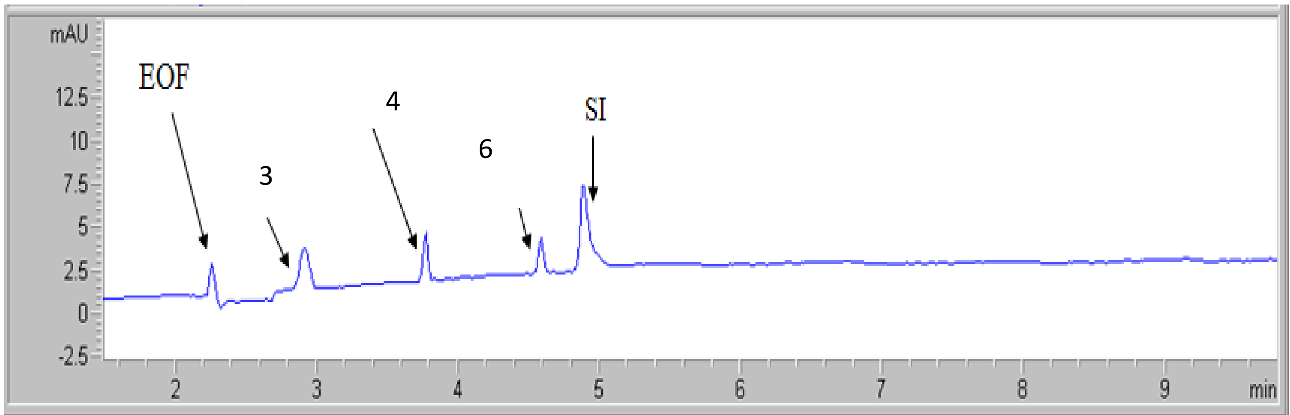

Figure 19. Sample A: 3 Paracetamol, 4Acetylsalicylic Acid,6 Atropine

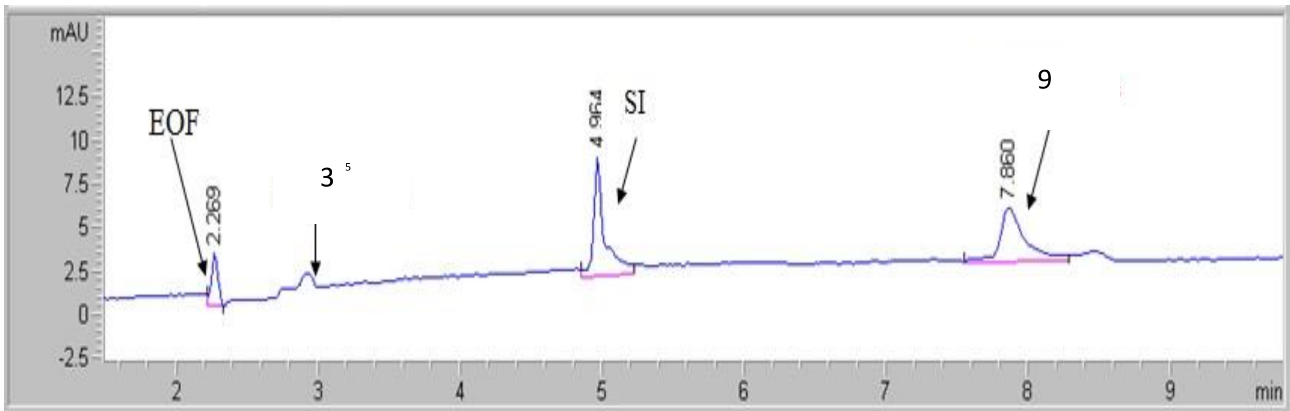

Figure 20 Sample B3 Paracetamol, 9 Diphenhydramine

Table 14results in simulated street drugs

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	Campione simulato A	Campione simulato B	Campione simulato C	Campione Simulato D
Caffeine	x	x	47,0 mg/g	x
Lidocaine	x	x	35,0 mg/g	x
Paracetamol	24,0 mg/g	10,5 mg/g	x	x
Acetilsalicilic Acid	21,0 mg/g	x	x	x
Scopolamine	x	x	x	5,10 mg/g
Atropine	34,3 mg/g	x	x	x
Tetramisole	x	x	x	4,70 mg/g
Quinidine	x	x	15,2 mg/g	x
Diphenhydramine	x	30,5 mg/g	x	x

-

- *Accuracy*

To complete the validation of the method, tests have been performed of accuracy that has been evaluated by performing recovery tests by adding a standard solution of known concentration of each analyte to solutions of the samples previously analyzed.

The additions were made considering three different concentrations: 5 mg / mL, 10 mg / mL and 15 mg / mL.

The recovery values found were always superior to 88% for all analytes, then the accuracy of the method is good.

These results are reported in Table 13

Table 15 accuracy

	Recovery %		
	added 5 µg/mL	**added 10 µg/mL**	**added 15 µg/mL**
Caffeine	89	90	88
Lidocaine	108	93	97
Paracetamol	97	92	98
Aaceylsalicylic Acid	105	90	88
Scopolamine	90	95	94
Atropine	88	90	89

5.4. Conclusions

The illicit use of drugs of abuse has always been a serious health risk. However, the toxic action and special events that can be observed in the individual adverse that makes use of common street drugs are often a result of the synergy of drugs with the substances to be "cut".

The presence of adulterants is often neglected by a clinical point of view-toxicological so it is important to have precise and selective methods for the analysis of these substances, commonly added to street drugs, in particular: caffeine, acetaminophen, atropine, scopolamine, lidocaine, diphenhydramine, levamisole, acetylsalicylic acid, quinidine. In this thesis has been developed a method in capillary electrophoresis, with detector photodiodes, innovative and reliable using the principles of the MEKC (Micellar electrokinetic Chromatography) for the analysis of adulterants in samples of street drugs simulated.

The system proposed MEKC showed high efficiency, low cost, and versatility. Moreover, given the reduced consumption of organic solvents, can be considered a technique "green", which aims to minimize the environmental impact.

The pretreatment procedure of the sample is simple and fast and allows to obtain high recoveries with a good purification of the matrices of the interference.

The preparation of the BGE is rapid and highly reproducible. For the validation of the method were performed numerous tests, both on standard solutions, both on simulated samples of interest. The results are satisfactory, having obtained a good linearity (R2> 0.9991) and a high precision for all analytes in exams (RSD values of less than 6.03%) and a good accuracy (recoveries higher than 88%).

The method MEKC proposal for analysis of adulterants in simulated street drugs seems, therefore, suitable to be applied to the analysis of real samples seized by the Police Forces.This type of investigation can be useful for monitoring the trends of consumption for the purpose of human health, in the case of new discoveries of adulterants and contaminants 5

6. DEVELOPMENT OF A CONFIRMATORY HPLC MS/MS METHOD FOR THE ANALYSIS OF GLUCOCORTICOIDS IN HUMAN URINE

6.1. Introduction

Glucocorticoids are steroid hormones, that inhibit the process of inflammation. Natural glucocorticoids are produced in the adrenal glands located immediately above the kidneys in the cortex as a defensive reaction to the damage or injury of the tissues. Physiologically, they contribute in several metabolic regulations, above all in the glucose and lipid metabolism. Synthetic glucocorticosteroids used in medicine are analogs and derivatives of natural hormones. Glucocorticosteroids have found wide use in contemporary sport as powerful anti-inflammatory agents for the treatment of bronchial asthma and for the therapy of acute and chronic soft tissue and joint injuries. Synthetic corticosteroids can produce a number of serious side effects as they are more active than their natural analogs. The misuse of coticosteroids can lead to immunodeficiency; hyperglycemia due to increased gluconeogenesis, insulin resistance, and impaired glucose tolerance; reduced bone density (osteoporosis, osteonecrosis); weight gain due to increased visceral and truncal fat deposition (central obesity) and appetite stimulation; hypercortisolemia (also known as, exogenous Cushing's syndrome); muscle breakdown (proteolysis).

The effects on the central nervous system are less considered but very important. The prolonged use of glucocorticoids can produce excitatory effect on central nervous system (euphoria, psychosis) and impaired memory and attention deficits[50]

Glucocorticoids act on the frontal lobes amygdala and hippocampus. Along with adrenaline, these enhance the formation of flashbulb memories of events associated with strong emotions, both

positive and negative[51]. In fact, the block of either glucocorticoids or noradrenaline activity impaired the recall of emotionally relevant information. Additional sources have shown subjects whose fear learning was accompanied by high cortisol levels had better consolidation of this memory . The effect that glucocorticoids have on memory may be due to damage specifically to the CA1 area of the hippocampal formation. In multiple animal studies, prolonged stress (causing prolonged increases in glucocorticoid levels) have shown destruction of the neurons in this area of the brain, which has been connected to memory performance[52]. Glucocorticoids have also been shown to have a significant impact on vigilance (attention deficit disorder) and cognition (memory).[53] Long-term exposure to glucocorticoid medications, i.e asthma, has been shown to create deficits in memory and attention both during and after treatment, a condition known as "steroid dementia."[54]

Particularly dangerous is the uncontrolled and incorrect use of these compounds by sportsmen during competitions. As a result, synthetic corticosteroids were added to the list of banned doping substances by the World Anti-Doping Agency (WADA)[55] . These rules state that glucocorticoids levels must be detectable at 30 ng/mL.

Several methods can be found in the literature for the detection of glucocorticoids: radioimmunoassay[56], [57], fluorimetric[58], [59], and HPLC with UV detection [60],[61]. Despite their rapidity, these methods lack the sensitivity and selectivity needed for doping control. Glucocorticosteroids can also be assayed by gas chromatography with mass spectrometry[62],[63]. A significant limitation of this method is the necessary and laborious stage of preparing volatile methoxyiminetrimethylsilyl derivatives. Glucocorticosteroids are currently assayed by HPLC combined with mass spectrometry, where there is no need for the derivatization stage[64],[65]. This methods have high sensitivity and selectivity bu the literature currently lacks reliable data on the simultaneous screening analysis of the main glucocorticosteroids, including isobars.

The aim of the present work was to develop a development of a confirmatory method for the analysis of 22 glucocorticoids in human urine according to World Anti-Doping Agency (WADA) criteria.

Abstract

Initially isobaric analytes (that require chromatographic separation) were selected to identify the best stationary and mobile phase conditions to allow chromatographic separation.
A C18 column and a gradient of aqueous and organic solvents for a reversed-phase chromatography was used.
I evaluated the flow rate and gradient profile to obtain baseline separation of the chromatographic peaks. Finally I included the remaining analytes to check the suitability of the chromatographic method.
Concerning MS, a full optimisation of source parameters and collision energy values was performed.
For each analyte, a product scan spectrum was acquired to identify the most suitable ion transitions to be used for identification, which was then performed in SRM mode in the final method.
A mixed-mode SPE cartridge was used for sample pre-treatment, following deconjugation using β-glucuronidase. Validation of the method in terms of linearity, precision and accuracy was carried out.

6.2. **Experimental**

Chemicals

Standard stock solutions were purchased
from Cerilliant (Round Rock, TX, USA). All standards were methanolic solutions, its deuterated internal standard (IS) that were dissolved in methanol/water (1:1). Ammonium acetate was obtained from Sigma Aldrich (St. Louis, MO, USA). Ammonium hydroxide and concentrated formic acid were from Fisher Scientific (Pittsburgh, PA, USA). HPLC grade methanol was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA). Human blank plasma was from the University of Utah blood bank.

Preparation of working solutions

Calibrator working solutions containing all analytes were prepared by diluting standard stock solutions with methanol/water (50:50, v/v). Separate working solutions were prepared in the same way for QC samples. A deuterated internal standard (IS) working solution was prepared in methanol/water (50:50, v/v) at a concentration of 0.1 µg mL^{-1}. All working solutions were stored at -20°C. Stock solutions were stable for at least 4 months when stored at -20 °C (as assessed by HPLC assays).

Preparation of calibration standards and quality control samples

Calibration standards were prepared daily by adding the appropriate aliquot of calibrator working solutions and 30 µL of deuterated IS working solution to 1 mL of blank urine Calibrators were at the followingurine concentrations: 0.2, 0.5, 1.0, 5.0, 20.0, 50.0, 75.0 and 100.0 ng mL^{-1}In the same way, quality

control (QC) samples were prepared by adding the appropriate amount of QC working solutions and 30 μL of deuterated IS working solution to 1 mL of blank urine. QC samples were at three concentration levels: low QC contained 0.6 ng mL^{-1}; medium and high QC were at a concentration of 10.0 and 85.0 ng mL^{-1}, respectively, for all analytes.

Equipment

LC–MS/MS analyses were performed using an ACQUITY UPLC® system (Waters, Milford, MA, USA) coupled to a Quattro Premier XE™ triple quadrupole mass spectrometer (Waters). Solid phase extraction (SPE) was carried out on Oasis MCX cartridges (60 mg, 3 mL) (Waters) using a Vac Elut manifold. A Thermo Scientific (Waltham, MA, USA) IEC FL40 floor centrifuge and a Zymark (Hopkinton, MA, USA) TurboVap® evaporator were also used.

Data were handled with MassLynx™ (v 4.1) software (Waters).

LC-MS/MS conditions

The chromatographic separation was achieved on a Supelco (Bellefonte, PA, USA) Discovery® HS F5 column (2.1 x 50 mm, 3 μm) held at 40°C. The mobile phase consisted of 10 mM ammonium acetate containing 0.1% formic acid (A) and methanol (B). The flow rate was kept constant at 0.25 mL min^{-1} and a gradient program was run. Initial mobile phase conditions were 90% A and 10% B; B was increased linearly from 10 to 70% in 6 min, held at 70 % for 0.50 min, decreased back to the initial mobile phase condition of 10% B in 0.30 min and finally held at 10% for 4.0 min to re-equilibrate the column (total chromatographic run time was 10.80 min). Injections were performed with an autosampler maintained at 4°C; injection volume was 12 μL.

The mass spectrometer was operated in electrospray positive ionization (ESI+) mode and performed multiple selective reaction monitoring (MRM). Capillary voltage was set at 3.00 kV and source and desolvation temperature were set at 100°C and 350°C, respectively.

Nitrogen was used as the desolvation gas at a flow rate of 800 L h^{-1} while argon was

used for collision. Cone voltage and collision energy were optimized for each analyte along with the selected MRM transitions. three product ions were monitored for each analyte; the most abundant one was used for quantification while the other one was used as qualifier. Dwell time was 200 ms for all the other MRM transitions monitored.

Sample pre-treatment: solid phase extraction

Sample pre-treatment was carried out by SPE on mixed-mode cation-exchange cartridges conditioned with 2 mL of methanol followed by 2 mL of water. A 1-mL aliquot of urine fortified with the analytes was spiked with 30 µL of deuterated IS working solution (0.1 µg mL^{-1}) and acidified with 1 mL of ammonium acetate (100 mM, pH 5.0). The resulting mixture was briefly vortexed and centrifuged at 2800 rpm for 10 min to remove any particulate material. The supernatant was loaded onto previously conditioned cartridges that were then washed with 2 mL of ammonium acetate (100 mM, pH 5.0) and 2 mL of methanol. Analytes were eluted with 2 mL of 10% ammonium hydroxide in methanol and the eluate was evaporated to dryness under air at 40°C. Finally, the residues obtained were reconstituted in 75 µL of 0.1% formic acid and transferred into autosampler vials.

Method validation

The method was validated following the U.S. Food and Drug Administration (FDA) guidelines for bioanalytical method validation[66] [139].

- *Linearity and lower limit of quantitation*

Linearity was evaluated over the 0.2-100 ng mL^{-1} concentration range NOXM by analyzing in duplicate the calibration standards subjected to the previously

described sample preparation procedure. The analyte/IS peak-area ratios were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}) and the calibration curves were constructed by means of the least-square method.

The lower limit of quantitation (LLOQ) was defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. It was assessed by analyzing six fortified samples (each prepared from a different urine source) and determining precision (expressed as percentage relative standard deviation (RSD%)) and accuracy (expressed as percentage of the nominal concentration). The

samples were accepted as LLOQ if RSD% was ≤ 20% and if the mean measured concentration was within ± 20% of the theoretical concentration.

- *Extraction yield, precision and accuracy*

Extraction yield was determined for each analyte at the low, medium, and high QC concentrations (6 replicate each). QC samples were subjected to the previously described pre-treatment procedure and analyzed along with unextracted standard solutions prepared at the same theoretical concentrations. The average analyte peak areas of the extracted samples were compared to those of the unextracted standard solutions and the extraction yields were calculated and expressed as percentage value. The data obtained are reflective of the combination of extraction recovery of the analytes and matrix effect and they can also be referred to as overall process efficiency, according to the definition of Matuszewski et al.[67] [140].

Intraday accuracy and precision were obtained by analysing low, medium and high QC samples five times within the same day; interday accuracy and precision were evaluated by analysing the three QC levels 6 times over three different days for a total of 18 results per concentration. Intra- and interday precision were both expressed as percentage relative standard deviation (RSD%). Intra- and interday accuracy were calculated by dividing the mean measured concentrations of the analytes by the theoretical concentrations and were expressed as percentage value.

- *Selectivity*

Selectivity was assessed by the analysis of plasma samples from six different healthy donors. Each sample was extracted and analysed to determine if any potential interference from endogenous components was present at the retention time of the analytes and the ISs. In addition, three replicates for each urine source were spiked with the ISs and one replicate was spiked with the ISs and the analytes at a concentration corresponding to the LLOQ. To be acceptable, the mean peak-area ratio of any signal at the retention time of the analytes to the corresponding IS must not exceed 20% of the mean analyte/IS peak-area ratio at LLOQ. Moreover, the area of any

peak at the retention time of the ISs must be less than 5% of the mean peak area of the ISs in the LLOQ samples.

- *Stability*

Stability experiments were performed with low and high QC samples (three replicates for each concentration level) stored under various conditions. The stability of the analytes in urine samples was evaluated after three freeze-thaw cycles and at room temperature. For the assessment of freeze-thaw stability, frozen QC samples were allowed to completely thaw unassisted at room temperature and then were frozen again for at least 12 h. This was repeated for a total of three times. Room temperature stability was evaluated on QC samples left at room temperature for 24 h prior to analysis. Stability was also tested in processed samples stored on the autosampler (+4°C) for 5 days and at -20°C for 7 days. The analytes were considered stable under the tested conditions if the mean concentrations found in the stored samples did not differ from the theoretical concentrations by more than ±5%.

6.3. **Results and discussion**

Development of the chromatographic conditions

The investigated analytes are all quite polar molecules, characterized by Considering the relatively low lipophilicity of each compound we decided to try two different approaches: in the first one we used a C18 column and a gradient of aqueous and organic solvents for a reverse phase chromatography; in the second one we use an HILIC column and a with reversed-phase type eluents to further the liquid-liquid partition chromatography.

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About the HILIC we used an Acquity UPLC BEH HILIC 1.7 um 2.1 x 100mm Column. Mobile phase was A (acetonitrile & 20 mM ammonium acetate) B (purified water & 20 mM ammonium acetate).

Unfortunately this strategy cannot be used because the column have no retention at all on analytes. Even with long time conditioning and changing gradient all analytes elutes togheder after one minute.

A HILIC stationary phase was also tried, but no significant improvements were obtained.. A gradient program was applied to optimize resolution of the analytes and total run time.

MS/MS parameters were optimized by infusing standard solutions of each analyte directly into the source along with the mobile phase at initial composition. As the most abundant fragment ion corresponded to the loss of water for all analytes, three parent-product ion transitions were monitored for each compound to improve method selectivity; the most intense transition was used for quantification and the other one was employed to confirm peak identity. it was the closest eluting peak; for all other analytes the corresponding deuterated analogue was employed.

Fig. 2

Chromatograms of a standard solution of the analytes (0.5 ng mL^{-1}) and the ISs. For each analyte three MRM transitions are shown (quantification transition is provided first).

Development of the SPE procedure

As for chromatographic method development, the lipophilicity of the analytes s challenged the optimization of the extraction conditions. Various SPE cartridges and different procedures were tested. C18 sorbent gave low recovery (< 30%) even though the samples were basified before loading to stabilize the analytes in their deprotonated, more lipophilic, form. Moderately hydrophobic cartridges (C2) could not su fficiently retain hat eluted during the washing step. Better results in terms of retention of the compounds were obtained on C8 and hydrophilic-lipophilic balance (HLB) cartridges (loading the former in basic conditions). However, none of the elution solvents tested (methanol, acetonitrile, acidified methanol) gave satisfactory extraction yields that were especially low for. Eventually, mixed-mode cation-exchange polymeric sorbent proved to be suitable for the extraction of the analytes fr om the matrix. The compounds of interest in their protonated form were highly retained by ionic interaction and the elution with basified methanol gave encouraging results. Nonetheless, it was necessary to carry out some tests to optimize the recovery of

G. Among the solutions investigated, 10% ammonium hydroxide in methanol gave extraction yields higher than 70% for all the analytes. In addition, thanks to the retention mechanism, it was possible to wash the cartridge with 100% organic solvent without affecting the recovery, thus removing also hydrophobic interferences and obtaining cleaner extracts.

The chromatograms of a blank urine sample subjected to the SPE procedure (**Fig. 3)** show that no significant peaks are present at the retention times of the analytes. The representative chromatograms of a urine sample fortified with the analytes (low QC) and subjected to the SPE pre-treatment is reported in **Fig 4**.

Fig. 3

Chromatograms of a blank urine sample. Three MRM transitions are shown for the analytes and the ISs (quantification transition is reported first).

Fig. 4

Chromatograms of an urine sample fortified with the analytes and the ISs. Concentrations correspond to low QC. Three MRM transitions are shown for each analyte (quantification transition is reported first).

Method Validation

Calibration curves were constructed by means of the least-square method and a 1/x weighting factor was applied. Good linearity ($r^2 > 0.995$) was obtained over the studied concentration ranges. The linearity range, slope, intercept and correlation coefficient for each analyte are summarized in **Table**. The LLOQ was 0.2 ng mL^{-1} for and 0.5 ng mL^{-1}. Precision and accuracy at the LLOQ assessed in 6 fortified samples from different urine sources satisfied the

acceptance criteria (i.e. RSD% ≤ 20% and deviation from the theoretical concentration within ± 20%). Extraction yield, precision and accuracy assays were carried out for each analyte at the low, medium, and high QC concentrations (6 replicate each); mean results are provided in **Table 3**. Extraction yield values calculated herein are reflective of the combination of matrix effect and extraction recovery of the analytes. Results were satisfactory, being always higher than 80%. Precision and accuracy were also acceptable: RSD values were always lower than 8.8% and accuracy ranged between 87.0% and 105.4%.

- *Selectivity*

The analysis of urine samples from 10 different healthy volunteers showed no evidence of unacceptable endogenous interference at the same MRM transitions and retention times of the analytes and the ISs, proving the selectivity of the method toward endogenous compounds.

- *Stability*

Stability of the analytes was assessed in urine samples stored at room temperature for 24 h and after three freeze-thaw cycles. Stability was also evaluated in processed samples stored on the autosampler (+4°C) for 5 days and at -20°C for 7 days. The mean observed concentrations of the analytes in the stored samples were always within ± 5% of the nominal concentrations, indicating that the analytes are stable under all tested conditions.

Table 2

Linearity parameters.

Table

Validation parameters: extraction yield, precision and accuracy data.

6.4. **Conclusions**

An analytical method based on the use of liquid chromatography coupled with tandem mass spectrometry and on a solid phase extraction procedure for sample clean-up has been developed for the simultaneous determination of 22 glucocorticoids in human urine. Few other methods have been reported for the detection of some of the analytes [136,137], but to the best of knowledge this is the first one that includes all of them.

The method has been validated according to the WADA criteria with satisfactory results and it will be used to investigate the glucocorticoids misuse in sports and discourage its use for doping. Acknowledgements

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FOOTNOTES

Some of the analytical methods developed during the Ph.D. program and presented herein, have been object of scientific meeting communications:

2013

- Emanuele Morganti, Maria Assunta Santoro, Maria Addolorata Saracino, Roberto Mandrioli, Maria Augusta Raggi
  Fast MEKC Method for the Analysis of Adulterants Commonly Present in Street Drugs
  13° SAYCS (Sigma-Aldrich Young Chemists Simposium), Riccione, 27-30 Ottobre 2013 (Abstract Book P22)

- Emanuele Morganti, Laura Plizza, Roberto Mandrioli, Maria Augusta Raggi
  A Novel CE-Stacking Approach for the Analysis of Ethinylestradiol and Dexamethasone Residues in Meat Samples
  13° SAYCS (Sigma-Aldrich Young Chemists Simposium), Riccione, 27-30 Ottobre 2013 (Abstract Book P23)

- Francesca Bugamelli, Rosaria Trematore, Emanuele Morganti, Maria Augusta Raggi *Determination of Phenylbutazone by means of HPLC with Amperometric Detector*
  13° SAYCS (Sigma-Aldrich Young Chemists Simposium), Riccione, 27-30 Ottobre 2013 (Abstract Book P5)

- Vittorio Volterra, Maria Addolorata Saracino, Roberto Mandrioli, Francesca Bugamelli, Emanuele Morganti, Maria Augusta Raggi
  Uso di agomelatina in stati depressivi e monitoraggio terapeutico
  11° Congresso SIPB (Società Italiana di Psichiatria Biologica), Napoli, 19-21 Settembre 2013
  (Abstract Book P.194)

- Maria Addolorata Saracino, Emanuele Morganti, Roberto Mandrioli, Francesca Bugamelli, Nadia Ghedini, Maria Augusta Raggi
  An original method for the analysis of gamma-hydroxybutyric acid (GHB) in biological matrices
  22nd NMMC (National Meeting on Medicinal Chemistry), Roma, 10-13 September 2013
  (Abstract Book P.AT.04)

- Maria Addolorata Saracino, Chiara Marcheselli, Francesca Bugamelli, Emanuele Morganti, Maria Augusta Raggi

Club drugs: analysis of ketamine and norketamine in dried blood spots by HPLC-DAD-MEPS
24° International Symposium of Pharmaceutical and Biomedical Analysis - Recent Developments in Pharmaceutical Analysis (PBA-RDPA 2013), Bologna, 30 June - 3 July 2013 (Abstract Book P 1-16)

2012

- Emanuele Morganti, Roberto Mandrioli, Maria Addolorata Saracino, Maria Chiara Pieri,
  Ernst Kenndler, Maria Augusta Raggi
  Determination of GHB by Means of Capillary Electrophoresis with Indirect UV Detection
  XII Giornata di Chimica dell' Emilia Romagna, Ferrara, 17 Dicembre 2012
  (Abstract Book pag. 66)

- Roberto Mandrioli, Emanuele Morganti, Migena Kasimi, Ernst Kenndler, Maria Chiara Pieri, Maria Augusta Raggi
  Un metodo originale per l'analisi di GHB in plasma umano mediante CE con rivelazione UV indiretta
  12° SAYCS (Sigma-Aldrich Young Chemists Simposium), Riccione, 1-3 Ottobre 2012 (Abstract Book O 14)

- Roberto Mandrioli, Emanuele Morganti, Maria Addolorata Saracino, Maria Chiara Pieri, Lorenzo Somaini, Maria Augusta Raggi
  Capillary electrophoresis with indirect UV detection for the Therapeutic Drug Monitoring (TDM) of GHB in human plasma
  21st NMMC (National Meeting on Medicinal Chemistry), Palermo, 17-20 July 2012
  (Abstract Book P33 pag. 121)

- Stefano Manfredini, Silvia Vertuani, Gemma Malisardi, Marina Marini, Provvidenza Maria Abruzzo, Cosetta Marchionni, Maria Augusta Raggi, Francesca Bugamelli, Emanuele Morganti, Alessandra Modesti, Tania Gamberi, Carla Ferreri, Antonella Pini, Alessandro Ghezzo, Filippo Fortuna
  Tocotrienol supplementation: a novel approach to the complementary treatment of Friedreich's Ataxia
  ChimAlSi 2012, IX Italian Congress of Food Chemistry. "Food, Functional Foods and Nutraceuticals", Ischia (NA), 3-7 June 2012 (Abstract Book P-116)

- Maria Addolorata Saracino, Emanuele Morganti, Michele Protti, Maria Augusta Raggi
  Antioxidants as modulators in health and disease: an analytical point of view
  NPCF 6, 6° meeting Nuove Prospettive in Chimica Farmaceutica, Riccione (RN), 15-17 Aprile 2012 (Abstract Book pag. 146)

Other analytical methods resulted from the research performed during the three years of

the Ph.D. program has been object of publication:

- I. Neri, C. Gurioli, M. A. Raggi, M.A. Saracino, E. Morganti, F Bugamelli, F. De Ponti, S. Vaccari, A. Patrizi, R. Balestri
  Detection of D-penicillamine in skin lesions in a case of dermal elastosis after a previous long-term treatment for Wilson's disease;
  Journal of the European Academy of Dermatology and Venereology, 29 (**2015**) 383-386

- M. A. Saracino, C. Cannistraci, F. Bugamelli, E. Morganti, I. Neri, R. Balestri, A. Patrizi, M. A. Raggi
  A novel HPLC-electrochemical detection approach for the determination of D-penicillamine in skin specimens;
  Talanta, 103 (**2013**) 355–360

However, as it is not strictly related to the determination of psychoactive compounds, it has not been included in the present thesis work.

Other manuscripts are in an advanced stage of preparation and will soon be sent to international journals.

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