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Nano-liquid chromatography for enantiomers separation of baclofen by using vancomycin silica stationary phase

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ABSTRACT

The chiral separation of baclofen (Bac) was obtained by nano-liquid chromatography tandem mass spectrometry (nano-LC–MS/MS) using a 100 μm I.D. fused silica capillary column packed with silica particles chemically modified with vancomycin. Various experimental parameters, such as composition (buffer concentration, water content, organic modifier) and pH of the mobile phase and sample solvent were investigated for method optimization. In order to increase the sensitivity an on-column focusing procedure was applied. Acceptable separation of Bac enantiomers was obtained in less than 11 min eluting in isocratic mode, with 90:10 MeOH/water (v/v) containing 10 mM ammonium acetate at pH 4.5. These optimized experimental conditions were applied to the analysis of human plasma samples spiked with racemic mixture of Bac. The use of a Buckypaper disc as sorbent membrane allows one to recover both enantiomers with yields ≥ 65%. The method was fully validated, following the identification criteria of the European Commission Decision 2002/657/EC.

1. Introduction

Still today, chirality is a key topic of research because of the overriding importance of enantiomers in various areas such as environmental, food, pharmaceutical, and above all human health science. In this regard, it is worth mentioning that more than a half of the produced and prescribed drugs have one or more chiral centres [1] and, as a result, they exhibit one or more couples of enantiomers.

It is well-known that drug/receptor interaction is greatly stereoselective. Consequently, only one of the two enantiomers can give the wanted pharmacological activity, whereas the other one may display inactivity, lower potency, toxicity, and even undesirable side effects. Moreover, in the pharmaceutical industry, the importance of single enantiomer preparation on drug pharmacodynamics or pharmacokinetics is still not completely understood, but in many countries, the regulatory agencies, engaged in the registration of new drugs, focus their efforts in legislating for the registration of the single enantiomers. In this regards, pharmaceutical industries are asked to provide complete information about the stereochemistry and stereoselectivity of all chiral products as well as the stereoselective analytical techniques for their determination. In this context, US Food and Drug Administration and other health regulatory agencies have issued definite guidelines for both the marketing of chiral drugs and their enantiomeric separation [2].

Baclofen (4-amino-3-p-chlorophenylbutyric acid) (Bac), a γ-amino-butyric acid (GABA) analog, is an inhibitory neurotransmitter acting as a selective $GABA_B$ receptor agonist [3]. Since its introduction in 1967, this drug is widely used as a skeletal muscle relaxant in the treatment of spastic disorders correlated with cerebral and spinal cord paralysis. Over the course of time, the therapeutic range has been extended to the treatment of intractable hiccups [4], tics in children with Tourette syndrome [5], tardive dystonia, tetanus, cerebral palsy, and regional pain syndromes [6,7]. Moreover, recently, it has been proved that Bac is effective in the therapy of gastroesophageal reflux [8] and in suppressing alcohol dependence [9].

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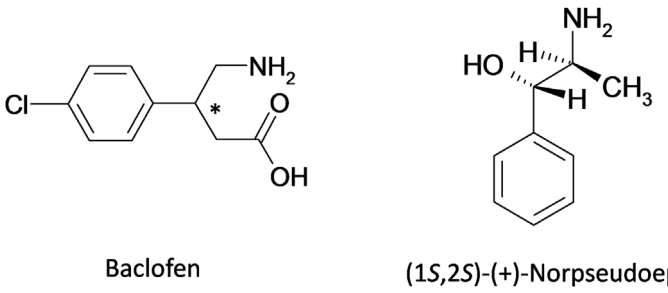

Fig. 1. Chemical Structure of baclofen and IS.

Bac is clinically administered as a racemic mixture under the commercial names Irex® or Lioresal®. Taken orally, it is rapidly absorbed from the gastrointestinal tract, reaching maximum plasma concentrations between 30 and 90 min and, differently from natural amino acids, it is able to pass the blood–brain barrier. Its half-life is about 3–4 h; Bac is almost completely recovered unchanged in urine [10].

Pharmacodynamic studies have reported that only the *R*-enantiomer exhibits stereospecific activity at $GAGA_B$-receptors [11,12] with an activity about 100 times greater than the *S*-enantiomer. In some cases, the latter may exhibit toxicity and antagonize the effects of the *R*-enantiomer [13].

Therefore, considering the different pharmacodynamic behavior of the two Bac enantiomers, the development of separative methods is essential for pharmacological and pharmaceutical purposes. Such methods, in combination with suitable sample preparation, could be extremely useful in therapeutic drug monitoring (TDM), particularly in case of intoxications or severe effects.

Different analytical techniques, relying on the direct resolution approach, have been used for the separation of Bac enantiomers. Besides gas chromatography (GC) [14], different solutions have been experimented utilizing high-performance liquid chromatography (HPLC): a Pirkle type chiral stationary phase (CSP) [15], ligand exchange mechanism [16], inclusion complexation with chiral crown ethers [17], and affinity interaction by using amylose 3,5-dimethylphenyl carbamate [18] and teicoplanin [19].

Bac enantiomers have also been separated employing the indirect resolution method after derivatization with different reagents [20]. However, this strategy suffers from some drawbacks such as the need of reagents with high enantiomeric purity, long time of analysis, potentially different response to the detector, etc. Another effective approach, tested working by reversed-phase HPLC, has been the addition of a cyclodextrin (CD) to the mobile phase [21]. CDs and their derivatives have also been profitable when used for the separation of Bac enantiomers by capillary electrophoresis (CE). Compared to the HPLC-based method [21] the addition of α-CD [22], β-CD [23] and highly-sulfated-β-CD [24] to the background electrolyte (BGE) implicates lower costs of analysis due to the minute volumes of mobile phase consumed. Chiral crown-ethers, added to the BGE alone [17] or in combination with CDs were equally efficient for the chiral separation of Bac. In order to increase the analytical sensitivity matching the Bac concentrations in biological fluids, most of the mentioned applications include derivatization of the two enantiomers and laser-induced fluorescence (LIF) detection. In only one study related to the investigation of a pharmaceutical formulation, Bac enantiomers were analyzed by CE-MS with a BGE supplemented with sulfobutylether-β-CD. The CS, negatively charged, moved in the direction opposite to that of the analytes, thus avoiding the MS [25].

Although the miniaturized chromatographic or electrophoretic techniques offer several advantages over the conventional ones (e.g. reduced cost, short analysis time, highly enantioselectivity, simplicity, and low chiral selector consumption) to our best knowledge, Bac enantiomers have never been separated by using nano/capillary LC or CEC. In only one paper, Bac was appropriately derivatized and analyzed in CE-LIF in plasma samples [26].

In this study, a high specific analytical method based on nano-LC–MS has been optimized for the chiral separation of Bac using a fused silica capillary packed with CSP-vancomycin silica. The novel method was validated and applied to human plasma samples spiked with a racemic mixture of the drug. After protein precipitaticion, samples were subjected to a clean-up step, utilizing buckypaper (BP) [27] as a sorbent membrane to perform rotating-disc SPE, and the enantiomers were analyzed by nano-LC–MS/MS.

2. Experimental

2.1. Chemicals and reagents

R-(+) (Cas number, 63701-55-3) and *S*-(-) (CAS number, 63701-56-4) enantiomers of Bac, its racemic form (CAS number, 1134-47-0) and (*1S,2S*)-(+)-Norpseudoephedrine (CAS number, 492-39-7) (internal standard, IS) were obtained from Sigma (St. Louis, MO, USA) (purity ≥98%). Fig. 1 depicts the chemical structures of Bac and IS.

The following chemicals were of analytical grade and were obtained from Carlo Erba (Rodano, Milan, Italy): methanol (MeOH), ethanol (EtOH), 2-propanol (2-prOH), 1-propanol (n-prOH), ammonia solution (30%, w/w), glacial acetic acid (99.8%, w/w) (AcH) and formic acid (99.0%, w/w) (Hfo). Acetone and acetonitrile (ACN) were from Romil (Cambridge, UK). HPLC ultrapure water (filtered at 0.2 μm and packaged under nitrogen) was provided by VWR (International PBI S.r.l. Milan, Italy).

Stock solutions of Bac and IS (1.0 mg/mL) were prepared with ultrapure water and MeOH, respectively and kept at −18 °C. Working standard solutions, at concentrations lower than 10 μg/mL, were composed by combining suitable volumes of the stock solutions and diluting with 2-prOH.

500 mM of ammonium acetate or ammonium formate were set up by diluting the proper volume of acids with ultrapure water and titrating with 5 M ammonia to the desired pH.

Polar organic mobile phases were daily obtained by mixing suitable amounts of buffer solution or ultrapure water and organic solvents in 10 mL volumetric flasks. The standard and working mixtures and plasma samples were stored at −20 °C. The mobile phases, when not in use, were stored at +4 °C.

2.2. Instrumentation

An ultrasonic bath model FS 100b Decon (Hove, UK) was used to sonicate solutions and fused silica columns during the packing procedure.

A Stereozoom 4 optical microscope (Cambridge Instruments, Vienna, Austria) with illuminator was used to check the conditions of the capillary and to monitor the capillary packing method and the fused silica tip emitter for the MS interfacing.

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An HPLC pump (Perkin Elmer Series 10, Palo Alto, CA, USA) was utilized to pack the capillaries.

Fused silica capillary tubing (100 μm I.D. or 25 μm I.D. × 363 μm O.D.) was obtained from Composite Metal Service (Hallow, UK).

A Crison Basic pH 20 (Crison Instruments SA, Barcelona, Spain), with a combined electrode and a temperature sensor, was used for pH determination. Accurate pH measurement were obtained by applying a three-points calibration with certified buffer solutions at pH 4.01 and 7.00 and 9.21. (Crison Instruments SA, Spain).

2.3. Chiral capillary column preparation

A silica-based vancomycin-chiral stationary phase (Vanco-CSP) was packed into 100 μm I.D. fused silica capillary. Vancomycin (CAS number: 1404-93-9, Sigma-Aldrich, St. Louis, MO, USA) was chemically bonded onto LiChrospher 100 diol-silica particles with a 5 μm diameter and 100 Å pore size (Merck Darmstadt, Germany) following a synthesis procedure previously reported by our group [28].

The chiral columns were packed as previously described using the slurry packing procedure [29].

Due to thermal instability of Vanco-CSP at high temperature, both frits were realized employing different silica-based particles, such as LiChrosorb® 10 μm RP-18, 100 Å (Merck Darmstadt, Germany) material, which was heated with an electrical wire at about 650 °C x 6 s for frit preparation.

Key parameters of the packing procedure are summarized below: the pump and slurry solvents were MeOH and acetone, respectively, while distilled water was used during the sintering process; the maximum pressure was 35 Mpa (about 5100 psi). The CSP was packed for 25.0 cm length, while the effective lengths were 26.5 for UV and 30.0 cm for MS detection, respectively.

2.4. Nano-liquid chromatography system

Nano-LC separations of enantiomers were carried out with laboratory-assembled instrumentation as previously reported [30]. Preliminary experiments were performed by coupling the separation system with a UV detector and only later-on with the MS. MeOH was pumped to the injection valve utilizing a Rheos 2000 micro-pump (Flux Instruments, Basel, Switzerland). The typical nano-flow rates (nL/min) were obtained using a passive split system. In this respect, stainless steel T (Vici Valco, Houston, TX, USA) was joined to the HPLC injection valve through a 5 cm × 500 μm I.D. stainless steel tube while a PEEK™ tubing of 50 cm × 130 μm I.D. was connected to the pump. The remaining port was positioned at the waste position with 70 cm x 50 μm I.D. PEEK™ tubing. The split solvent was recycled as pump solvent.

A modified LC injection valve (Enantiosep, Münster, Germany) was used for all experiments. Its configuration included an external loop, of approximately 30 μL, which was employed for both sample loading and mobile phase reservoir. Because the injection was based on the pressure-pulse driven stopped-flow injection method [31], the sample volume injected was governed by the time and by the mobile phase flow rate [32]. The applied flow rate was measured by connecting a 10 μL syringe (Hamilton, Reno, NV, USA) to the outlet column through a Teflon® tube (TF-350; LC-Packing, CA, USA) and the mobile phase volume was measured for approximately 5 min. In order to reduce the band broadening effect, the inlet column was directly connected to the modified valve.

A Spectra 100 UV instrument (Thermo Separation Products, San Jose, CA, USA) was utilized for the on-column UV detection. Data were acquired at 195 nm, 20 Hz and rise time 0.5 s.

The Rheos micro-LC pump was controlled by Janeiro II-XV software (Rev.2.0), (Flux instruments), whereas the UV data were recorded by Spectra System SN4000 System Controller Eight Port (Thermo Separation Products, San Jose, CA, USA) and reprocessed by ChromQuest version 3.0 software (Thermo Finnigan, San Jose, CA, USA). The temperature control was achieved by working in a continuous conditioning room (25 °C).

The nano-LC system was also coupled with an MS detector, LCQ™ ion-trap (Thermo Finnigan San Jose, CA, USA). A laboratory assembled nano-spray-ESI interface was utilized for nano-LC–MS as previously reported [33]. Briefly, the outlet column was joined to the fused silica tip emitter (25 μm I.D. × 375 μm O.D. × 10.5 cm) throughout a stainless steel union with zero dead volume (VICI VALCO Instruments, Houston, USA). The union joined an external power supply for the spray voltage of 1.8 kV (CZE1000 R, Spellman High Voltage Electronics, NY, USA). The interface was housed in a support comprising an XYZ linear translation stage. The exact position of the tip emitter in front of the MS orifice was controlled by an analogical-video system. The tip emitters were laboratory arranged and forged in the appropriate shape by using a very simple rotating disk supporting fine emery paper.

The positive ionization mode was selected and MS chromatograms were attained in the range 100–250 *m/z*, acquiring both in full and MS/MS scan mode.

The MS parameters were adjusted in order to record the optimum signal-to-noise ratio. In this respect, the MS tune was set in automatic mode infusing 0.1 μg/mL of the Bac racemic mixture in the mobile phase at 1 μL/min. The following conditions were applied: capillary voltage, 16 V; capillary temperature, 190 °C; tube lens offset, 5 V; automatic gain control (AGC), 2.5×10^8; the number of microscan, 3 and maximum injection time, 95 ms.

For MS/MS experiments, $[M+H]^+$ was fragmented using He collision into the ion trap. The normalized collision energy (%) was 38 and 30 for Bac and IS. During the method validation, any chromatographic parameter, i.e. retention time, peak height and peak area was acquired in extracted precursor or fragment ion peak chromatogram mode. The mass spectra and the ion chromatograms were recorded and handled with Xcalibur™ 2.0 (Thermo Finnigan).

2.5. Sample preparation

An aliquot of 500 μL of control drug-free human plasma (kindly donated by Prof. Gentili, University of Rome "La Sapienza", Rome, Italy) was thawed at room temperature. Blank and plasma samples, fortified with the standard racemic mixture of Bac and IS, were extracted combining a deproteinization step and an SPE clean-up procedure.

2.5.1. The deproteinization step

Briefly, Bac racemic standard including the IS (at 0.8 μg/mL) was poured out into a 15-mL polypropilene conical centrifuge tube (Falcon®, Corning, NY, USA) and the solvent was eliminated under a nitrogen stream. Afterward, 500 μL of plasma was transferred into the same tube and the mixture was vortexed (Reax 2000, Heidolph Instruments GmbH & CO, Germany) for 30 s and kept at rest for 1 min. Subsequently, 2 mL of MeOH was added to induce protein precipitation, vortexing for 1 min and centrifuging at 4000 rpm for 10 min on a universal 320R centrifuge refrigerated at 0 °C (Hettich AG, Bäch, Switzerland). The supernatant, carefully transferred into 50 mL pirex glass beaker, was evaporated to dryness at 35 °C under a gentle nitrogen stream. The procedure was applied to the same volume of plasma without the racemic Bac and IS.

2.5.2. The clean-up step: rotating disc SPE based on BP as a sorbent membrane

The SPE clean-up step was performed on a slightly modified procedure proposed by Gentili's group [34] and based on the use of BP as a sorbent membrane [27,35]. Briefly, BP is a porous flexible felt (about 100 μm of thickness), composed of self-supporting

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entangled, unoriented, oxidized multi-walled-carbon nanotubes (MWCNTs). In order to prepare the SPE device, a BP sheet of $17.5 \times 22.5\ cm^2$ was purchased from Nanolab Inc. (Waltham, USA), and carefully cut with scissors to form 25-mm diameter discs. Each BP disc was introduced into a circle-shaped polypropylene mesh pouch and the edges were united with heat.

The so assembled device was immersed in an aqueous solution of nitric acid (65%, v/v) for 2 h and, then, rinsed with ultrapure water to remove any acidic trace, including residues of catalyst and surfactant potentially occurring on the pristine BP material. The oxidation reaction with nitric acid was repeated with consecutive steps until 8.5 h. Finally, carbonaceous impurities were washed off with MeOH. At the end of this activation procedure, a 7 mm long magnetic bar was housed into a narrow polypropylene tube fixed on the top side of the pouch to assure the disc rotation. Just before its use, the BP device was conditioned in 10 mL pirex glass beaker with ultrapure water for 10 min.

Each dry extract from the previous step (both blank and spiked) was dissolved with 20 mL of ultrapure water in a 50 mL pyrex beaker under the ultrasonic bath (1 min). The extraction device was immersed and left under continuous magnetic stirring at 100 rpm for approximately 24 h to assure complete analyte adsorption on the BP membrane. Afterward, the device was pulled out with pliers, dried thoroughly with absorbent paper and finally placed in a clean 50 mL pirex glass beaker for the desorption procedure. The complete extraction of the analytes took place in 5 mL of methanol under continuous magnetic stirring at 100 rpm for 40 min. For an effective desorption, two consecutive extractions were carried out using 3 and 2 mL of methanol for 20 min each. The fractions were collected in 10 mL Erlenmeyer glass flask and evaporated at 37 °C employing a rotavapor R-200 equipped with a B490 heating bath from Buchi Labortechnik (Flawil, Switzerland) and a vacuum pump (Laboport® series, KNF Neuberger Inc., Trenton, NJ, USA). The residue was reconstituted in 200 μL of 2-prOH, ultrasonicated for 20 s and then injected for the nano-LC analysis.

3. Results and discussion

3.1. Enatiomeric separation of baclofen

Macrocyclic glycopeptides, such as vancomycin, teicoplanin, avoparcin, ristocetin A etc., represent a widely used powerful CS class commonly employed for the enantiosepation of a range of compounds. In LC or in CEC, vancomycin is covalently immobilized to the silica or to the polymeric supports. Consequently, the CS is not free to rotate assuming a different configuration as in CE mode. A multimodal interaction (affinity) with analytes is established involving π–π, electrostatic, hydrogen, inclusion in the hydrophobic cavity, dipole stacking, van der Walls and steric interactions [36].

Although Bac enantiomers have been separated utilizing a CSP-teicoplanin by HPLC [19], based on our experience and considering the amino acid-like structure of the investigated enantiomers, a CSP-vancomycin attached to silica was selected and studied with nano-LC [37]. The presence of carbonyl group, lone pair on nitrogen and oxygen atoms, together with benzene ring and chloro atom as electron withdrawing can interact with the complementary groups present on the CS structure. The inclusion basket, containing several asymmetric centres, can also provide the chiral discrimination of the Bac enantiomers.

In this study, baseline separation of Bac enantiomers was obtained employing a polar organic mobile phase in the isocratic elution mode. The method was optimized, in term of enantioresolution, peak shape, retention time, composition (buffer concentration, water content, organic modifier) and pH of the mobile phase and sample solvent.

In this respect, mobile phases containing a buffer at pHs ranging between 2.5 and 8.5, buffer concentration in the range of 5–25 mM, water content from 5 to 20% (v/v) and different organic solvents, i.e., EtOH, 2-prOH, n-prOH, acetone and ACN (10% v/v) were evaluated. Optimum conditions, offering the best Bac enantioseparation at a reasonable analysis time, were obtained by using a MeOH-based mobile phase (i.e. 90:10 MeOH/water, v/v containing 10 mM ammonium acetate at pH 4.5) at 500 nL/min. This mobile phase was also used for method validation and human serum analysis.

3.2. Sensitivity improvement: on-column pre-concentration

The use of columns with lower I.D. offers some advantages related to chromatography, e.g., higher efficiency and higher mass sensitivity [38] and the possibility to address the analytical chemistry towards sustainable chemistry. However, when real samples have to be analyzed, the low injected sample volumes represent a limitation in achieving the needed detection sensitivity.

In this study, the sensitivity was enhanced using MS and a large sample volume injection. Although with capillary techniques the injected volume is low (10–100 nL), a valuable approach for increasing the sensitivity is the use of an on-column focusing strategy. With this methodology, utilizing suitable experimental conditions, a relatively high sample volume (order of microliters) can be injected without affecting the column efficiency, peak shape, and chromatographic separation. Therefore, in this study, an appropriate sample solvent dilution was investigated.

The dilution solvent was selected considering both Bac solubility and the lowest eluotropic strength considering the mobile phase. As reported by our group [39], the lowest ionic strength generates an increase of solute affinity with the Vanco-CSP.

Racemic Bac and I.S. were dissolved in different solvents such as water, ACN, MeOH, EtOH, iso-prOH, n-prOH, acetone and mobile phase; the mixtures were then injected (about 500 nL) for the nano-LC analysis.

Evident band broadening was observed when the mobile phase or water were employed as a solvent, while the use of alcohols or acetone offered better chromatographic efficiency and enantiomers resolution. The best compromise in term of low band broadening, sensitivity and solubility were obtained with 2-prOH. Therefore, the sample was dissolved in this solvent and nano-LC separations were performed injecting different volumes in the range 250–2900 nL. Considering the peak height and peak width at half height ($H/w_{1/2}$) ratio versus the injected sample volumes, the highest peaks' compression effect was observed at 1750 nL (210 s injection time at 500 nL/min) that was applied in next experiments. Finally, analyzing the racemic Bac mixture, the instrumental LOD and LOQ (signal to noise ratio, 3:1 and 10:1, respectively) were 57/62 and 150/155 (first and second eluted enantiomer) ng/mL, respectively.

Fig. 2 shows the total ion chromatogram (TIC), base peak, and extracted ion chromatograms of the separation of a standard racemic mixture of Bac and IS under the optimized conditions.

3.3. Optimization of the extraction procedure

The hydrophilic properties of Bac and its low solubility in organic solvents, especially in hydrophobic solvents, precluded any possibility of developing a successful procedure based on dispersive liquid-liquid microextraction (DLLME). Although some parameters such as type and volume of extraction solvent and disperser solvent, extraction time, pH, and salt addition were studied, no significant results were obtained.

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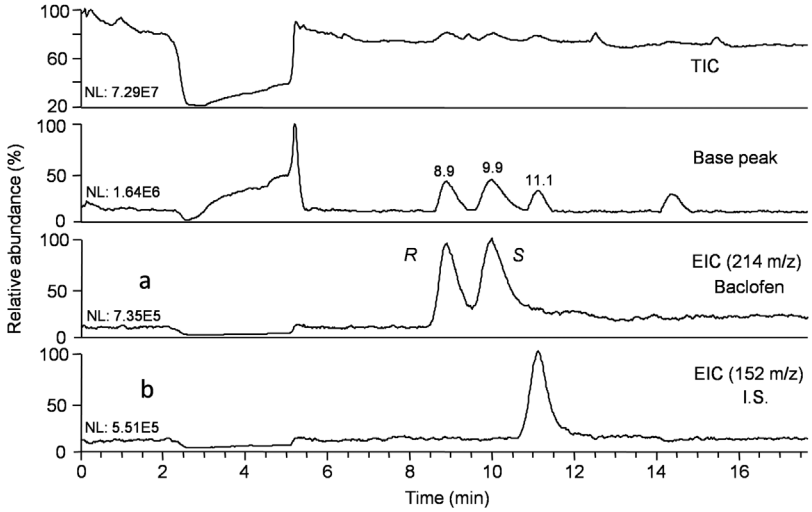

Fig. 2. Total ion, base peak and extracted ion chromatograms of a standard mixture a) 214 *m/z* baclofen and b) 152 *m/z* I.S. by nano-LC–MS system. Experimental conditions: capillary column, 100 μm I.D. packed length, 25.0 cm; MS interface length, 30.0 cm; stationary phase, 5 μm diol silica –CSP-Vancomycin; mobile phase, 10 mM NH_4Ac pH 4.5 in MeOH/H_2O (90:10, v/v); temperature, 25 °C, 500 nL/min; sample: racemic mixture of baclofen 0.8 μg/mL and 0.4 μg/mL IS in 2-prOH. Injection volume: 1750 nL. For MS conditions, see section 2.4.

For this reason, after a classical deproteinization step with methanol, a part of the work was addressed to the development and optimization of an original SPE clean-up step.

3.3.1. Optimization of the deproteinization step

Based on previously published works [27,34], a preliminary screening study was undertaken by evaluating two aspects to induce protein precipitation: type of organic solvent and the acidic conditions.

In this regard, ACN or EtOH or MeOH were separately added at different proportions in relation to 500 μL of plasma (1/4, v/v and 1/8, v/v sample/solvent) in the presence or absence of 0.5% (v/v) formic acid. Finally, 2 mL of MeOH without acid addition allowed a good protein precipitation and better recovery of the target drug.

3.3.2. Optimization of the SPE clean-up step

Carbon nanostructured materials have found an important collocation within the field of sample preparation and, among the several allotropic forms, single-walled and multi-walled carbon nanotubes (SWCNTs and MWCNTs) have been used for classical SPE on the cartridge as well as for dispersive and magnetic SPE [40]. On the other hand, despite having great potential, the CNTs-based membranes [41] have still been little experimented [27,34]. Among the different possible assemblies, buckypaper (BP) is a lightweight self-aggregate of CNTs compressed in a thin sheet. Like free CNT, BP exhibits van der Waals, π−π stacking and hydrophobic interactions which make it a very good sorbent for recovering nonpolar or moderately polar and aromatic organic compounds.

In this study, BP has been submitted to a strong acidic treatment with HNO_3 to introduce hydroxyl, carbonyl, and carboxyl groups on defects and holes in the edges of CNTs. In this way, the increased hydrophilicity and wettability of BP improved its affinity towards water-soluble compounds such as Bac.

Considering the developed methodology from previous publications [27,34],some preliminary experiments were carried out with ultrapure water (20 mL), fortified with a mixture of Bac in racemic form and I.S. Extraction efficiency of BP can be controlled by several parameters, for instance, the type and volume of extraction solvent, extraction time, and last but not least, the oxidation degree.

All the optimized experiments were done in duplicate. Different assays were initially carried out using different extraction solvent as MeOH, ACN, acetone and 50:50 MeOH/ ACN (v/v).

The total extraction volume was 5 mL, divided into two successive extractions (3 + 2 mL) each of 20 min of extraction time. MeOH produced the highest peak area absolute value, while extraction time longer did not provide further improvement (data not shown). Applying this extraction methodology, the optimal oxidation degree was identified as the one producing the highest affinity of Bac towards the sorbent material. Controlling the strong-acid reaction time (up to twelve hours), every two hours the modified BP was used to evaluate the recovery in terms of absolute peak area. A significant increase in extraction efficiency was after eight hours of oxidation treatment. A further increase of the reaction time did not show any improvement.

3.4. Mass spectrometry quantification method

According to the European Commission Decision 2002/657/EC: (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32002D0657) and its subsequent Guidance document SANCO/12571/2013. (http://ec.europa.eu/food/plant/docs/plant_pesticides_mrl_guidelines_wrkdoc_11945_en.Pdf), MS detection is indispensable for unambiguous identification of target analytes in real samples. In this respect, a preliminary fragmentation study was performed to identify diagnostic product ions with a relative intensity greater than 10%. The product ion (*m/z*) transitions observed for blaclofen were as follows: 214 > 197 ($[M+H-NH_3]^+$), 214 > 196 ($[M+H-H_2O]^+$), 214 > 179 (likely $[M+H-Cl]^+$); for IS: 152 > 134 ($[M+H-H_2O]^+$). Fig. 3 reports the MS/MS spectra of Bac and IS.

For quantification, the peak area of the most intense product ion related to that of the IS was evaluated. The 214 > 196 *m/z* and 152 > 134 *m/z* were selected for Bac and IS, respectively, respectively.

3.5. Nano-LC–MS/MS validation and analysis of real plasma sample

Once optimized, the whole BP-SPE-Nano-LC–MS/MS method was subjected to validation. Peak area repeatability, LOD, LOQ, linear calibration, recovery, precision and trueness were the investigated parameters.

For the intra-day and inter-day trueness and precision evaluation, spiked plasma sample at two concentration levels (level 1,

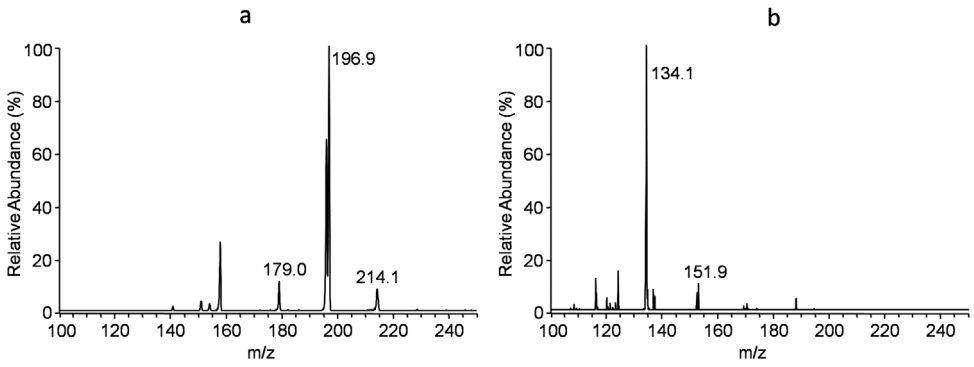

Fig. 3. The MS/MS spectra of a) baclofen and b) IS For futher information see section 2.4.

Table 1
Repeatability and reproducibility of the analytical method.

		Peak Area (RSD, %)			
		Level I (0.8 μg/mL)		Level II (0.4 μg/mL)	
		intraday (n = 6)	interday (n = 18)	intraday (n = 6)	interday (n = 18)
1st peak	R (+)-Baclofen	8.2	10.3	9.5	11.4
2nd peak	S (-)-Baclofen	9.7	12.1	10.3	13.4

Table 2
Studied linear dynamic range of enantiomers of baclofen in spiked plasma sample before the sample pretreatment BP-SPE and analyzed by nano-LC–MS/MS.

	Analytes y = mx + n	linear dynamic range (ng/mL)	Regression equation (n = 6)				
	$m \pm t \cdot S_m (10^{-2})$		$n \pm t \cdot S_n (10^{-2})$	R^2	LOD^a_{method} (ng/mL)	LOQ^b_{method} (ng/mL)	
1st peak	R (+)-Baclofen	100-500	2.7 ± 1.3	1.8 ± 2.1	0.9921	40	85
2nd peak	S (-)-Baclofen	100-500	2.9 ± 1.4	1.5 ± 1.9	0.9918	40	85

m slope, Sm standard deviation of the slope, n intercept, Sn standard deviation of the intercept, R^2 determination coefficient.;
a) Calculated as the concentration associated to a S/N ratio of 3;
b) Calculated as the concentration associated to a S/N ratio of 10;
t = (Student's t test) = 2.78, α = 0.05.

Table 3
Results of the precision and accuracy study of the BP-SPE Nano-LC–MS/MS method of baclofen in spiked human plasma. For experimental condition see material and method section.

Analytes	Spiked level (ng/mL)	Found [a] (ng/mL)	Accuracy (RSD,%)	t
R (+)-Baclofen	200.0	182.2 ± 29.4	91 (17)	1.09
	400.0	383.0 ± 65.8	95 (16)	1.38
S (-)-Baclofen	200.0	178.0 ± 26.9	89 (15)	1.25
	400.0	377.0 ± 68.2	94 (18)	1.29

a) Average value ± confidence interval (five determinations, 95% confidence value).
t = (Student's t test) = 2.78, α = 0.05.

0.8 μg/mL and level 2, 0.4 μg/mL) were injected six times each (n = 6) in three consecutive days (n = 18). As reported in Table 1 RSD% values for the intra- and inter-day precision of the peak areas were in the range 8.2–10.3 and 10.3–13.4%, respectively.

The calibration curves of each enantiomer of Bac were set up by extracting spiked free drug plasma sample (including the IS) at six levels of concentration (n = 6) using the BP-SPE procedure. Each level was in triplicate and injected three times for nano-LC analysis. Table 2, reports the obtained results.

As can be observed, a linear MS signal response at the evaluated concentration range with a determination coefficients (R^2) higher than 0.991 for both enantiomeric forms was obtained.

In this work, the method LOD (S/N = 3) and LOQ (S/N = 10) values were 40 and 85 ng/mL for each enantiomer, respectively.

In the literature, the methods for Bac quantification in human plasma offer higher sensitivity but, being achiral analysis, the signals of both enantiomers are concentrated in a single chromatographic peak. For instance, a classical SPE on cartridge combined with LC–MS produced a LOQ of 25 ng/mL [42] while a conventional LC system coupled to high-resolution MS (HR-MS) allowed one to reach a LOQ value of 3 ng/mL [43]. However, also chiral analyses carried out by a conventional LC-UV system, using α-cyclodextrin in CE-LIF [22], CSP ligand-exchange based [44] and Chirobiotic T based chiral column [19], provided LOD values surprisingly lower than 20 ng/mL for both enantiomers. Notwithstanding the superior sensitivity achieved on a conventional chromatographic column, the presented method shows method limits absolutely compatible with a TDM application. As a matter of fact, considering a common daily treatment of 60 mg Bac dose, in racemic form, its post-ingestion plasma concentration was estimated in about 700 ng/mL [42], which is greatly higher than LOQ reached in this work.

Lastly, trueness, precision and recovery of the method were studied for each enantiomers. In this regard, drug-free plasma samples were spiked with the racemic mixture of Bac and IS at two

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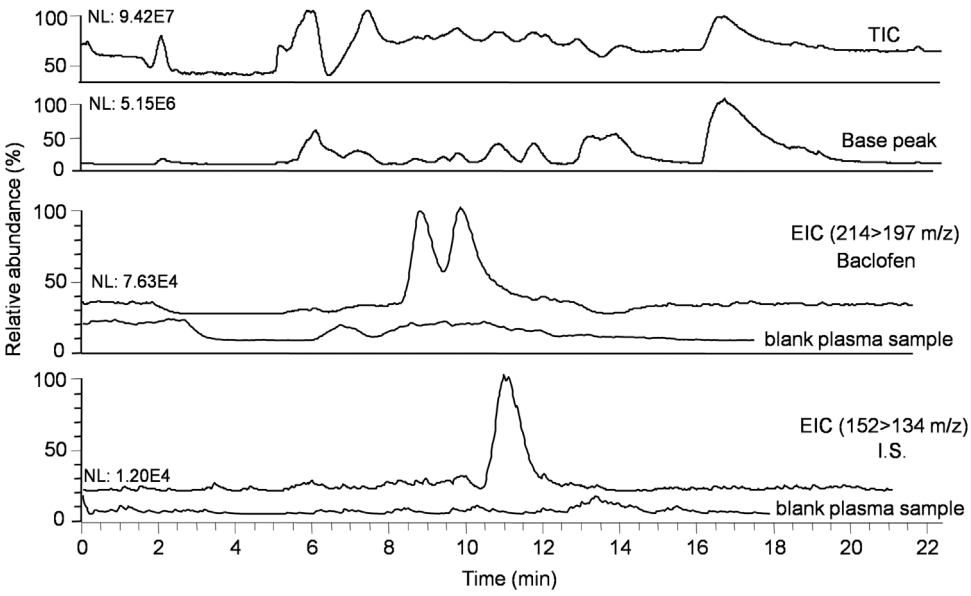

Fig. 4. Total ion chromatogram, base peak and MS quantitative transition of spiked human plasma sample after BP-SPE-nano-LC–MS/MS method. Experimental conditions: racemic mixture of baclofen, 400 ng/mL, IS, 800 ng/mL. For further information see section 2.4.

concentration levels by performing 3 times extraction (n = 3). The found concentration was calculated from the previous calibration curves (Table 2) and compared with a Student's t test, matching the experimental t-value with the tabulated one for n = 5 (t_4 = 2.78, p = 0.05). As shown in Table 3, all experimental t-values were lower than t_4, with a trueness in the range of 89–95% and precision (RSD, %) less than 20% in all each enantiomers. At both spike levels, the average recovery of the two enantiomers were 65% and 72%, respectively.

Fig. 4 shows the TIC, base peak and the extracted ion chromatogram (EIC) of Bac and IS in spiked human plasma sample after the BP-SPE-nano-LC–MS/MS.

4. Conclusions

In this study, a lab-made nano-LC–MS/MS method was used for the separation of Bac enantiomers on a 100 μm I.D. capillary column packed with the CSP-vancomycin attached to silica stationary phase. Acceptable resolution was achieved in less than 11 min. After a suitable deproteinization step of human plasma sample, a SPE procedure making use of CNT-based membrane was applied to clean-up the extract from the previous step. The method was validated as suggested by the European Commission Decision 2002/657/EC, obtaining good recovery, accuracy in the range 89–95% with and RDSs lower than 20%.

Compared to previous methodologies based on conventional chromatography [22,42–44], the proposed nano-LC method exhibits a lower sensitivity (LOQ =85 ng/mL) but still perfect compatibility with a TDM application in a common daily Bac dosage [42]. On the other hand, the use of minute volumes of mobile phase for the nano-LC analysis results advantageous both in terms of costs and operator health, representing a valid eco-sustainable alternative to the classical HPLC waste of organic solvents.

Finally, to the best of our knowledge, the rotating disc-SPE procedure combined with nano-LC–MS/MS system is applied, for the first time, to the determination and quantification of enantiomers of Bac in a human plasma sample.

Declaration of Competing Interest

None.

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