In vitro and in vivo quantification of chloroprocaine release from an implantable device in a piglet postoperative pain model

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Background: The pharmacokinetic properties and clinical advantages of the local anesthetic chloroprocaine are well known. Here, we studied the pharmacokinetic profile of a new hydrogel device loaded with chloroprocaine to investigate the potential advantages of this new strategy for postoperative pain (POP) relief.

Materials and methods: We performed both in vitro and in vivo analyses by considering plasma samples of four piglets receiving slow-release chloroprocaine. To quantify chloroprocaine and its inactive metabolite 4-amino-2-chlorobenzoic acid (ACBA), a HPLC–tandem mass spectrometry (HPLC-MS/MS) analytical method was used. Serial blood samples were collected over 108 hours, according to the exposure time to the device.

Results: Chloroprocaine was consistently found to be below the lower limit of quantification, even though a well-defined peak was observed in every chromatogram at an unexpected retention time. Concerning ACBA, we found detectable plasma concentrations between T₀ and T₁₂h with a maximum plasma concentration (Cmax) observed 3 hours after the device application. In the in vitro analyses, the nanogel remained in contact with plasma at 37°C for 90 minutes, 3 hours, 1 day, and 7 days. Chloroprocaine Cmax was identified 1 day following exposure and Cmin after 7 days, respectively. Additionally, ACBA reached the Cmax following 7 days of exposure.

Conclusion: A thorough review of the literature indicates that this is the first study analyzing both in vivo and in vitro pharmacokinetic profiles of a chloroprocaine hydrogel device and is considered as a pilot study on the feasibility of including this approach to the management of POP.

Keywords: postoperative outcome, hydrogel device, chloroprocaine, ACBA, pharmacokinetics

Introduction

The mechanism of local anesthetics (LAs) in blocking the transmission of painful stimuli to the brain is well known.¹ Chloroprocaine, an ester-type LA, is less cardiotoxic than most others, but it is characterized by an extremely short half-life.² New delivery systems based on hydrogel devices may effectively and safely extend the LA’s analgesic action.³,⁴ Thanks to their unique properties, including biocompatibility, flexible methods of synthesis, network structure, and stability of the incorporated bioactive molecules, hydrogel devices have sparked particular interest in use in drug delivery applications.³ Currently, a wide number of natural polymers (ie, chitosan, pectin, and alginate) are employed for hydrogel preparation due to their capability of mimicking biological structures and the fact that they are well tolerated inside the human body.³,⁶

Postoperative pain (POP) remains a challenge.⁷ POP is a common feature in all surgical procedures, and it can have a profound impact on patients’ outcomes. LAs represent a cornerstone for effective postoperative analgesia⁸ because they block...
nociceptive stimulus from the periphery and provide pain relief both at rest and during movement. However, LAs have limited duration of action, and even with modern long-acting formulations, analgesic efficacy is limited to the first day following surgery. Unfortunately, POP continues much longer than the first day, and as soon as the effect of the analgesic block decreases, nociceptive and hyperalgesic mechanisms can start again. Since prolonged uncontrolled pain in the perioperative period can lead to immediate and long-term complications (including pain chronicization), there is strong interest in prolonging nociceptive blockage. Catheters are used to provide continuous infusion of LAs but, despite their efficacy, they require additional efforts for management by care providers and patients, and complications can occur.

New strategies for prolonged and controlled release of LAs are required, together with the use of dedicated LAs with optimal pharmacokinetic profiles for continuous infusion (ie, low toxicity, rapid metabolism, and rapid onset/offset of analgesic effect).

Chloroprocaine has an optimal pharmacokinetic profile, and it is promising from the perspective of continuous infusion in the postoperative period. Furthermore, we have recently demonstrated that a continuous intrawound infusion of chloroprocaine can also reduce perilesional inflammation in an animal model.

Finally, nanotechnology meanwhile offers new potential advantages in terms of controlled and sustained release of drugs over several days.

We present data on a chloroprocaine-loaded hydrogel device that might offer revolutionary advantages for postoperative analgesia through its specific pharmacokinetic profile, supported by a continuous hydrogel device administration. In this study, we analyzed both in vitro and in vivo (piglets receiving slow-release chloroprocaine) pharmacokinetic profiles of the chloroprocaine-loaded device.

Materials and methods

Materials

Alginate-based hydrogels were obtained by calcium cross-linking starting from an internal gelation mechanism. Hydrogels were produced from an alginate (alginic acid sodium salt powder, 180947, lot MKBJ0727V; Sigma-Aldrich Co., St Louis, MO, USA) solution with an initial concentration of 3.36% (w/v). For HPLC analysis, high-purity water (CHROMASOLV®, 34877; Sigma-Aldrich Co.) was used for the preparation of solutions and suspensions. The drug (chloroprocaine powder, Lot: #140376; Sintetica Company, Mendrisio, Switzerland), pre-suspended in water and mixed with a diluted solution of alginate (0.5% w/v), was added to the initial solution. The final concentrations were 36 mg/mL alginate loaded with 75 mg/mL of chloroprocaine. To prevent bacterial contamination, ethanol-washed alginate was produced by suspending 10 g alginic acid sodium salt in 50 mL of absolute ethanol. The procedure was repeated three times. In each step, alginate was suspended in ethanol for 5 minutes, left to sediment for 10 minutes, subsequent to which the supernatant was removed. Following the final wash, the remaining ethanol was allowed to evaporate for 48 hours. All the processes (including preparation of alginate solution) took place under laminar flow. Solutions and water were filtered with 0.22 μm filters. The hydrogels were produced within an ad hoc mol (14 cm×2 cm×0.4 cm) for implantation and testing, unless diversely specified.

In vitro chloroprocaine release profile

UV analysis

A chloroprocaine calibration curve was obtained for a physiological-like solution (NaMgCa medium, 145 mM NaCl, 1.5 mM MgCl₂, and 2.5 mM CaCl₂) measuring solution absorbance with UV-spectroscopy technique (UV/visible Spectrophotometer; Jenway, Staffordshire, UK) in a range of 200–500 nm. NaMgCa medium was developed with the aim of mimicking human plasma concentration considering not only monovalent ions (Na⁺) but also divalent ions (Ca²⁺ and Mg²⁺). Medium composition presents a Na⁺ concentration that is isotonic to human plasma and the identical concentration of Mg²⁺ and Ca²⁺ in human plasma. From a stock drug solution in NaMgCa medium with a concentration of 1.5 mg/mL, different solutions in the concentration range of 2.5–120 μg/mL were prepared by subsequent dilution. All measurements were done in 10 mm quartz cells (108-QS; Hellma Analytics, Müllheim, Germany). A calibration curve was obtained considering the absorbance peak at 291 nm.

Drug release was performed from hydrogel samples with 15.4 mm diameter and 3 mm thickness in 1 mL of medium. Samples were put in contact with NaMgCa medium for 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, and 24 hours, collecting the medium after each time point and replacing it with fresh medium.

All measurements were calculated in duplicate and in at least two distinct experiments.

HPLC/tandem mass spectrometry (HPLC-MS/MS) analysis

HPLC-MS/MS (TSQ Quantum Access; Thermo Fisher Scientific, Waltham, MA, USA) analytical method was
used to quantify chloroprocaine and its inactive metabolite 4-amino-2-chlorobenzoic acid (ACBA) in plasma and acidified methanol, following in vivo and in vitro tests, respectively. In brief, four nanostructured devices containing chloroprocaine and four unfilled nanogel devices (used as a reference) remained in contact with plasma at 37°C for 90 minutes, 3 hours, 1 day, and 7 days (four samples) for HPLC-MS/MS analysis.

Mobile phases A (ultrapure water, Millipore Direct-Q™ system) and B (CH₃OH, 34860-2.5 L-R; Lot: # STBF5815V; Sigma-Aldrich Co.) were both acidified with formic acid (33015-1L; Lot: # SZBF1540V).

Chloroprocaine chlorohydrate powder (Lot: #140376; Sintetica Company) and its inactive metabolite (ACBA) were obtained from Sigma Aldrich Co. (217719-5G; Lot: # S54349V) and used to prepare the standard solutions. Lidocaine (20 mg/mL; Bioindustria L.I.M S.p.a, Fresonara, Italy) was used (following dilution) as an internal standard (IS). Stock solutions of chloroprocaine and ACBA were prepared in ultrapure water (chloroprocaine: 1 mg/mL; ACBA: 1 mg/mL).

The separation of the two analytes was performed using a Thermo Scientific Accela system, a quaternary pump coupled with an autosampler, and a Kinetex® 2.6µ C18 100 Å Column (33015-1L; Lot: # SZBF1540V). Chloroprocaine and ACBA analysis for “in vitro tests”

The preliminary analyses showed a high concentration of chloroprocaine and its metabolite (mg/mL) following exposure of the plasma to the device loaded with the drug, as a consequence of its release.

Before proceeding with the analysis, it was necessary to dilute the samples to a ratio of 1:10,000 with acidified CH₃OH (0.1% HCOOH) following processing them, according to the standardized procedures reported subsequently.

Calibrators (A–F) were prepared for quantification and quality controls (QcH, QcM, and QcL) directly in CH₃OH/ H₂O, at the concentrations specified subsequently, to compare signals from comparable matrices. The linear correlation coefficients (R²) of the calibration curves obtained from those calibrators were consistently higher than 0.99 for both chloroprocaine and ACBA.

In vivo tests

The hydrogels were tested in vivo using piglets as the animal model (pigs weighing 20 kg).

All animal procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) – University of Pavia. The animal care and experimental procedures respected local, national, and European Union guidelines for protection of animals used for scientific purposes (Directive 2010/63/EU – revising Directive 86/609/EEC).

Two different types of tests were performed as follows:

1. In each pig, two incisions were made in the abdominal region, one for the control without the hydrogel and one with the loaded hydrogel.
2. Two incisions were made in the same region, and a hydrogel without the drug (control) was placed in one incision and in the other the loaded hydrogel was placed. The incision was performed by simulating a laparotomy (15 cm), cutting the skin, the abdominal fat, the abdominal muscles, and the peritoneum. Prior to the hydrogel insertion, the peritoneum was sutured. During the surgery, the pigs were anesthetized and all the materials used were sterile. The animals were routinely monitored for general appearance, activity, and healing of the implant sites and were sacrificed after 21 days. No pigs were lost during the study.
The surgery was performed simulating a laparotomy (15 cm), cutting the skin, the abdominal fat, the abdominal muscles, and the peritoneum (Figure 1A). The peritoneum was then sutured (Figure 1B), the device was inserted (Figure 1C), and finally all the other tissues were sutured (Figure 1D).

**Working solution preparation for the detection of chloroprocaine and ACBA analysis for “in vivo tests”**

Plasma samples of pigs with implanted nanostructured hydrogel devices were analyzed to evaluate chloroprocaine release.

Calibrators and the calibration curves in pigs’ plasma collected immediately prior to treatment (reference plasma) were prepared as described in the following sections.

Chlorprocaine chlorohydrate powder was weighed and dissolved in water to obtain two distinct stock solutions (M₁ and M₁QC: 1 mg/mL) to be used for the preparation of the six calibrators and three quality control working standard solutions – labeled A, B, C, D, E, F, and QcH, QcM, and QcL, respectively – by a serial dilution procedure.

Analogously, ACBA stock solution (1 mg/mL in CH₃OH) and the related working standard solutions for the calibrators and quality control preparations were prepared.

The stock solutions M₁ and M₁QC (chloroprocaine and ACBA) were stored at −20°C until use, whereas the working standard solutions were prepared on a daily basis.

**Preparation of calibrators, quality controls, and plasma samples**

Starting with 90 µL of reference plasma and 10 µL of each working solution, six calibrators (A–F) and three quality controls (QcH, QcM, and QcL) were prepared, each containing both chloroprocaine and the inactive metabolite ACBA at the desired concentrations: F 15.65 ng/mL, E 31.25 ng/mL, D 62.5 ng/mL, C 125 ng/mL, B 250 ng/mL, A 500 ng/mL; QcH 400 ng/mL, QcM 200 ng/mL, and QcL 20 ng/mL.

The IS (lidocaine) and methanol were added to each sample (100 µL of pig’s plasma) to promote protein precipitation. After mixing via vortex and centrifuge separation, the supernatant was recovered and dried under a weak stream of nitrogen. Next, the precipitate was suspended in acidified methanol and directly injected into the column.

Both the corresponding calibration curves of chloroprocaine and ACBA demonstrated an excellent linearity in the considered concentration range (15.65–500 ng/mL), characterized by a correlation coefficient ($R^2$) consistently higher than 0.99.

![Figure 1](https://www.dovepress.com/)

**Figure 1. Different steps of surgery.**

**Notes:** (A) Incision of skin, abdominal fat, abdominal muscles, and peritoneum. (B) Suture of peritoneum. (C) Device insertion. (D) Suture of all other tissues.
Pharmacokinetic profile of chloroprocaine from an implantable device

Acceptance criteria of an analytical run
As reported in the “Guideline on bioanalytical method validation” (EMA/CHMP/EWP/192217/2009 Rev.1 Corr.*), the back calculated concentrations of the calibration standards should be within ±15% of the nominal value, with the exception of the lower limit of quantification (LLOQ), for which it should be within ±20%. At least 75% of the calibration standards (1 over 6), with a minimum of six, are required to fulfill this criterion. If one of the calibration standards does not meet these criteria, this calibration standard should be rejected and the calibration curve without this calibration standard should be re-evaluated, and regression analysis should be performed. The accuracy values of the Qc samples should be within ±15% of the nominal values. At minimum 67% of the Qc samples and 50% at each concentration level should comply with this criterion. If these criteria are not fulfilled, the analytical run should be rejected, and the study samples re-extracted and analyzed.

Results
In vitro evaluation of chloroprocaine-loaded hydrogel release UV spectroscopy and HPLC-MS/MS analytical method was performed for in vitro evaluation of the chloroprocaine release from the nanostructured hydrogel device.

UV analysis of chloroprocaine release
For initial quantitative evaluation of the drug in release medium, UV spectroscopy was employed to correlate the concentration of the drug with the absorbance. Chloroprocaine spectra at different concentrations in medium are displayed in Figure 2. For concentrations above 50 µg/mL, absorbance resulted in out-of-scale peaks. The spectra present characteristic peaks of different absorbance intensities.

The chloroprocaine-loaded hydrogel sample demonstrated an initial release rate of 22.5 mg/h, and in the following hours, a constant rate of ~8.0 mg/h. The release decreased after 5 hours and was prolonged until 24 hours, at which point the detection limit was reached (Figure 2B).

Evaluation of chloroprocaine and ACBA metabolite release by HPLC-MS/MS
For HPLC-MS/MS detection, four nanostructured devices containing chloroprocaine and four unfilled nanogel devices (used as reference) remained in contact with plasma at 37°C for 90 minutes, 3 hours, 1 day, and 7 days (four samples). The plasma samples were analyzed via HPLC-MS/MS to assess chloroprocaine and ACBA concentrations.

The obtained concentrations for chloroprocaine and its ACBA metabolite were 0.522–5.436 and 2.84–8.348 mg/mL, respectively (Figure 3).

The analyses produced a peak of maximum plasma concentration \( C_{\text{max}} \) of chloroprocaine after 1 day of exposure to the device (5.436 mg/mL) and a minimum \( C_{\text{min}} \) after 7 days (0.522 mg/mL).

Otherwise, ACBA reached \( C_{\text{max}} \) after 7 days (8.348 mg/mL).

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Figure 2 (A) Chloroprocaine UV spectra at different concentrations in NaMgCa medium. (B) Chloroprocaine release from hydrogel device measured at different time points.

Notes: A standard curve \( y = 62,194x + 0.0837; R^2 = 0.9958 \) was obtained from the absorbance of the band at 291 nm for chloroprocaine, as it was the only one clearly detectable for the range of concentrations analyzed (2.5–75 µg/mL). It was not possible to apply UV spectroscopy to evaluate concentrations below 2.5 µg/mL, the LLOQ.

Abbreviations: h, hours; LLOQ, lower limit of quantification.
Chloroprocaine plasma concentrations were undetectable in all the reference samples; in contrast, traces of ACBA were found in all the samples.

In vivo evaluation of the chloroprocaine-loaded hydrogel release
For each of the four piglets (named Gelly 2, Gelly 3, Gelly 4, and Gelly 5), 11 plasma samples were analyzed and identified as basal, T₀, T₉₀min, T₃h, T₆h, T₁₂h, T₂₄h, T₄₈h, T₆₉h, T₉₆h, and T₁₀₈h, according to the length of exposure (measured in hours) to the device. The basal sample was never in contact with the device (Figure 4), whereas the T₀ sample remained in contact with the device for less than 3 hours.

As expected, signals of ACBA, lidocaine, and chloroprocaine were absent in the plasma of the untreated piglets.

No quantifiable amount of chloroprocaine was identified in any of the plasma samples.

The detected ACBA plasma concentrations are reported in Table 1. The LLOQ was 15 ng/mL.

Plasma concentrations were detectable (≥LLOQ), primarily between T₀ and T₁₂h.

The T₀ plasma concentration of Gelly 2 (although revalued several times) was an outlier (3,209.42 ng/mL).

The peak of maximum ACBA plasma concentration (Cₘ₉₉) was observed 3 hours following the device application (Table 1).

Although the chloroprocaine plasma concentrations were consistently below the LLOQ, a well-defined peak in the chromatograms of each animal (between T₉₀min and T₁₂h) was detected, albeit at a different retention time from that expected.

In order to identify their well-defined retention times, some working solutions of ABCA, lidocaine, and chloroprocaine were at first added to a piglet-free drug plasma and analyzed; the related chromatographic peaks were obtained at 2.82, 2.73, and 1.90 minutes, respectively (Figure 5).

On the contrary, unexpected peaks at 2.58 minutes (differing from 1.90 minutes) were observed in the chloroprocaine mass channel during the chromatographic runs of Gelly 2, Gelly 3, Gelly 4, and Gelly 5, and they were observed between T₃h and T₆h.

Discussion
LAs are useful in treating acute POP, although severe side effects are related to their systemic usage (lidocaine and bupivacaine). Less toxic LAs (eg, chloroprocaine) have been developed, but present short half-lives. To prolong drug effectiveness and minimize drawbacks, LA drug delivery systems have been developed.

Currently, hydrogels are being widely exploited in biomedical applications as drug delivery systems. An innovative aspect of the present work regards the use of alginate, commonly employed to enhance biocompatibility of PLGA microspheres or as coating to tailor drug release, as a principal material for hydrogel device development.

Alginate is an anionic naturally occurring biopolymer, typically extracted from brown algae, that has many possible applications in different biomedical applications. The favorable properties of alginate, including biocompatibility and ease of gelation, enable its use as a matrix for the entrapment and delivery of proteins, drugs, cells, wound healing, and tissue engineering.

The usage of an anionic polymer could also have the advantage of creating electrostatic interactions between polymer and drug, which may reduce drug diffusion coefficients, prolonging the release time. Although alginate is inherently non-degradable in mammals as they lack the...
enzyme (ie, alginase) which can cleave the polymer chains, it has been reported that the degradation of alginate gels can be controlled by the release of the divalent ions involved in the gel cross-linking into the surrounding media via exchange reactions with sodium ions.25 Furthermore, as already evidenced by other studies, the partial oxidation of alginate hydrogels can produce an easy degradation in vivo,26 which constitutes further evidence supporting the clinical utilization of alginate as a device that will not need to be removed after its implantation. All these features, including its anatomical

Table 1 ACBA plasma concentrations in four piglets following application of the nanostructured drug-loaded hydrogel

| Sample name | ACBA (ng/mL)                      | Basal | 3 hours | 6 hours | 12 hours | 24 hours | 48 hours | 72 hours | 96 hours | 102 hours | 108 hours |
|-------------|-----------------------------------|-------|---------|---------|----------|----------|----------|----------|----------|-----------|-----------|
| Gelly 2     | N.C.                              | 3,209.4 | 477.01  | 228.22  | 69.16    | N.C.     | N.C.     | N.C.     | 15.53    | N.C.      | N.C.      |
| Gelly 3     | N.C.                              | 109.25  | 524.14  | 216.31  | 55.50    | N.C.     | 15.09    | 19.53    | N.C.     | N.C.      | N.C.      |
| Gelly 4     | N.C.                              | 19.67   | 470.44  | 378.73  | 83.00    | N.C.     | N.C.     | N.C.     | N.C.     | N.C.      | N.C.      |
| Gelly 5     | N.C.                              | 393.53  | 1330.4  | 313.69  | 44.38    | N.C.     | N.C.     | N.C.     | N.C.     | N.C.      | N.C.      |

Abbreviations: ACBA, 4-amino-2-chlorobenzoic acid; N.C., not calculable.
compatibility with soft tissues, make alginate suitable for the controlled release of LAs as an alternative to the use of synthetic biodegradable polyesters.

Through an UV and an HPLC-MS/MS system, we analyzed in vitro and in vivo release of an experimental chloroprocaine-loaded hydrogel sample. First, in vitro results demonstrated an initial release rate of 22.5 mg/h and in subsequent hours, a constant rate of ~8.0 mg/h. The release decreased after 5 hours and was prolonged until 24 hours. Further analysis associated the decrease in chloroprocaine concentration with an increase in ACBA concentration, indicating additionally an in vitro drug degradation.

From an in vivo analysis, we found that at the specified sampling time, LA’s concentration was never detectable. Conversely, ACBA was quantified in almost all plasma samples, with a peak of $C_{\text{max}}$ observed 3 hours following the device application.

The $T_{\text{max}}$ concentration value of Gelly 2 (although revalued several times) was found not to align with that of the others. We assume that blood was incorrectly collected immediately following the nanodevice hydrogel device application rather than prior to the application.

Moreover, the presence of a molecule similar to chlorprocaine was detected through molecular weight and the same fragmentation pattern, although differed structurally. This may have resulted from specific interactions with the stationary phase of the analytical column and the mobile phases of another chlorprocaine metabolite undergoing an in-source fragmentation producing chlorprocaine.27–29

Figure 5 Chromatograms of a piglet’s plasma sample, added with chloroprocaine, ACBA, and lidocaine, as an internal standard. Abbreviations: ACBA, 4-amino-2-chlorobenzoic acid; + c ESI, a positive, centroid scan, acquired from ions generated by an electrospray ion source (ionization mode); min, minutes.
In vitro, the ACBA metabolite reached the C\textsubscript{max} after 7 days, whereas chloroprocaine obtained a C\textsubscript{max} following only 1 day of exposure to the device, and a minimum (C\textsubscript{min}) after 7 days. These results suggest that the drug degraded slowly over time. As was the case in the analysis of reference samples, quantifiable traces of the metabolite emerged, and it was assumed that the device released some material, initially present on the surface of contact with the plasma.

The presented data suggest that the hydrogel device maintains the structural characteristics of drugs. Therefore, the initial rapid drug release may be effective in treating POP in the initial postoperative hours, while a reduced rate of release may be effective to prevent chronic pain in the subsequent period.

In the near future, we intend to repeat the analyses on the reference samples, starting from devices for which no release is expected, placed only in contact with serum and then water. Finally, we will validate the pharmacokinetic results by substituting the procaine for the chlorprocaine. Doing so will highlight any differences between the two drugs in terms of release and degradation processes.

By following a precision medicine approach, drug loading will be modulated depending on the type of pain to be managed. We are hopeful that such translational research will soon result in the clinical utilization of the hydrogel device.

**Limitations of the study**
This study reports qualitative results of a device implanted in piglets, to evaluate chloroprocaine release, for potential future management of human POP. Piglets, as larger animals, are considered the most similar pharmacokinetic model to human being in terms of experimental response, although the cost of piglets compared to rats necessitates a smaller sample size. This limitation, as well as the absence of previous literature data regarding analog in vitro and in vivo results, does not allow us to perform a power statistical analysis or to support any power calculation. Accordingly, the present study is to be considered a pilot, and our expectation is that future, larger investigations will allow us to move closer to the ultimate goal of human application in the clinical setting.

**Conclusion**
The experimental chloroprocaine-loaded hydrogel sample was developed to produce a new effective analgesic method, which will contribute to improving POP relief.

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**Disclosure**
The authors report no conflicts of interest in this work.

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