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Neurotoxicity of Adjuvants used in Perineural Anesthesia and Analgesia in Comparison with Ropivacaine

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Abstract

Background and Objectives—Clonidine, buprenorphine, dexamethasone, and midazolam (C,B,D,M) have been used to prolong perineural local anesthesia in the absence of data on the influence of these adjuvants on local anesthetic (LA)-induced neurotoxicity. Therefore, the impact of these adjuvants on ropivacaine (R)-induced death of isolated sensory neurons was assessed.

Methods—The trypan blue exclusion assay was used to assess death of sensory neurons isolated from adult male Sprague-Dawley rats. Drugs were applied, alone or in combination, for 2 or 24 hrs at 37°C.

Results—Neuronal viability was halved by 24 hr exposure to R (2.5 mg/mL), far exceeding the neurotoxicity of C, B, D, or M (at 2–100 times estimated clinical concentrations). Plain M at twice the estimated clinical concentration produced a small but significant increase in neurotoxicity at 24 hr. After 2 hr exposure, high concentrations of B, C, and M increased the neurotoxicity of R; the combination of R+M killed over 90% of neurons. Estimated clinical concentrations of C+B (plus 66 µg/mL D) had no influence on (i) R-induced neurotoxicity, (ii) the increased neurotoxicity associated with the combination of R+M, or (iii) the neurotoxicity associated with estimated clinical concentrations of M. There was dose-response neurotoxicity with 133 µg/mL D combined with R+C+B.

Conclusions—Results with R re-affirm the need to identify ways to mitigate LA-induced neurotoxicity. While having no protective effect on R-induced neurotoxicity *in vitro*, future research with adjuvants should address if the C+B+D combination can enable reducing R concentrations needed to achieve equi-analgesia (and/or provide equal or superior duration, in preclinical *in vivo* models).

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Introduction

Local anesthetics (LA) are widely used for peripheral nerve blockade. Ropivacaine is commonly chosen instead of shorter-duration agents such as mepivacaine or lidocaine in an effort to extend the duration of postoperative analgesia, and to prolong surgical block. While advantages of longer-duration blocks are numerous, particularly in the postoperative setting, the possibility of both local and systemic toxicity precludes a simple increase in concentration and/or volume of administration as a means of increasing analgesic duration. In an effort to expand the narrow therapeutic window of local anesthetics, several perineural adjuvants have been studied with the goal of prolonging the duration of analgesia for a given LA dose, reducing the LA dose and/or minimizing other effects (e.g., motor blockade). These perineural adjuvants include clonidine,^{1–3} buprenorphine,^{4–6} dexamethasone,^{7–9} and midazolam^{10,11} (abbreviated hereafter as C+B+D+M when referring to drug combinations). These adjuvants have gained recent but limited clinical use based on Grade A, Level 1B evidence that they increase perineural analgesic duration.

From the patient care perspective, in the context of peripheral nerve damage after LA blockade¹² (with an unknown effect of perineural adjuvants), toxicity to neurons in culture should be considered a potentially relevant predictor, given that up to 3% of patients have complications that can persist up to 6 months after nerve blocks with LA.¹³ Although these perineural adjuvants are commonly used clinically, their neurotoxicity profiles have yet to be investigated. In addition, given the toxicity of LA, it is unknown whether these adjuvants can mitigate the toxic impact of LA. The present study was performed to begin to address this issue.

Toxicity was assessed with the trypan blue exclusion assay in dissociated rat dorsal root ganglion (DRG) neurons following exposure to test agents alone and in combination with each other and/or with ropivacaine (R). Our hypothesis was that estimated clinical concentrations of C ± B ± D ± M would have minimal toxicity alone, and would attenuate R-induced toxicity.

Methods

The research protocol was approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC, Pittsburgh, Pennsylvania, United States). Animal care and handling were in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council).

Animal selection and housing

Male Sprague-Dawley rats (7–9 wk, 275–300 g, Harlan, Indianapolis, IN) were used in all experiments. Prior to use, rats were housed in an AAALAC-accredited animal care facility with unrestricted food and water on a 12:12-hour light-dark cycle for a minimum of 3 d after shipment from the vendor.

Harvest of primary afferent neurons, incubation, and drug exposure

Isolated sensory neurons were used to assess the potential neurotoxicity of adjuvants used for peripheral nerve block. This is because injury at the cell body is readily detected with a number of techniques including the trypan blue exclusion assay employed in the present study (see below). Furthermore, use of the isolated sensory neuron cell body *in vitro* maximizes the sensitivity of toxicity assays. This is because there are no barriers to diffusion limiting drug access to the target of interest. In addition, the cell body is likely more vulnerable to toxic challenge than distal axons (the site of drug administration *in vivo*) due to differences in surface-to-volume ratio and the distribution of cellular contents/organelles

that may influence the response to toxic challenge. Nevertheless, these factors may also lead us to over-estimate the potential toxicity of LA and adjuvants used in peripheral nerve blocks.

Bilateral lumbar (L3-L5) DRG were harvested, dissociated, and neurons cultured as previously described¹⁴ on poly-D-lysine coated cover slips (Becton Dickinson Biosciences, Bedford, MA). After 16–18 hr in culture with Neurobasal® medium (Gibco BioResearch Lab, Gaithersburg, MD), cover slips were then bathed within their individual culture wells with choline (as the control) or study drug (or drug combination). In a series of exploratory experiments, these control and test solutions were applied for a range of 30 min to 24 hr, with incubation conditions of 37°C, 5% CO₂, and 90% humidity. Test solutions were then rinsed with normal bath solution (NBS, containing in g/L: NaCl, 7.6; KCl, 0.224; CaCl₂, 0.275; MgCl₂, 0.057, HEPES, 2.6; Glucose, 1.8)¹⁴.

Neurotoxicity Assay

Neurotoxicity was assessed with the trypan blue exclusion assay. Following wash of the last test solution, cover slips were flooded for 10 min in 0.4% trypan blue (0.1% after dilution in NBS). Trypan blue was then washed with NBS, and cover slips were evaluated (within the culture dish well) with light microscopy for trypan blue staining. The fraction of trypan blue-stained neurons was estimated as previously described¹⁴ by counting all neurons, stained and unstained, in a single pass through each cover slip at 40X magnification on an inverted microscope (Olympus CK2 Phase, Olympus Optical Co., Ltd., Japan). Trypan blue staining and cell counting was performed at room temperature.

While there are limitations with the use of the trypan blue exclusion assay as a means of assessing neurotoxicity, which include its relative insensitivity and the absence of information provided about the underlying mechanism of neuronal death (i.e., necrosis or apoptosis), we chose to employ this approach in the present study because we felt the strengths of the assay outweighed its limitations. These included: (i) it is a widely used assay¹⁵ that we have previously employed to assess neurotoxicity in sensory neurons; (ii) it is fast, (iii) the signal is relatively unambiguous, and (iv) we did not want to make any assumptions about the mechanism of neuronal death.

Drug preparation

R was used in the present study for two main reasons: First, as an S-enantiomer, it is accepted to have less cardiac toxicity than bupivacaine, and therefore has largely replaced bupivacaine for the generation of long-duration nerve blocks in some clinical practices. Second, because a long-duration block is the goal when R is used, and since adjuvants are used to further extend the duration of block, adjuvants are often employed in combination with R. Drug concentrations for R ± C ± B ± D ± M (weight/vol and molar) are listed in Table 1; in the text, these are presented as weight/vol. Drugs were pharmaceutical-grade, preservative-free, commercially-available preparations (ropivacaine HCl: Naropin® 10 mg/mL, APP Pharmaceuticals, Schaumburg, IL; clonidine HCl: Duraclon® 100 µg/mL, Xanodyne Pharmaceuticals, Inc. Newport, KY; buprenorphine hydrochloride 0.3 mg/mL, Ben Venue Laboratories, Bedford, OH; dexamethasone sodium phosphate 10 mg/mL, [preservative-free, with no benzyl alcohol] APP Pharmaceuticals, Schaumburg, IL; and midazolam HCl 5 mg/mL, Ben Venue Laboratories, Bedford, OH). In culture, 1200 µL of drug (or choline) solution was mixed with 600 µL of culture medium; the final pH of all solutions after mixture with culture medium, before contact with cells, was 7.1 – 7.4 (ColorpHast® Indicator Strips, EMD Chemicals Inc., Gibbstown, NJ). R concentrations (2.5 mg/mL) did not change; C ± B ± M were tested at “high-concentration” and “estimated

clinical concentration;" D was tested at "high concentration" and at "subclinical concentrations" (detailed in the next section).

Concentration selection for C±B±D±M to match or approach clinical doses in the literature

For C, previous anecdotal observations suggest that concentrations of 2 µg/mL are sedating clinically¹⁶, and the same concentration was sedating in previous *in vivo* rat studies in our laboratory (unpublished observations). Therefore 1 µg/mL was selected for the present study. For B, there are no known reports of its use in continuous infusions via perineural catheter. However, previous anecdotal observations¹⁶ of the results of combining R+C+B in routine clinical nerve blocks suggested that B may have efficacy when used with C in a ratio of 0.5–1 µg/kg of C and 1.5–3 µg/kg of B. Therefore, we opted for an "estimated clinical" B concentration of 3 µg/mL (maintaining a C+B wt/vol ratio of 1:3). For D, we were not aware of perineural infusions of this drug (alone or in combination). Concentrations of D that ranged between 220 and 363 µg/mL (resulting in a total of 8 mg of D per injection) have been used in single-injection local anesthetic mixtures in clinical studies.^{7–9} While none of these authors cited previous pre-clinical safety data on these concentrations when combined with local anesthetics, we opted for conservatively low D concentrations of 66.6 µg/mL and 133 µg/mL for combination studies based on the estimation that if given as a 24-hour perineural infusion at 5–10 mL/hr, these concentrations would yield a total daily dose from 8–32 mg. Finally for M, 2 doses were chosen for the "estimated clinical" adjuvant combinations. Doses of 50 µg/kg of M have been used with bupivacaine as single-injection blocks.^{10,11} For a 70–100 kg patient, this would extrapolate to 3.5–5.0 mg; if this dose were evenly divided over a 24 hr infusion, which translates to an infusion rate of ~15–21 µg/hr. If the M concentration was fixed at 33.3 µg/mL, a 5–10 mL/hr infusion would lead to a 24 hr dose of 4–8 mg, while a 5–10 mL/hr infusion of 16.6 µg/mL would lead to a 24 hr dose of 2–4 mg. Therefore, concentrations of 33.3 and 16.6 µg/mL were chosen for M concentrations for the 24 hr exposure, alone and in combination.

Exploratory experiments (data not shown), utilizing multiple coverslips from single rats, were conducted to determine the timing for single and combination drug experiments that would enable detection of both positive and negative interactions. Based on these results, single and combination drug experiments were performed at 2 and/or 24 hr of drug exposure on neurons from 4 rats per manipulation. The drug concentrations used are listed in Table 1. The person counting neurons was blinded with respect to treatment of each cover slip.

Data analysis

Each drug/combination was applied to at least two cover slips per animal where the average proportion of "dead" neurons was used as the data point per animal. Choline chloride was used as a control for changes in osmolality associated with the addition of active drugs/combinations. Choline control treated cover slips were included in every test run to monitor the overall health of the preparation. Experimental animals were excluded and replaced when trypan blue staining in the choline control cover slips from these preparations was > 2 SD from the mean derived during exploratory experiments. Seventeen rats were used; data from 1 animal was excluded based on this criterion.

Differences between treatments and choline controls were assessed using a one-way ANOVA and the Holm-Sidak adjustment for multiple comparisons. Data were analyzed with SPSS® v17.0 for Windows (SPSS, inc., Chicago, IL).

Results

Single Drug Tests

A 24 hr exposure to R (2.5 mg/mL), and high concentrations of M (66.6 µg/mL) or B (20 µg/mL) resulted in significant ($p < 0.01$, $n = 4$, Figure 1) increases in the proportion of dead neurons. In contrast, a lower “estimated clinical” concentration of M (33.3 µg/mL), and high concentrations of C and D had no detectable influence on cell death.

Ropivacaine and Single-Adjuvant Neurotoxicity after 2 hr Incubation

High concentrations of single adjuvants C and M each increased R-induced neurotoxicity (Figure 2, $p < 0.01$, $n = 4$). The neurotoxicity associated with R+B and R+D was not significantly greater than with R alone.

Drug Combination Neurotoxicity after 24 hr Incubation

In 24 hr exposure experiments with estimated clinical concentrations of adjuvants (Figure 3a), all combinations of R+M were neurotoxic, and the increase in neurotoxicity associated with the R+M combination was not influenced by the further addition of the C+B+D combination. In the absence of M (Figure 3b), neurotoxicity associated with the combination R+C+B+D (with D at 66.6 µg/mL) was comparable to that resulting from incubation with R alone. However, doubling the D concentration to 133 µg/mL resulted in an increase in neurotoxicity that was significantly ($p < 0.05$) greater than that associated with R alone (Figure 3b).

Discussion

In these experiments, we first confirmed that a concentration of R administered clinically (2.5 mg/mL) is neurotoxic to isolated sensory neurons. High concentrations of clinically-packaged, preservative-free adjuvants, C, B, D and M were significantly less neurotoxic than R. Next, we found that high-concentration single adjuvant combinations with R (specifically R+C, R+B, and R+M) significantly increased R-induced cell death with only 2 hr exposure. In the absence of R, estimated clinical concentrations of the combination of C+B+D+M had no detectable influence on sensory neuron cell death after 24 hr exposure. However, even a low concentration of M in combination with R resulted in a significant increase in neurotoxicity relative to that associated with R alone. This effect of R+M was not influenced by the addition of C+B+D. Finally, increasing the concentration of D from 66 to 133 µg/mL in the combination of estimated clinical concentrations of R+C+B resulted in a further increase in neurotoxicity. These observations reinforce the need for additional pre-clinical testing prior to clinical use of drug combinations.

While we believe that the results from the study of isolated sensory neurons *in vitro* have important implications for the use of LA and adjuvants *in vivo*, there are three issues associated with the use of isolated sensory neurons *in vitro* that may have resulted in an over-estimate of neurotoxicity: (i) the isolated neurons are (by definition) previously injured, making them potentially more vulnerable to a second challenge; (ii) there are no barriers to diffusion or vasculature to facilitate drug removal, resulting in higher concentrations of active drug at the neuronal membrane in the present study than are likely to be encountered *in vivo*; and (iii) the cell body, which was the target for drug application in the present study, may be more vulnerable to toxic challenge than distal axons (the site of drug administration *in vivo*), due to differences in surface-to-volume ratio and the distribution of cellular contents/organelles; these factors may influence the response to toxic challenge. The impact of the first of these issues may have clinical relevance in light of the possibility that there

may be an increase in neurotoxicity observed *in vivo* when LA are used in the context of previous injury or diseases such as diabetes mellitus.

The neurotoxicity of R and of all clinically-used LA ^{14,17–20} further highlights the need to identify novel approaches to mitigate LA neurotoxicity, via direct or indirect approaches. For example, if toxic increases in intracellular Ca^{2+} ultimately mediate LA-induced neuronal death as previously suggested¹⁴, adjuvants such as verapamil which can attenuate Ca^{2+} influx may be a direct approach to attenuate neurotoxicity. While none of the adjuvants assessed in the present study attenuated R-induced neurotoxicity, if they facilitate the use of lower concentrations of LA, they may still provide an indirect strategy to reduce LA-induced neurotoxicity. However, given evidence from the present study that there is dose-response neurotoxicity with “subclinical” concentrations of D (i.e., with 133 $\mu\text{g/mL}$ but not 66 $\mu\text{g/mL}$) exacerbating R-induced neurotoxicity; careful parametric analyses would be required to maximize the therapeutic potential and safety of any combination.

The results of this study also highlight the need for safety data on drug combinations used, in addition to data on drugs used in isolation. That is, while neurotoxicity associated with single-drugs C, B, D, or M alone was minimal (if any), even after 24 hr exposure at concentrations far exceeding those described in clinical use, this was clearly not the case when these adjuvants were combined with R. For example, the combination of R+C resulted in >80% cell death after only a 2 hr exposure. Single-adjuvants (C, B, D, or M) in 2 hr exposure experiments were used at concentrations > 2 times that estimated to be used clinically; results from the 24 hr experiments (using estimated clinical concentrations) suggest that use of these adjuvants in combination (without R) should be safe. The one outlier in this regard was M which not only produced a small but significant increase in cell death after 24 hr at a concentration only twice that estimated has been reported clinically, but also profoundly worsened R-induced neurotoxicity at both 2 hr (at high concentrations) and 24 hr (at estimated clinical concentrations). Adding a low concentration (17 $\mu\text{g/mL}$) of M to R resulted in a significant increase in cell death relative to that associated with R alone. These results suggest that M should not be used in combination with any LA for peripheral nerve block, pending further preclinical safety research to safely declare otherwise.

Results with R+D raise the possibility that the toxicity of this combination may be both time- and concentration- dependent. That is, the increase in neuronal death associated with exposure to the combination of D at 666 $\mu\text{g/mL}$ with R for 2 hr was not significantly greater than that of R alone. In contrast, a “subclinical concentration” of D (133 $\mu\text{g/mL}$, but not 66 $\mu\text{g/mL}$) when used in combination with R+C+B, resulted in a significant increase in cell death after 24 hr. This observation reinforces the need for additional pre-clinical testing prior to clinical use of a drug combination.

Future research should address the following considerations. First, given the confirmation of LA-induced neurotoxicity, it is important to re-evaluate the safety of catheter infusions of LA, given the relatively widespread use of this approach in combination with the prolonged exposure of neural tissue to relatively high concentrations of a LA. Second, it is important to identify mechanisms underlying the actions of adjuvants when used for peripheral nerve blocks. The adjuvants studied were (i) comprised of compounds that are approved for use in humans, and (ii) largely adapted from the spinal analgesia literature for which there was strong mechanistic rationale. There is evidence that C can block voltage-gated Na^{+} channels ²¹ and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels ²², in addition to alpha-adrenergic receptors. Similarly, while there is evidence of functional G-protein coupled receptors in peripheral nerves²³, it is unknown whether activation of these receptors contributes to LA-induced peripheral nerve block. Mechanistic understanding of the actions of these compounds may suggest more effective strategies for both increasing

LA-efficacy and decreasing LA toxicity. Third, further efforts are needed to confirm or reevaluate the mechanisms of LA toxicity, now in the context of multiple-drug combinations. This will be especially important if other adjuvants are shown to mitigate the neurotoxic actions of LAs. Fourth, a dual strategy should be developed to (i) directly mitigate LA-induced toxicity, and (ii) more effectively increase the potency and duration of LA block. Fifth, because epinephrine (2.5 – 5 µg/ mL) is routinely co-administered with LA in many practice settings, it will be important to determine the impact of epinephrine on LA-induced neurotoxicity alone and in combination with other adjuvants.

In summary, while our *in vitro* results are consistent with the suggestion that C and B are safe at estimated clinical concentrations, our results with M suggest that this compound should not be combined in any perineural mixture with LA, pending further preclinical research. Results of the present study also suggest that attention should be directed toward exploring the time-dependent and concentration-dependent basis for neurotoxicity associated with D combined with R.

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Figure 1.

Cell death as a function of treatment with ropivacaine (R) and *high-concentration* (see Table 1) perineural adjuvant single-drugs (C: clonidine, B: buprenorphine, D: dexamethasone, M: midazolam) without R, after 24 hr. Two concentrations of M were tested (33.3 and 66.6 $\mu\text{g}/\text{mL}$, M33 and M66, respectively). Based on ANOVA with the Holm-Sidak correction for multiple comparisons, M66, C, B, and R were cytotoxic to neurons at 24 hr, but M33 and D were not. * $P \leq 0.012$ (versus choline), † $P < 0.001$ (versus choline and all other treatments), ‡ $P < 0.001$ for B being less neurotoxic than R, and $P \leq 0.007$ for B being more neurotoxic than all treatments other than R.

Figure 2.

Cell death as a function of treatment and time, for ropivacaine (R) combinations **with high-concentrations** (Table 1) of single adjuvants clonidine (C), buprenorphine (B), dexamethasone (D), and midazolam (M) after 2 hr of drug exposure. Based on ANOVA with the Holm-Sidak correction for multiple comparisons, R-M and R-C were significantly more neurotoxic than choline (* $P < 0.001$) and were more neurotoxic than R, R-D, and R-B (* $P < 0.01$). R-B was neurotoxic compared to choline control († $P = 0.046$). Based on *post hoc* comparisons, C and M as single-adjuvants worsened R toxicity (each $P < 0.001$), D and B did not. ‡ $P < 0.01$ for R-B being less cytotoxic than each of R-C and R-M).

Figure 3.

Cell death as a function of treatment after 24 hr exposure to plain ropivacaine (R) and clonidine (C) – buprenorphine (B) – dexamethasone (D) - midazolam (M) adjuvant combinations (in estimated clinical concentrations, Table 1) without and with R. Concentrations of M are listed in the figure as 16.6 $\mu\text{g/mL}$ (M17) and as 33.3 $\mu\text{g/mL}$ (M33); concentrations of D are similarly listed as 66.6 $\mu\text{g/mL}$ (D66) and as 133.3 $\mu\text{g/mL}$ (D133). Of note, D66 and D133 are technically lower concentrations than those that have been reported clinically in single-injection blocks (see Methods text for details) **Figure 3a** shows all R-M combinations, with plain R as a reference for *post hoc* comparisons. Based on ANOVA with the Holm-Sidak correction for multiple comparisons, M17 and M33

combined with RPV were profoundly neurotoxic (independent of the presence of C-B-D); * $P < 0.001$ for each R drug (plain or mixture) being more significantly neurotoxic than choline control, and † $P < 0.001$ for plain R being less cytotoxic than all R combinations.

Figure 3b shows the cytotoxicity outcomes after 24 hr exposure for the listed adjuvant combinations in “estimated clinical” concentrations (Table 1), without and with R 2.5 mg/mL (with no combinations of R and M). Based on ANOVA with the Holm-Sidak correction for multiple comparisons, C-B-D-M17 and C-B-D-M-33 were not cytotoxic, while all R (plain or in combination) mixtures were (* $P < 0.001$). C-B-D66 did not worsen the cytotoxicity of plain R, but C-B-D133 did worsen the neurotoxicity of plain R († $P = 0.045$).

Table 1Single-Drug and Combination Incubation Experiments at [†]2 hr and/or [‡]24 hr

Drug	Drug concentration during incubation	Micromolar drug concentrations
^{†‡} R	2.5 mg/mL	9127
[‡] C	[§] 66.6 µg /mL	251
[‡] B	20 µg /mL	40
[‡] D	667 µg /mL	1291
[‡] M	66.6 µg /mL	184
[‡] M33	33.3 µg /mL	92
[†] R + C	2.5 mg/mL + [§] 41.7 µg /mL	9127 + 157 = 9284
[†] R + B	2.5 mg/mL + 20 µg /mL	9127 + 40 = 9167
[†] R + D	2.5 mg/mL + 667 µg /mL	9127 + 1291 = 10,418
[†] R + M	2.5 mg/mL + 66.6 µg /mL	9127 + 184 = 9311
[†] R + M33	2.5 mg/mL + 33.3 µg /mL	9127 + 92 = 9219
[†] R + M17	2.5 mg/mL + 16.6 µg /mL	9127 + 46 = 9173
[‡] R + C + B + D + M33	2.5 mg/mL + 1 µg/mL + 3 µg/mL + 66.6 µg/mL + 33.3 µg/mL	9127 + 3.8 + 6 + 129 + 92 = 9358
[‡] R + C + B + D + M17	2.5 mg/mL + 1 µg/mL + 3 µg/mL + 66.6 µg/mL + 16.6 µg/mL	9127 + 3.8 + 6 + 129 + 46 = 9312
[‡] R + C + B + D67	2.5 mg/mL + 1 µg/mL + 3 µg/mL + 66.6 µg/mL	9127 + 3.8 + 6 + 129 = 9266
[‡] R + C + B + D133	2.5 mg/mL + 1 µg/mL + 3 µg/mL + 133 µg/mL	9127 + 3.8 + 6 + 258 = 9395
[‡] C + B + D + M33	1 µg/mL + 3 µg/mL + 66.6 µg/mL + 33.3 µg/mL	3.8 + 6 + 129 + 92 = 231
[‡] C + B + D + M17	1 µg/mL + 3 µg/mL + 66.6 µg/mL + 16.6 µg/mL	3.8 + 6 + 129 + 46 = 185

R: ropivacaine; C clonidine; B: buprenorphine; D: dexamethasone; M: midazolam

[†]Tested in 2 hr experiments (n=4 rats per treatment).[‡]Tested in 24 hr experiments (n=4 rats per treatment).[§]The maximum achievable C concentration in R-C was less than that in C (plain) due to the need to add R (3.75 mL of the 10 mg/mL preparation) to 6.75 mL of C (100 µg /mL) to create 10 mL of the combined drug solution, before diluting in culture medium to achieve the listed concentration of C.