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1 **Controlled Release of Doxorubicin from pH-responsive Cockle Shell-derived**
2 **Nanoparticle and its Pharmacokinetics in Dogs**

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26 **Abstract**

27 Stimuli-responsive cockleshell derived nanocarrier (CSNP) have huge potential in
28 drug targeted delivery. They can be employed for targeted site-specific drug delivery
29 due to its responsive nature to stimuli such as change in pH which minimizing its
30 systemic off-targeted effect to due to excessive releases of doxorubicin. We use a
31 simple top-down method to synthesis CSNP carrier from biological waste of
32 cockleshell, which were negatively charged with higher loading capacity when
33 conjugated with DOX. This study is aimed at demonstrating the *in vitro* release
34 mechanism of DOX from CSNP under the influence of change in pH and to develop
35 a bioanalytical method for pharmacokinetics of the synthesized CSNP-DOX in dogs.
36 Apart from drug release kinetic of CSNP evaluation, a high-performance liquid
37 chromatography bioanalytical method was developed and validated for the
38 pharmacokinetics of CSNP-DOX determination. Six dogs were divided into two
39 groups to receive CSNP-DOX and free DOX 30 mg/m² i.v respectively. At
40 predetermined time interval, blood was sampled and processed for DOX
41 concentration. The CSNP-DOX with high encapsulation efficiency and a mean
42 diameter of 34.0 ± 3.4 nm was used. The *in vitro* release profiles demonstrated by
43 DOX release from CSNP-DOX-loaded were pH dependent in nature which follows a
44 Higuchi mathematical model equation. Pharmacokinetic parameters were determined
45 with an excellent bioanalytical method having high extraction yield and linearity of
46 89.87% and 0.997. CSNP-DOX increases the t_{1/2} and AUC_{0-t} of DOX as compared to
47 dogs given free DOX. Our data further reveal a sustained release of DOX from
48 CSNP under the influence of change in pH. However, we developed a rapid
49 bioanalytical method for cumula al model. Based on these novel results, CSNP
50 reveal to have promising ability to prolong release of DOX in circulation which tends

51 to reduce cytotoxic DOX release quantification which was further expressed on
52 kinetic mathematic

53 **Keywords:** pH-triggered release; CSNP-DOX; Bioanalytical assay;
54 Pharmacokinetics; Dog

55 **1. Introduction**

56 Stimuli-responsive nanocarriers are known to have the ability to improve the
57 therapeutic index of chemotherapeutics upon delivery [1]–[3]. In recent times,
58 stimuli-responsive nanocarriers have received significant attention in pharmaceutical
59 industries due to the fact that, tumour microenvironment has different pH as when
60 compared to the surrounding normal tissues, paving way for targeted drug delivery
61 [3], [4]. Stimuli-responsive nanocarriers has great potentials in maintaining drugs
62 therapeutic concentration in circulation since higher concentration of
63 chemotherapeutics are associated with cytotoxicity due to early peak concentration
64 which is followed by linear release in circulation [5]–[8]. Targeted drug delivery
65 strategies direct drug to specific pathological sites with minimal or no adverse effect
66 on the surrounding normal cells and tissues by altering the pharmacokinetic
67 parameters of the drugs [9]. Currently, so many novel stimuli-responsive
68 nanocarriers are designed for anticancer delivery, although many of them
69 encountered numerous setbacks ranging accumulative toxicity in tissue leading to
70 their decline in clinical application [10]. In this regard, Cockleshell calcium
71 carbonate nanoparticles (CSNP) could serve as smart nanocarriers due to its facile
72 pH-responsive trigger release of drugs in weak acidic microenvironment. These
73 property have drawn the attention of research in the field of drug and gene delivery
74 towards improve drug therapeutic index [11]. CSNP can be used as drug carrier for

75 the delivery of chemotherapeutic agents in order to improve the anti-cancer potency
76 of the agents and reduces systematic off-targeted effects.

77 Doxorubicin (DOX) is a potent chemotherapeutic drug currently used for the
78 treatment of metastatic cancers in dogs [12], [13]. However, due to the lack of cell
79 specificity, heterogeneous blood supply and interstitial pressure in solid tumours
80 prevent drug dispersion and penetration into the tumour thus, leading to its decline in
81 clinical application [5], [13], [14]. Moreover, the mechanism of toxicity is
82 complex, so very difficult to pinpoint single pathway due to the involvement of
83 multiple processes [15]. Although, lipid peroxidation, oxygen free radical release by
84 oxidative reaction within cell mitochondria are famous in induced tissue injury [16].
85 Moreover, the bioavailability of DOX at the tumour site is often poor due tumour
86 interstitial pressure, heterogeneous blood supply network hindering DOX penetration
87 to tumour site, thus, necessitate additional doses, consequently leading to tissue
88 damage due early concentration peak in circulation [17]. It is important to state that
89 dogs are used as translational model in drug development and discovery, since they
90 possess similar anatomical and physiological status with other mammals as
91 compared to small laboratory rodents [18]. Hence, they serve as the most preferred
92 model for therapeutic evaluation of new drugs [19].

93 In a way to remedy the problems associated with DOX is by encapsulating it on a
94 nanocarriers with distinct physicochemical properties which can alter the
95 pharmacokinetic profile and bioavailability of DOX, thereby modulating the drug's
96 therapeutic efficacy and safety [20], [21]. For instance, passive targeting or enhanced
97 permeability and retention (EPR) effect of nanoparticles enables the accumulation of
98 its active ingredient in the tumour site [22]. In addition to site-specific targeting,

99 well-structured nanocarriers with a high drug payload and stimuli-triggered release
100 of drugs are crucial towards preventing repeat drug dosing [23].

101 However, to prolonged drug blood circulation time, stimuli-responsive nanocarriers
102 are know to mask the DOX activity in circulation which only become activated when
103 triggered by stimuli [22], with physical and chemical stimuli such as temperature,
104 light, ultrasonic, ionic, redox and enzymes have been employed in the delivery of
105 chemotherapuetics [9]. Althought, pH-trigger stimuli are widely employed in the
106 delivery of anticancer drugs taking advantage of the tumour microenvironment [22].

107 Currently, gold, liposomes and silver nanoparticles demonstrate increased plasma
108 concentration and localization of the drugs at the targeted site [22]. Despite all these
109 uniques advantages, most nanocarriers are non-biodegradable with poor loading
110 capacity thus required multiples doses in order to deliver required concentration
111 which could initiate systemic toxicity and immunogenic responses [22]. Hence,
112 inorganic, biogenic and biodegradable materials are generally preferred due to
113 toxicological concerns. Since most nanocarriers use in nanomedicine posses the
114 ability to maintain the therapeutic dose level of a particular drug at the targeted point
115 of interest with a sustained release concentration in the systemic circulation [9].
116 Although, our major concern is that most of the common carriers commercially
117 available are synthetic from organic materials, which could have cumulative
118 cytological effects on the biological system [24].

119 Calcium salt are found abundantly in sea-shell in form of calcium carbonate
120 aragonite polymorph, which are currently employed in tissue regeneration and drug
121 developments [25], [26]. Since calcium carbonate are biogenic and biodegradable in
122 nature, its nanocrystals can be use in delivering bioactive proteins, hydrophilic and

123 hydrophobic drugs [24], [27]. Moreover, they exhibit high loading capacity with
124 DOX and with other insoluble anticancer agents due to the hydrazone bond carbon
125 linkage and porous nature of the CSNP [25], [28]. It was also demonstrated that
126 CSNP-DOX is less cytotoxic when compared to the free-DOX on normal cells lines
127 [29], [30]. However, the information on the kinetic release mechanism would
128 provide a better understanding in predicting the possible toxicities of CSNP-DOX
129 which is lacking in scientific literature.

130 In this study, CSNP-DOX complex was prepared using precipitation approach as
131 applied in our previous studies [28], with the amine functional ending of DOX,
132 structurally linked to the carboxyl end of the CSNP through a hydrazone bond to
133 form the complex which dissociate in weak acidic microenvironment by protonation of
134 carboxylic group ending of CSNP. The drug release kinetics of CSNP-DOX was
135 determined via bio-analytic method developed with the pharmacokinetic parameters
136 obtained to confirm that CSNP has the ability to regulate and prolong DOX in blood
137 circulation. To the best of our knowledge, this work was the first study to develop a
138 bioanalytical method which was used to determine the pharmacokinetics parameters
139 of DOX from CSNP in dog's plasma circulation which has never been reported in
140 scientific literature. Based on these results, biogenic CSNP polymorph can be use in
141 the delivery of DOX sparing the life of cells in their physiological state in dogs to be
142 given long-term repeated doses for therapeutic purposes.

143 **2. Results**

144 **2.1 Cockleshell nanocarrier synthesis and characterization**

145 The CSNP and CSNP-DOX produced have an average particle size of 28.0 ± 1.2 nm
146 and 34.0 ± 3.4 nm in diameter respectively. CSNP and CSNP-DOX appears to be
147 homogeneous and spherical in shape as shown in Figure 1, with a mean surface zeta

148 potential of – 19.2 mV and – 32.4 mV and polydispersity index of 0.132 and 0.312
149 respectively (Table 1). However, an encapsulation efficiency of 92.7% and a loading
150 capacity greater than 70%.

151 **2.2 Release kinetic studies**

152 The DOX release kinetic from CSNP was evaluated using the snakeskin dialysis bag
153 in phosphate buffer (pH 7.4) and citrate acid buffer (pH 4.8, 5.5 & 6.0) at 37 °C. The
154 amount of DOX in the medium was determined based on a calibration curve ($r^2=$
155 0.991), $Y = 5.998X + 0.129$ Y absorbance, X = concentration ($\mu\text{g mL}^{-1}$)] (Figure 2).
156 It is important to note that initial burst release was observed at a faster rate within the
157 first 4.5 hours (8%) followed by a slower sustained release at the remaining 8 days.

158 Free-DOX was used as a positive control in the release kinetic assay (Figure 3). At
159 pH 7.4, the release of DOX from CSNP was sustained and relatively slows when
160 compared to the free-DOX solution. About 52.6% (3.7 mg) of free-DOX was rapidly
161 released in the first 96 hours, while only 13.7% (0.9 mg) of DOX was recorded from
162 CSNP-DOX sample within the first 96 hours. The CSNP-DOX in acidic medium
163 show a greater DOX release rate than in pH 7.4 in response to the pH change. In the
164 first 96 hours, about 25% (1.8 mg) of DOX was detected, with an increasing rate of
165 drug release over time as compared to the drug release pattern of CSNP carrier in the
166 pH 7.4. The data generated were fitted into three different kinetic equations and
167 according to the coefficient of determination (R^2), the Higuchi equation was best
168 fitted compared to the first order and zero order regression models as shown in Table
169 2. In which the mechanism of DOX release is described by Higuchi equations; which
170 mean that the release fashion is as a result of steady diffusion due to slow
171 degradation of the nanoparticle as shown in figure 4.

172 **2.3 General observation on feed intake, heart rate and haematology**

173 There was a general decrease in feed consumption in the two groups after 24 hours
174 intravenous administration of both free-DOX and CSNP-DOX. However, the heart
175 rates during the period were within the normal range in both groups with no
176 statistically significant changes were observed in the haematological profile within
177 48 hours with $p > 0.05$ in all of the parameters as shown in Table 3. All of the dogs
178 were clinically healthy with no clinical evidence of systemic disturbance observed.

179 **2.4 Analytical method development and validation**

180 The method developed was optimized to reduce cross-matching using an organic
181 solvent (acetonitrile HPLC grade) and buffer solution (pH 4.6) at different
182 concentrations. Elution of the Chromatographs with the baseline at low wavelengths
183 indicates that the method was selective for the detection of DOX with a clear
184 resolution between DOX peak and that of the internal standard as shown in figure 5.
185 The excellent extraction yield from the plasma was evident by the low interference
186 from other protein molecules peaks on the chromatogram. The retention times
187 observed for DOX and daunorubicin was 4.4 and 5.4 minutes respectively (Figure 5).

188

189 **2.4.1 Linearity of calibration curve**

190 A linear relationship was obtained from the concentration verse peak area ratio of
191 0.25 – 4 µg/mL with a coefficient of determination, slope and intercept value of
192 0.9973, 0.1253 and 0.0027 respectively (Figure 6).

193 **2.4.2 Extraction recovery yield, Limit of detection, limit of quantification and**
194 **coefficient of variation**

195 The average recovery extraction was determined in triplicate of the blank plasma was
196 89.87% recovery from 1 µg/mL of DOX (Table 4). The limit of detection (LOD) of
197 DOX at the ratio of 4:1 in the organic solvent was 549.90 ng/mL which corresponds
198 to signal to noise of the method developed with a lower limit of quantification (LOQ)
199 at 1666.0 ng/mL as shown in Table 5.

200 **2.5 Doxorubicin plasma to whole blood partition fraction**

201 Free-DOX has a significant higher fraction of DOX in plasma and red blood cells
202 1.862 ± 0.098 when compared the ratio of DOX partition from the CSNP-DOX
203 (0.420 ± 0.004). This indicated a low rate of DOX release from the nanocarrier due
204 to lack of external or environmental stimuli, which triggers the release from the pH
205 sensitive material carrier.

206 **2.6 Pharmacokinetics evaluation of free-DOX and CSNP-DOX in dogs**

207 **2.6.1 Animal dosing protocol and sample collection for plasma concentration**
208 **determination**

209 There was no evidence of any microbial growth upon incubation of the CSNP at 37
210 °C for 48 hours since all of the glassware used was sterile with the CSNP being
211 stored at 50 °C in the oven. In addition, a sub-toxic dose (30 mg/m^2) was given to
212 prevent any unwanted side effects such as vomiting and anaemia which could alter
213 the results.

214 **2.6.2 DOX plasma concentration and pharmacokinetics parameters**

215 The mean plasma concentration-time data from both free-DOX and CSNPDOX
216 administered to dogs are as shown in Table 6. The CSNP carrier tends to change the
217 level of DOX concentration in systemic circulation when compared to the
218 concentration in dogs given free-DOX. The level of DOX concentration decreases
219 rapidly within first 4 hours after administration which goes below the LOD after 48
220 hours. Subsequently, the CSNP alters the pharmacokinetic parameter when compared
221 to the group of dogs given free DOX. A substantial increase in the half-life ($t_{1/2}$), and
222 area under the curve (AUC) were observed with CSNP-DOX which when compared
223 to the free-DOX parameter, with an increase in the $t_{1/2}$, with a 6 fold differences in
224 the AUC. A significant wide volume of distribution of DOX ($6.83 \text{ mg}/(\mu\text{g}/\text{mL})$) was
225 demonstrated with free-DOX (3 times higher compared to the dogs given CSNP-
226 DOX) with much more rapid clearance rate of $0.14 \text{ mg}/(\mu\text{g}/\text{mL})$ which is 3.5 times
227 higher as compared to the dogs given CSNP-DOX as shown in Table 7. The DOX
228 plasma concentration following single dose administration of CSNP-DOX was
229 higher in all of the corresponding time intervals when compared to the dogs given
230 free-DOX with a calculated bioavailability using the AUC values of CSNP-DOX
231 against the free-DOX being 5.6% as shown in Table 7 below.

232 The retention time for DOX was 4.42 minutes with the highest plasma peak
233 concentration at $26.75 \pm 0.06 \mu\text{g}/\text{mL}$ and lowest plasma peak concentration at $2.22 \pm$
234 $0.15 \mu\text{g}/\text{mL}$ as the lowest detectable concentration which is equivalent to the 13.6%
235 of the DOX delivered as shown in figure 7.

236 **3. Discussion**

237 In this study, we hypothesized that CSNP is pH-stimuli responsive drug carrier that
238 decompose in acidic microenvironment, which trigger the release of DOX in a slow

239 sustained release fashion. The characteristics of these nanocarriers are of great
240 importance for stimuli trigger response and drug delivery. Here, we provide an
241 amazing report on the *in vitro* kinetic release mechanism, bioanalytical method
242 model and pharmacokinetics profile of CSNP-DOX in healthy dogs, which could
243 ameliorate the narrow therapeutic index and poor selectivity of DOX which have led
244 to the use of nanocarriers in the attempt to improve the chemotherapeutic potential of
245 DOX [14].

246 Unlike DOX alone, CSNP-DOX demonstrated a pH stimuli-responsive tool in *in*
247 *vitro* release at acidic pH of 6.0 and below medium and encourages time-dependent
248 DOX release from CSNP which could serve as basis for its application in anticancer
249 delivery. Since, pH-triggered release is an essential tool for anticancer targeted
250 delivery, with tumours possessing abundant lactic acid due to hypoxia [5], [7]. The
251 percentage of DOX concentration release in the acidic pH medium when compared
252 to the free-DOX in the neutral pH medium was quite adequate enough to elicit
253 optimal therapeutic effect as earlier reported by [11]. The release pattern
254 demonstrated by CSNP loaded with DOX was encouraging due to the sustained
255 release fashion observed in the neutral pH, when compared to the release profile
256 observed in pH 4.8 - 6.0 and free-DOX at physiological pH 7.4. This low pH
257 dependent responsive release pattern expressed by CSNP could reduce the off
258 targeted effects to proliferative healthy cells and improve selectivity of DOX to
259 tumour cells. Furthermore, the low percentage release pattern recorded makes it an
260 excellent nanocarrier when compared to the result of [11], who reported a higher
261 percentage release pattern which could be attributed to the analytical methods
262 employed. This further confirmed that the dialysis bag diffusion method is a more

263 accurate method for drug release kinetic assay as earlier suggested by [31]. In
264 addition, our findings were consistent with [8], [41] who found that the release
265 profile of encapsulated drugs on nanocarriers depends on the environment of the
266 receiving medium in which the nanocarrier is suspended.

267 As expected, the CSNP retained DOX at the neutral pH and rapidly released the drug
268 upon triggering by weak acidic pH in a similar release pattern as reported by [33].
269 This suggest that DOX are bonded to the carrier by polyelectrolyte ion and Schiff's
270 base linker which are easily separated in acidic pH medium due to deprotonation of
271 the amine and carboxyl group ending. In addition, [34] and [35], also reported that
272 proton donation as a result of pH interaction leading to ionic bond dissociation and
273 degradation/decomposition of the nanocarrier, which eventually leads to drug release
274 for pharmacological activity.

275 Liu et al. [9], works further clarify that, dimethylamine ions ($(\text{CH}_3)_2\text{NH}_2^+$) complex
276 formed by nitrogen-carbon bond (hydrazone group), which when hydrolyzed in
277 acidic conditions, from esters liberate DOX to the microenvironment.
278 Vijayakameswara et al. [36], stated that the end amine functional group on DOX are
279 sensitive to pH changes, causing dissociation of DOX from the carboxylic endings
280 on drug carriers. These explanations suggest that inorganic aragonite CSNP can be
281 used as a targeted smart nanocarrier in chemotherapy, since it could prolong DOX in
282 blood circulation thus, prevents adverse side effects to proliferating healthy cells as
283 seen with conventional therapy. However, the desorption process of CSNP was very
284 slow which directly affect the dissolution rate, thus agrees with the reports of
285 Svenskaya et al. [37].

286 The release kinetics pattern gives an inside view of the pharmacokinetic nature of the
287 nano-drug formulation [38]. The slow release observed could be unconnected to slow
288 degradability of the CSNP in the simulated mediums, which is an added advantage to
289 the DOX release from the carrier molecules, when compared to the release profile in
290 acidic medium. The smart pH-responsive control release demonstrated by the CSNP
291 is a proof of its potential as an ideal nanocarrier for delivering anticancer drugs as
292 earlier suggested by Wu et al. [39] & Zhou et al. [40]. However, when the rate of
293 drug release is slow from the nanocarrier, tends to alter the drug pharmacokinetics
294 which further offer a unique advantage towards the safety of the anticancer drug on
295 proliferating healthy cells as earlier reported by Peng et al. [41].

296 Drug release kinetics are influenced by many factors including the polymer matrix
297 structure, degradation of the polymer matrix, drug diffusion and carrier geometry
298 (Shaikh et al., 2015). The dissociation of DOX from CSNP follows the Higuchi
299 mathematical kinetic model which describe drug release pattern from the carrier
300 particles as reported by Shaikh et al. [42]. This study agrees with Shaikh et al. [42],
301 that, a carrier matrix with porous nature at the nanoscale, typically release its payload
302 drugs according to the Higuchi mathematical model thus explains the degradability
303 of the nanocarrier in the receiving medium.

304 However, it is essential to empirically develop a bioanalytical method for the
305 detection and quantification of analytes in biological samples. The benefit of the
306 method developed in this study, is that it tends to reduce the use of toxic solvents in
307 the extraction process and detection assay and associated with excellent selectivity
308 when compared to several other published methods [43]–[45].

309 Many of the methods developed previously for DOX recovery from the drug carrier
310 in plasma required the use acidic solvents [43], [44]. In comparison, the extraction
311 method developed in this study could easily separate DOX from the sphere matrix of
312 CSNP and from plasma protein with less acidic solvent combination. Conversely, in
313 the preliminary studies, we observed that detection of DOX was not possible without
314 buffering the mobile phase with an acid buffer at pH 4.7. Consequently, the method
315 was validated in accordance with the ICH and bioanalytical method guidelines [46].
316 The application of liquid-liquid precipitation method has the ability to recover
317 approximately 80% of DOX, which comparable with the earlier method developed
318 by Dharmalingam and Nadaraju [44], though with less acidic solvent.

319 The accuracy of the method developed was within the acceptable range of the
320 coefficient of variation by the standard of USFDA bioanalytical method for HPLC,
321 since it did not exceed 15% in all triplicate samples used in the validation
322 assessment. In addition, this method possesses excellent ability to separate and detect
323 DOX without interference of similar structural metabolites such as daunorubicin and
324 another analyte present in the plasma due to the distinct differences in their retention
325 times. Although, other scholars used fluorescent detectors in for detecting DOX
326 [47]–[49], the selectivity of our method aided in specific detection and quantification
327 of DOX devoid of other chromatographic peaks. However, the method used was
328 unable to detect very low concentrations that are less than 500 ng/mL, though this is
329 concentration is insignificant in clinical application in dogs [39]. The method
330 developed uses simple precipitation techniques, with a linearity that possesses a wide
331 limit of concentration and was reproducible using a simple, easily prepared mobile

332 phase with a short run time, making it applicable for quantification of DOX
333 concentration for pharmacokinetic studies from dog's plasma.

334 DOX concentration in plasma to whole blood cells proportion ratio has a great
335 significant importance in pharmacokinetics, with DOX in plasma to blood cells ratio
336 describing the distribution of DOX in circulation within the plasma and blood cells.
337 The dose selection used were on the basis of DOX toxicity in dogs as earlier reported
338 by Baldwin et al. [50].

339 The result of free DOX plasma concentration was consistent with that of Niu et al.
340 [51], that free-DOX are rapidly cleared from the plasma circulation with little seen in
341 tissues. However, variations in the plasma concentration and half-life and other
342 pharmacokinetic parameters are strongly dependent on the sensitivity of the
343 bioanalytical method employed in detection the analytes [52]. The differences in the
344 pharmacokinetic parameters of free DOX and conjugated DOX were similar to the
345 earlier report of Li and Huang [20] on the kinetics profile of free DOX compared to
346 the entrapped DOX on transporter nanocarrier in the treatment of cancer in rats
347 model. Likewise, the report of Shah [53], agrees with the half-life and plasma
348 concentration maximum observed in this study for free-DOX showing a high
349 concentration on DOX in plasma when compared to the encapsulated DOX
350 administered in healthy dogs. The low plasma concentration obtained from CSNP-
351 DOX was a direct backwards phase process of the DOX incorporated in the matrixes
352 which were released slowly into systemic circulation. These findings agree with the
353 statement of Arias [34], that only drug released from the carrier matrix are quantified
354 for pharmacokinetic analysis.

355 Moreover, the electrokinetics potential of the CSNP used as a carrier could have
356 influenced the interaction process and the amount of DOX in circulation at each
357 predetermined time point. Thus, agrees with earlier submission of Honary and Zahir
358 [54], that pharmacokinetics of the drugs delivered are altered by the colloidal zeta
359 potential of the delivery carrier.

360 The prolonged half-life recorded by CSNP-DOX in the dogs could be attributed to
361 the slow release as earlier demonstrated by CSNP-DOX in neutral pH 7.4 in the *in*
362 *vitro* release kinetics studies. The increased half-life observed with CSNP-DOX in
363 the dogs when compared to the dogs given free-DOX could increase DOX potency
364 and efficacy. Likewise, the prolonged circulation time demonstrated by CSNP
365 incorporated with DOX is a good characteristic for a controlled nanocarrier with
366 targeting ability. These findings concur with reports of Singh and Lillard [31], that
367 when biodegradable materials are used in drug delivery, a very slow release fashion
368 were demonstrated.

369 Similarly, the slow clearance rate was recorded with CSNP-DOX as when compared
370 to the free-DOX. This was clearly attributed to the delayed release of the DOX from
371 the nanoparticle which needs external stimuli, to trigger the release into the
372 circulation. However, the low volume of distribution demonstrated were consistent
373 with the reports of other scholars who use polymers in the delivery of DOX in the
374 absence of triggering stimulus that causes the release of DOX encapsulated in the
375 CSNP [55].

376 Furthermore, a significant increase in the half-life, AUC, and decrease in the volume
377 of distribution and clearance rate of DOX conjugated to the CSNP were consistent
378 with the report of Lu et al. [56], who documented increase of pharmacokinetic

379 parameters as a result of incorporation of DOX to micelles carrier when compared to
380 the parameters of free-DOX. These alterations in C_{max} and half-life of DOX
381 incorporated on CSNP agrees with the findings of Giodini et al. [57] as
382 pharmacokinetics parameters are essential parameters for improving the therapeutic
383 index. In addition, cytarabine and docetaxel loaded on CSNP in the mice and rats,
384 with improved therapeutic efficacy in the treatment of acute leukaemia and breast
385 cancer respectively [24].

386 However, not all of the changes in the pharmacokinetics parameters were solely due
387 to the DOX properties. Some changes could be associated with the nanocarrier
388 physiochemical properties as earlier cited by Caron et al.[58], who reported that the
389 distribution of drugs depends on the properties of the carrier molecules. Several
390 scholars consistently reported rapid clearance of DOX concentration in plasma of
391 different animal models in less than 24 hours after administration [56], which is
392 contrary to our findings when DOX is loaded to CSNP and administered
393 intravenously in dogs. The low clearance rate associated with increased half-life
394 observed in this study demonstrated prolonged circulation of DOX in the plasma.

395 **4. Conclusion**

396 In conclusion, CSNP loaded with DOX has influenced the retention of the DOX in
397 blood circulation and enhances its bioavailability, which corresponds to the
398 controlled and sustained release observed earlier in the *in vitro* release profile. The
399 successful pH-triggered release of the CSNP-DOX suggests a promising potential of
400 CSNP as a drug carrier conjugated with anticancer molecules for future clinical
401 application. However, Hiquchi mathematical model best fits the kinetics release
402 indicating a slow degradation of the CSNP in a physiological medium, with a simple

403 and rapid bioanalytical method was developed and applied in pharmacokinetics
404 studies of CSNP-DOX in healthy dogs. Furthermore, the pharmacokinetic parameters
405 confirm that CSNP has the ability to regulate and prolong DOX in blood circulation,
406 thus translating to an improved pharmacological property of the DOX. Based on
407 these results, CSNP may be useful in the delivery of DOX sparing the proliferating
408 cells in their physiological state in dogs given long-term repeated doses of therapy.

409 **5. Experimental (Materials and methods)**

410 **5.1 Drugs and reagents**

411 Dodecyl dimethyl betaine (BS-12) were purchased from Sigma-Aldrich Co. (Sigma-
412 Aldrich[®], St. Louis, MO, USA). Deionized water was used as diluent which has a
413 resistance of 18 M Ω taken from Milli R06 plus Q-Water system[®] (Organex, USA).
414 HPLC grade acetonitrile and methanol, acetic acid, trifluoroacetic acid were
415 purchased from Fisher Scientific (Fisher Scientific[®] UK). Doxorubicin hydrochloride
416 (CAS No.: 25316-40-9) (purity 99.6%) with pH 5.13 and daunorubicin (CAS No:
417 23541-50-6) (purity 99.7%), were purchased from Beijing Mesochem Technology
418 Co., Ltd. China. All other chemicals were of analytical grade and stored at 25 °C.

419 **5.2 Synthesis, drug loading and characterization of CSNP and CSNP-DOX**

420 Spherical aragonite CSNP was synthesized and characterized according to our
421 previous work [28], Briefly, 2 g of the micro size cockleshell powder was
422 precipitated in 50 mL of deionized water on a Telfon magnetic stirring machine at
423 1000 rpm for 1 hour at 25 °C. At the end of the preparation, the oven-dried
424 nanoparticles (27 °C for 72 hours) were further processed on a ball mill (BML-2”
425 Diahan Scientific[®] Korea) operated at 200 rpm for 48 hours before characterization.
426 For the preparation of CSNP-DOX, a co-precipitation method was employed to
427 incorporate DOX to the synthesized nanocarrier [28]. Characterization of the

428 synthesized nanocarrier and conjugated carrier for particle size, morphology and
429 surface charge was done using High-resolution transmission electron microscopy
430 (JEOL JEM 2100F HRTEM, Tokyo Japan) with an accelerating voltage of 100kV
431 and Zetasizer ZS (Malvern, ver. 7.02 UK) with a scattering angle of 90° as done in
432 our previous work [28].

433 **5.3 *In vitro* drug release**

434 The *in vitro* drug release assay was conducted using 0.01 M phosphate buffer pH 7.4
435 and citric sodium citrate acid medium pH 4.8, 5.5 and 6.0 to simulate physiological
436 microenvironment, tumour stroma microenvironment and endosomal of cancer cell
437 respectively. CSNP containing 7.2 mg DOX was aliquoted into 1 mL of phosphate
438 buffer before dispensed into a snakeskin® dialysis tube (10 kDa, MWCO, Thermo
439 Scientific®, USA), then suspended in 20 mL of pH 7.4, pH 4.8, 5.5 and 6.0 buffers
440 solution respectively. A free-DOX solution containing 7.2 mg DOX was established
441 in a similar manner without CSNP in accordance to the method of [59]. The CSNP-
442 DOX and free-DOX in the dialysis systems were constantly stirred on Telfon
443 magnetic stirrers at 100 rpm and 37 ± 0.5 °C. At different time intervals (0, 0.3, 0.5,
444 1.0, 2.0, 3.0, 5.0, 8.0, 24.0, 48.0, 72.0, 96.0, 120.0, 144.0, 168.0, 192.0 hours), 1 mL
445 aliquot was withdrawn from the systems for analysis which was immediately
446 replaced with an equal volume of fresh solvent medium. The experiment was done in
447 triplicate and analyzed using the zero-order equation, first order equation and
448 Higuchi equation as described by [60], [61]. The data were presented in mean ± SD.

449 **5.4 Analysis of doxorubicin concentration**

450 A reverse-phase HPLC was used to detect DOX concentration release as described
451 by Lu et al. (2015). The HPLC system consists of Separation Module 2690 from
452 Water Corp. (Milford, MA USA) with a stationary phase of Agilent Eclipse C₁₈

453 Column (4.6 mm x 250 mm, 5 μ m) (Santa Cala, CA, USA). The mobile phase was
454 run with in a combination of mobile phase A (methanol) and B (0.1% acetic acid) at
455 a flow rate of 1 mL/minute. The mobile phase was increased linearly from 40% to
456 90% in 5 minutes, followed by maintaining at 95% for 3 minutes and immediately
457 returns to 40% composition in 1 minute. The system was further maintained for 4
458 minutes before the introduction of the subsequent sample, thus making a total run of
459 13 minutes at 30 °C. A photodiode array detector (Waters W2998, Milford, MA,
460 USA) was set at a wavelength of 254 nm to detect DOX. Samples were analyzed in
461 triplicate at an injection volume of 80 μ L using Empower™ version 2 software.

462 For the pharmacokinetic study, daunorubicin was used as the internal standard (IS) of
463 the DOX release. The above-mentioned HPLC settings were applied for
464 pharmacokinetic study of CSNP-DOX except for the changes in the mobile phase.
465 The HPLC system was run using mobile phase C (acetonitrile + 0.1% acetic acid)
466 and D (0.1M disodium citrate acid pH 4.7). The mobile phase follows a linear
467 gradient: mobile phase C increases from 40% to 60% in 5 minutes, then to 70% in 5
468 minutes and maintained for 5 minutes then returns to 60% for 4 minutes and finally
469 returns to the initial mobile phase status after 1 minute.

470 **5.4.1 Preparation of stock solution internal standard and quality control**

471 One mg/mL of DOX and daunorubicin stock solutions were prepared using blank
472 plasma while DOX aqueous solutions of 1.25 - 2.5 μ g/mL range for quality control
473 and 0.25 - 4 μ g/mL for linearity were used. IS 1 μ g/mL was included in all the
474 samples before extraction process were performed on the plasma. The standard
475 calibration curve of DOX was generated from the signal ratio of DOX to
476 daunorubicin against the concentration of DOX. A DOX of 2 μ g/mL and 0.25 μ g/mL
477 was used as the upper and lower quality control.

478 **5.4.2 Method validation**

479 The percentage recovery, lowest limit of detection (LOD) and lowest limit of
480 quantification (LOQ) were determined from the ratio of the calibration curve slope
481 and recovery percentage DOX. The validation of the method was conducted
482 according to the international council of harmonisation (ICH) and bioanalytical
483 method guideline [46]. The selectivity of the protocol was determined by using blank
484 plasma from 3 healthy dogs spiked on DOX and daunorubicin to check where the
485 chromatographic peak interloped with that of the extraction solvent.

486 **5.4.3 Extraction of DOX and Daunorubicin from plasma**

487 The extraction of the drugs from the plasma was performed using liquid-liquid
488 extraction method. Briefly, 400 μL of acetonitrile: trifluoroacetic acid (95:5 v/v) was
489 spiked to 99 μL of blank plasma from clinically healthy dogs with 1 μL of the IS.
490 The mixture was then vortexed vigorously for 3 minutes and centrifuged at 5000 x g
491 for 15 minutes at 18 °C (Eppendorf® 5424r, Germany). 200 μL of the supernatant
492 was transferred into HPLC inserts for analysis with the blank plasma was used as a
493 control for the analysis. The extraction yield was evaluated at a low DOX
494 concentration within the limit of 0.25 to 4 $\mu\text{g}/\text{mL}$.

495 Extraction yield = $\frac{\text{Peak area of DOX in blank plasma}}{\text{Peak area of DOX in acetonitrile}} \times 100$

496

497 **5.5 Doxorubicin plasma concentration to blood partition**

498 The affinity of CSNP-DOX to whole blood and plasma were determined according
499 to the method of [62]. The protocol is determined by calculating the ratio of plasma
500 to blood DOX concentration which is used to predict the distribution and clearance
501 rate of the DOX-loaded on CSNP as compared to the free-DOX. Briefly, 10 $\mu\text{g}/\text{mL}$
502 (50 μL) of the DOX and its CSNP-DOX (equivalent) were spiked into the 450 μL of

503 whole blood separately and were incubated at 37 °C for 1 hour. The samples were
504 centrifuged at 5000 x g for 15 minutes at 18 °C to separate the plasma from the cells.
505 DOX was extracted from the plasma and analyzed by the established bio-analytical
506 method.

507 **5.6. Animal dosing protocol and sample collection**

508 Six healthy male canine (*Canis familiaris*) aged 9 – 36 months (mean 24.84 ± 6.6
509 months), weighing 10.00 – 16.30 kg (mean, 13.89 ± 1.60 kg) were acclimatized in
510 the research facility at the Faculty of Veterinary Medicine, Universiti Putra Malaysia
511 (UPM). The dogs were examined physically and clinically evaluated for their general
512 health status. All procedures involving animal care and use were approved by the
513 Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia
514 (UPM/IACUC/AUP-RO13/2016). Microbiological assessment of the synthesized
515 nanoparticles was evaluated before DOX were loaded and intravenously
516 administered. This was done by direct inoculation of 400 µL of the nanoparticles on
517 a general purpose medium (nutrient agar) and was incubated for 72 hours at 37 °C
518 after which microorganism growth on the media was examined.

519 All dogs were chemotherapeutic naive for 2 weeks prior to the experiment. The dogs
520 were fasted overnight prior to the experiment and fed 3 - 4 hours after the
521 administration of DOX and CSNP-DOX. Three dogs were enrolled into group A
522 which received CSP-DOX at 30 mg/m² intravenously via cephalic vein over 3 – 10
523 minutes. The remaining 3 dogs were enrolled into group B and were given free-DOX
524 at the dose of 30 mg/m² (1 mg/kg for dogs less 15 kg) through a side port on the
525 intravenous administration line and all the two groups were supported with 0.9%

526 sodium chloride (NaCl); 18.0 mL/kg/h intravenously via cephalic vein over 5 – 10
527 minutes, which are similar to the dose rate given in clinical schedules.

528 Blood samples (3 mL) were collected from the cephalic vein using a 22 G, ½ “needle
529 (Terumo[®], Belgium) into heparinized ethylenediamine tetraacetic acid (EDTA) tubes
530 for haematology and DOX plasma concentration quantification. The blood samples
531 were collected at points 0, 0.2, 0.5, 1, 2, 4, 6, 12, 24, 48, 60 hours after drug
532 administration and immediately centrifuged (10,000 x g for 15 mins at 18 °C) to
533 separate the plasma. The blood samples were stored at -80 °C before analysis. The
534 DOX plasma concentrations were determined using the developed protocol and
535 HPLC method above.

536 **5.6.1 Haematological analysis**

537 The haematological analysis was done using an automated haema-analyzer (Scil[®]
538 Vet ABC, USA) to procure the following parameters: white blood cells, red blood
539 cells, haemoglobin concentration, haematocrit, mean corpuscular volume, mean
540 corpuscular haemoglobin concentration and platelets.

541 **5.7. Release kinetics and pharmacokinetics parameters**

542 The results from the release kinetics were expressed as mean ± standard deviation
543 from triplicate data. The pharmacokinetic parameters were calculated using non-
544 compartmental IV infusion analysis with a PKSolver 2.0, Microsoft Excel add-in
545 [63], which was validated in *Computer Methods and Programs in Biomedicine*
546 *Journal* 2010. The following parameters were determined (i) maximum plasma
547 concentration (C_{max}), (ii) time to C_{max} (T_{max}), (iii) the area-under-the-curve between
548 0 and 72 (AUC_{0-72}), (iv) Area-under-the-curve between 0 and ∞ ($AUC_{0-\infty}$) and (v)
549 Apparent half-life in plasma ($t_{1/2}$) (vi) Mean residence time (MRT) (vii) Volume of

550 distribution at steady-state (V_{ss}) (viii) Systemic clearance (Cl) (ix) Elimination rate
551 constant (K). The relative bioavailability (Fr) was calculated according to the
552 following equation:

$$553 \quad Fr = AUCS/AUCR$$

554 Where AUCS(CSNP-DOX) and AUCR (free-DOX) are the AUC_{0-72} of each sample.

555 **Author's contributions**

556 DA and ZA contributed in developing the composite, all evaluation and data analysis
557 writing the manuscript; GTS and HCW contribute in the drug delivery and
558 manuscript writing, MHMN and HCW contribute in the release kinetics assay and
559 pharmacokinetic evaluation; ZA; GTS, MHMN, RM concise the original ideal of the
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561

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566 **Competing interests**

567 The authors declare that they have no competing interests.

568

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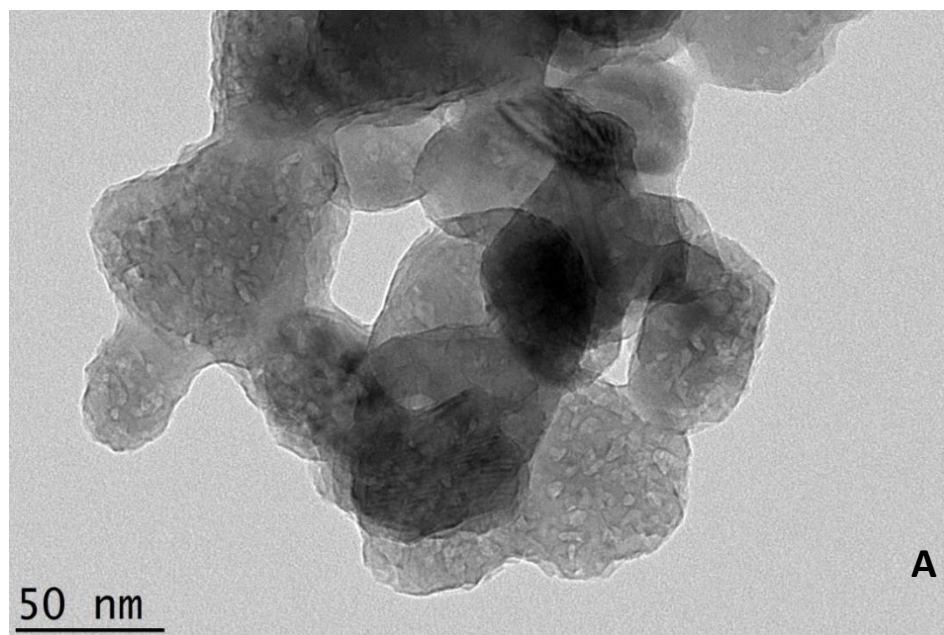
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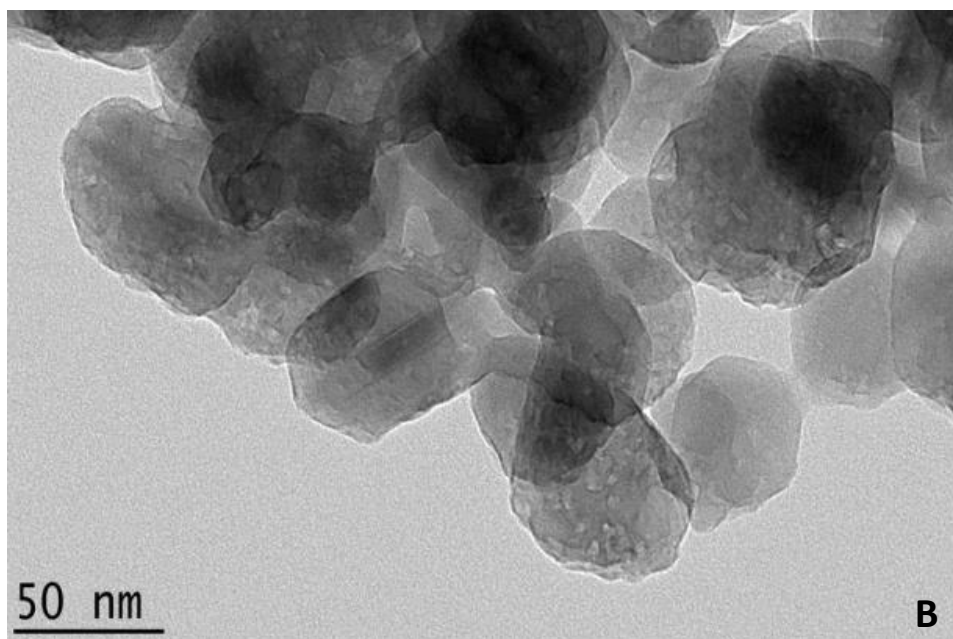
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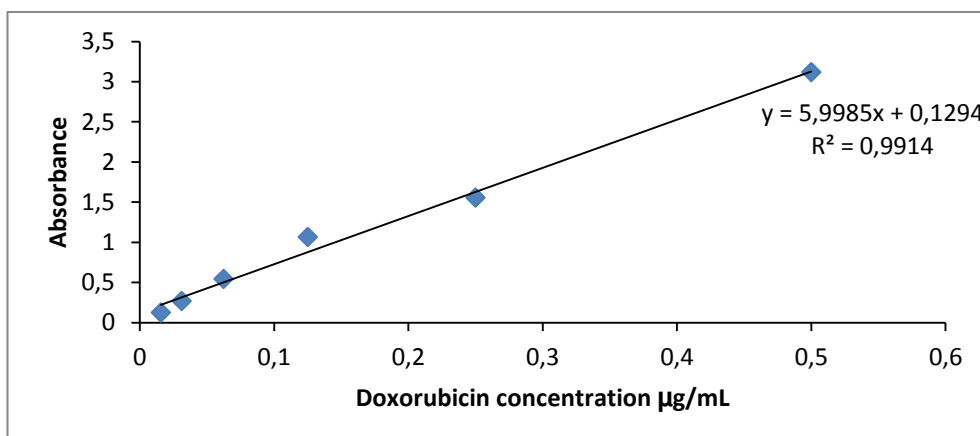
741 **Figures**





743

744 **Figure 1: Electron micrograph of A) CSNP and B) CSNP-DOX. The porous particle appeared**
 745 **to be spherical in shape with an average size of 28 ± 1.2 nm for CSNP and 34.0 ± 3.4 nm CSNP-**
 746 **DOX**
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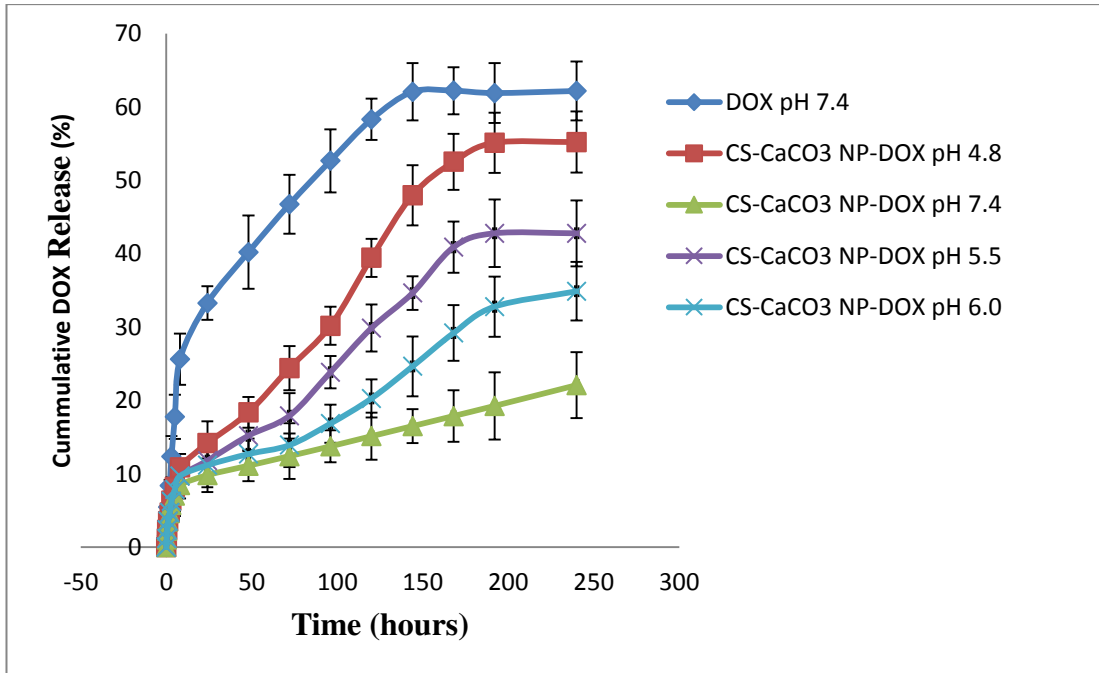


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749 **Figure 2: Calibration curve of DOX**

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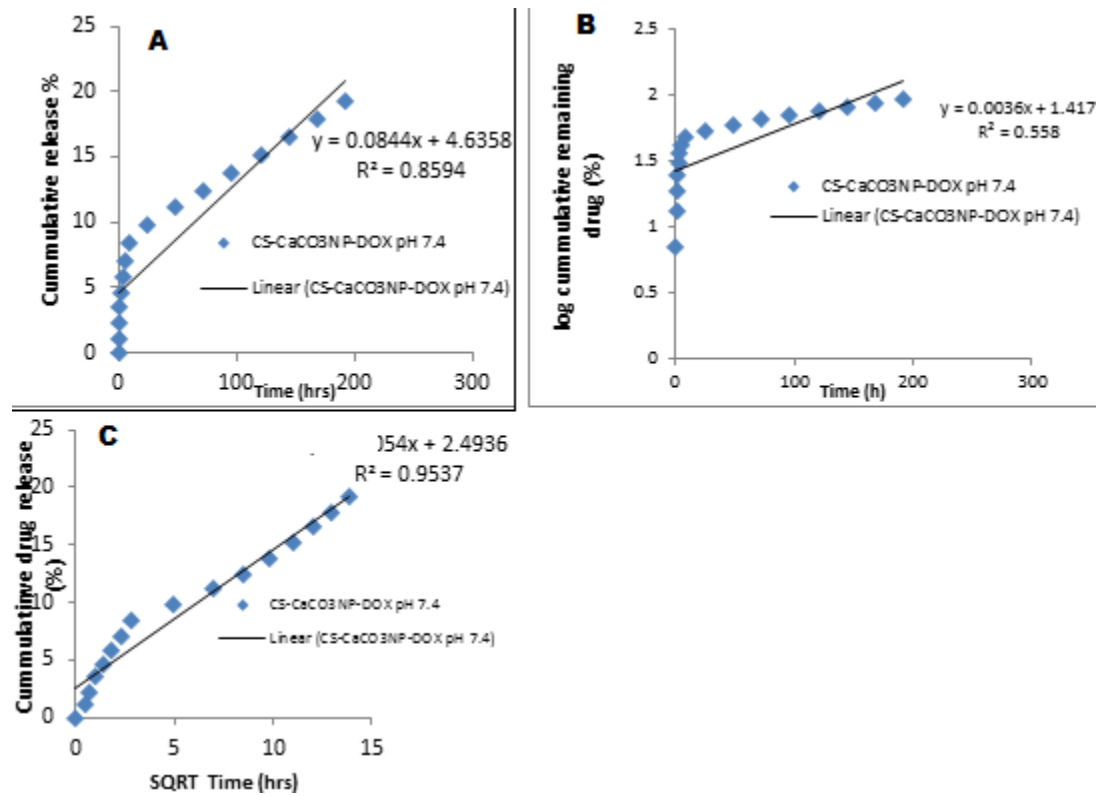


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753 **Figure 3: The Cumulative release of free-DOX and CSNP-DOX, in *in-vitro* release kinetic in**
 754 **different acidic and physiological pH medium. Triplicate data of each time point and value**
 755 **analysis as mean \pm SD, (n = 3)**

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757



758

759 **Figure 4: The kinetic release model of CSNP-DOX in pH 7.4 medium (A) Zero order kinetics**
760 **release model of CSNP-DOX (B) First order kinetic release model of CSNP-DOX (C) Higuchi**
761 **order kinetic release model of CSNP-DOX**

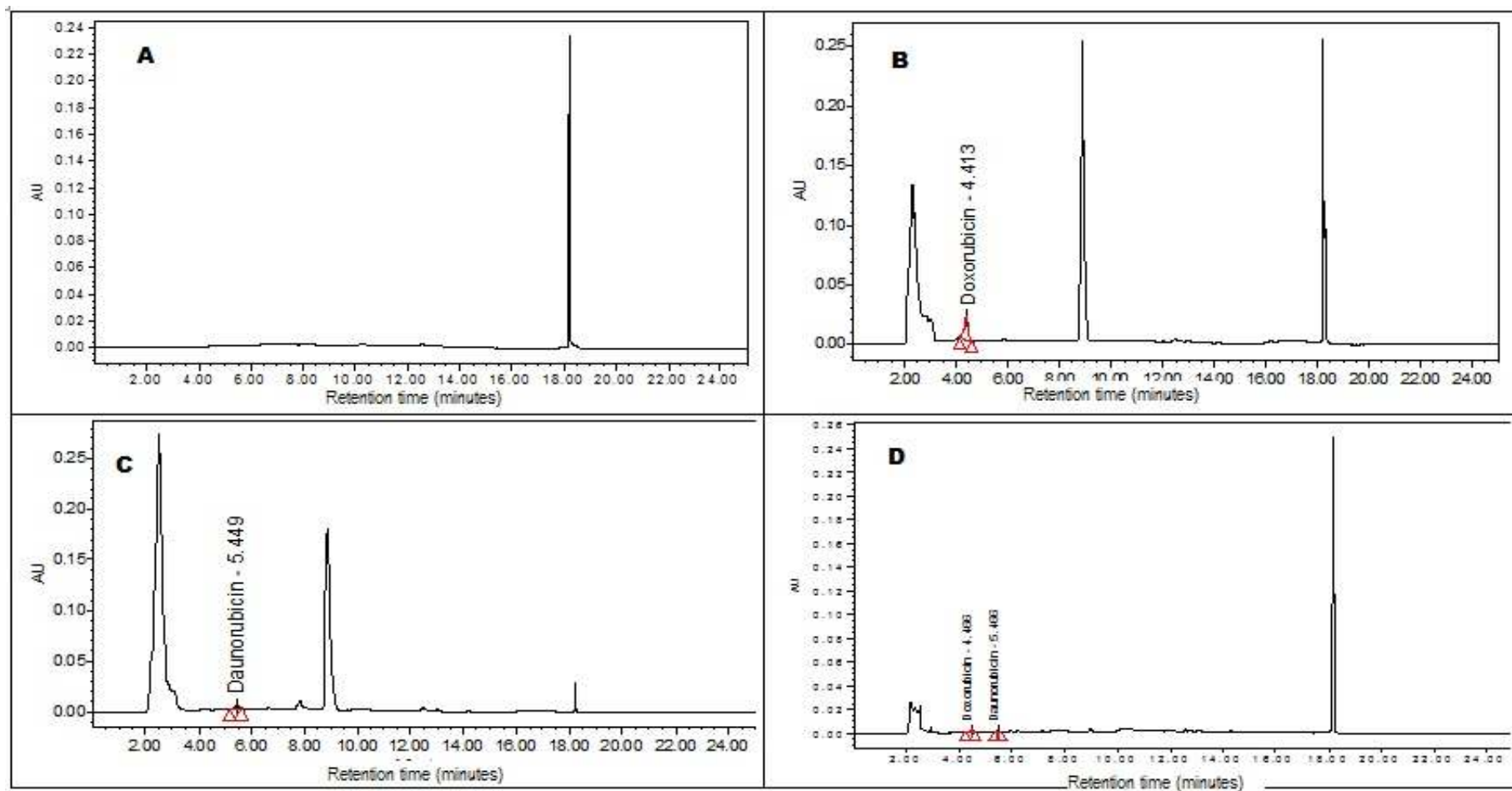


Figure 5: The chromatograms of blank plasma free-DOX, Daunorubicin (IS), DOX released from CSNP-DOX plasma from the canine. (A) Blank plasma from the canine (B) LLQ sample of DOX spiked on plasma (1.66 $\mu\text{g/mL}$), (C) Daunorubicin spiked on plasma (1.0 $\mu\text{g/mL}$), (D) Free-DOX release from CSNP-DOX after intravenous administration of 30 mg/m^2 at 60 hours

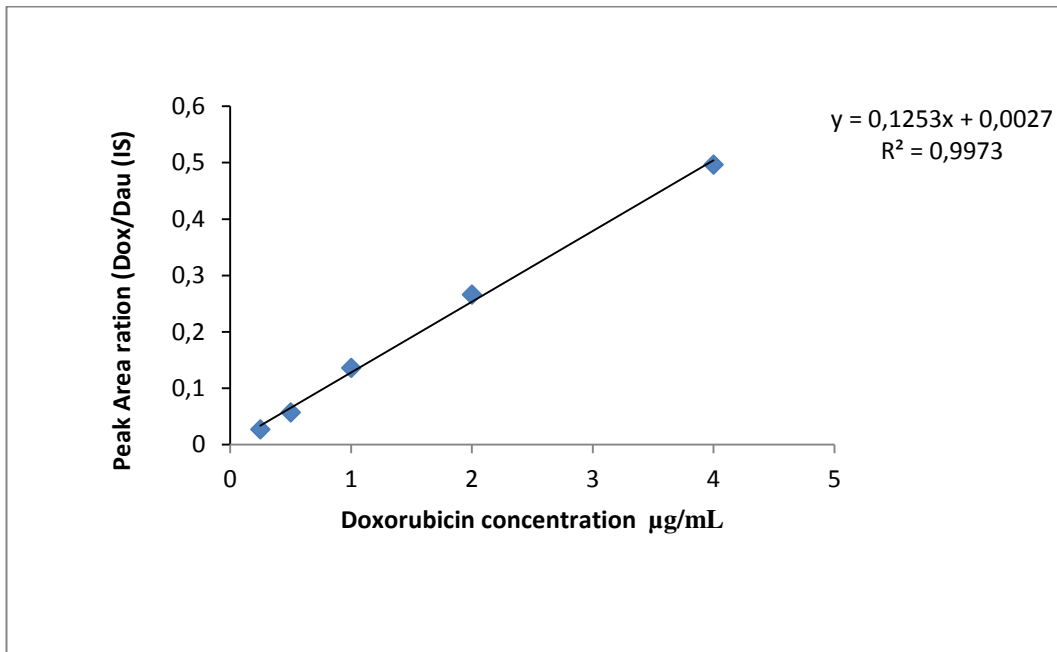


Figure 6: Linearity curve for the quantification of DOX from the peak ratio of DOX and daunorubicin

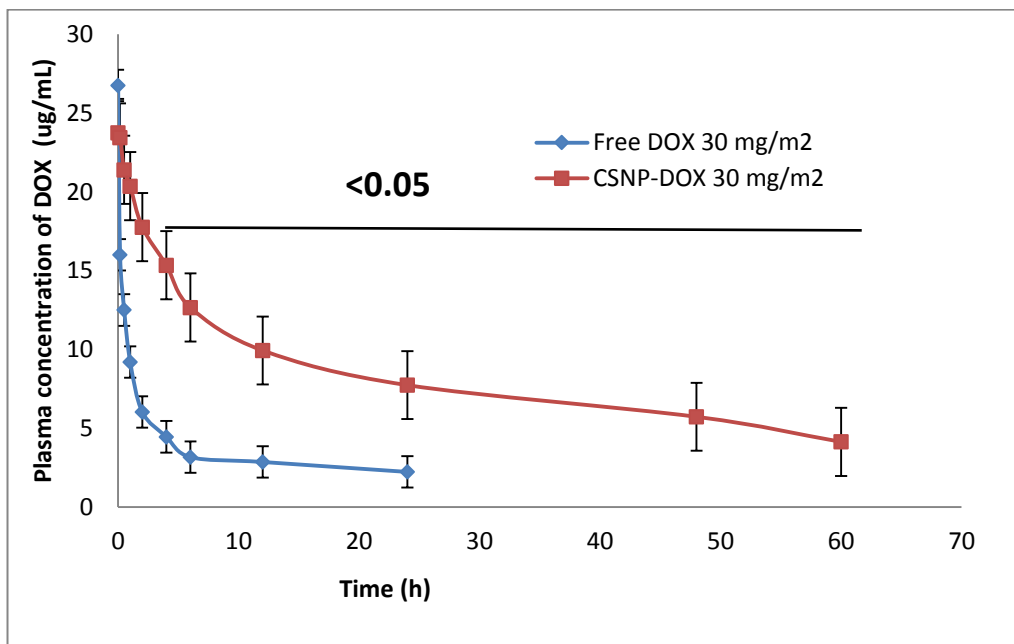


Figure 7: The plasma concentration time profile of DOX after free-DOX and CSNP-DOX i.v administration in dogs (n = 3) at a DOX dose 30 mg/m²

Tables

Table 1: Physicochemical properties of the CSNP and CSNP-DOX

Nanocarrier	Mean diameter size	Mean surface charge (mV)	Polydispersity index
CSNP	28.0 ± 1.2	- 19.2	0.132
CSNP-DOX	34.0 ± 3.4	- 32. 4	0.312

Table 2: Evaluation of the release kinetics based on zero order, first order and Higuchi equations

Coefficient of Determination (R ²)		
Zero order equation	First order equation	Higuchi equation
0.8594	0.5580	0.9537

Table 3: Haematological profile of free-DOX and CSNP-DOX single intravenous administration for pharmacokinetic studies in healthy dogs n = 3

Parameter	Free-Dox 30 mg/m ² (n =3)			CSNP-DOX 30 mg/m ² (n =3)		
	0 hours	24hrs	48hrs	0 hours	24hrs	48hrs
RBC 10 ⁶ /mm ³	7.08 ± 0.2	7.77 ± 0.1	5.82 ± 0.1	7.07 ± 0.32	7.1 ± 0.22	6.38 ± 0.2
HGB g/dl	15.07 ± 2	16.1 ± 1.5	14.4 ± 1.6	15.06 ± 1.59	14.1 ± 0.9	13.6 ± 0.8
HCT %	45.10 ± 5.3	46.8 ± 3.8	48.4 ± 2.9	45.76 ± 3.84	42.5 ± 2.8	40.4 ± 2.2
MCV μm ³	64.00 ± 5.7	60 ± 4.8	64 ± 4.3	64 ± 4.0	60 ± 4.2	63 ± 3.6
MCH Pg	21.23 ± 2.2	20.7 ± 1.5	21.3 ± 1.4	21.23 ± 1.56	19.8 ± 0.9	21.3 ± 1.9
MCHC g/dl	33.40 ± 0.5	34.4 ± 0.2	33.5 ± 0.1	33.4 ± 0.44	33.2 ± 0.5	33.6 ± 0.3
RDW %	15.40 ± 0.3	14.6 ± 0.1	15.2 ± 0.1	15.4 ± 0.26	15.5 ± 0.3	15 ± 0.2
PLT 10 ³ /mm ³	233.33 ± 118	187 ± 9.0	178 ± 82	233.3 ± 103.36	185 ± 99	163 ± 79.0
MPV μm ³	9.50 ± 1.4	10 ± 0.6	9.9 ± 1.0	9.5 ± 1.0	10.5 ± 0.9	10.7 ± 0.8
WBC 10 ³ /mm ³	9.10 ± 2.1	9.8 ± 1.8	10.6 ± 1.6	9.1 ± 2.71	8.9 ± 2.1	11.9 ± 2.8
LYM 10 ³ /mm ³	3.50 ± 0.9	4.2 ± 0.3	3.7 ± 0.1	3.5 ± 1.45	4.9 ± 0.2	4.1 ± 0.4
MON 10 ³ /mm ³	0.73 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.73 ± 0.31	0.8 ± 0.2	1.2 ± 0.1
GRA 10 ³ /mm ³	4.87 ± 2.9	5.6 ± 2.1	5.9 ± 2.0	4.86 ± 2.15	3.2 ± 1.5	6.6 ± 2.1
EOS 10 ³ /mm ³	0.32 ± 0.3	0.22 ± 0.1	0.34 ± 0.1	0.32 ± 0.1	0.14 ± 0.2	0.23 ± 0.2

All the values are expressed in mean and standard deviation, Student t-test analysis with p < 0.05 considered statistically significant.

Table 4: Percentage extraction yield of DOX from dog plasma

Concentration (μg/mL)	Calculated free DOX found (μg/mL)	Extraction recovery (%)
2.00	1.56	78.39
1.00	0.89	89.87
0.50	0.39	79.49
0.25	0.20	80.91

n = 6 for each sample concentration used for the analysis

Table 5: Analytical parameters of detection and quantification for the method developed for DOX quantification

Parameter	DOX
Concentration DOX added ($\mu\text{g/ml}$)	1.00
Mean SD	94.37 ± 11.01
Intercept (a)	0.002
Slope (b)	0.125
Correlation coefficient (r)	0.9973
Extraction recovery yield range (R%)	78.39 – 89.87
LOD (ng/mL)	549.96
LOQ (ng/mL)	1666.55
CV (%)	11.70

Where : LOD: Limit of detection; LOQ: Limit of quantification; CV: Coefficient of variation

Table 6: Plasma concentration values at different time interval for the two drug formulation from HPLC (n = 3) for each group

Time interval (h^{-1})	DOX ($\mu\text{g/mL}$)	CSNP-DOX ($\mu\text{g/mL}$)
0	26.75 ± 0.06	23.75 ± 1.35
0.16	16.0 ± 0.50	23.45 ± 1.22
0.5	12.50 ± 1.00	21.40 ± 2.22
1	9.20 ± 0.23	$20.36 \pm 2.10^*$
2	6.03 ± 0.13	$17.76 \pm 2.30^*$
4	4.45 ± 0.23	$15.34 \pm 1.90^*$
6	3.16 ± 0.40	$12.66 \pm 1.84^*$
12	2.85 ± 0.17	$9.93 \pm 0.93^*$
24	2.22 ± 0.15	$7.73 \pm 0.73^*$
48	-	$5.72 \pm 0.92^*$
60	-	$3.93 \pm 0.57^*$

Key: DOX: Doxorubicin, CSNP-DOX: Cockleshell derived nanoparticle loaded with doxorubicin and * < 0.05 which is considered statistical significant

Table 7: Pharmacokinetics parameters of CSNP-DOX and DOX alone following single dose 30 mg/m^2 intravenous administration (mean SD, n= 3) in dogs

Parameter	Unit	DOX	CSNP-DOX
$t_{1/2}$	H	30.96	35.59
T_{max}	H	0.00	0.00
C_{max}	$\mu\text{g/mL}$	26.75	23.75
AUC_{0-72}	$\mu\text{g/mL} \cdot \text{h}$	87.84	495.03
AUMC	$\mu\text{g/mL} \cdot \text{h}^2$	9130.29	33741.36
MRT	H	45.56	48.34
V_z	$(\text{mg})/(\mu\text{g/mL})$	7.57	2.21
Cl	$(\text{mg})/(\mu\text{g/mL})/\text{h}$	0.14	0.04
V_{ss}	$(\text{mg})/(\mu\text{g/mL})$	6.83	2.08
F	(mL/kg)	5.63	

Key: $t_{1/2}$: half-life; T_{max} : Time at maximum concentration; C_{max} : Maximum concentration; AUC: Area under the curve; AUMC: Area under the first-moment curve; MRT: Mean residence time; V_z : Volume of distribution; Cl: Clearance rate; V_{ss} : Volume of steady distribution; F: Relative bioavailability