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Mini-tablet combination for sustained release of clonidine hydrochloride and hydrochlorothiazide: Preparation and pharmacokinetics in beagle dogs

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Mini-tablets are increasingly gaining attention in solid dosage form design as multiple-unit systems combining different active compounds and providing a single or combined pattern of modified release for polypharmacy or combined treatments. A combination therapy of clonidine hydrochloride and hydrochlorothiazide achieves effective blood pressure control and reduction in adverse effects. However, the combination formulation of immediate release must be taken several times a day, which causes noticeable fluctuation of blood pressure and inconveniences to the patients. The present study was performed to develop a mini-tablet combination for sustained release of clonidine hydrochloride and hydrochlorothiazide independently, in which the two drugs and fraction doses were formulated into separate mini-tablets with different release patterns. The mini-tablets were prepared by a direct compression method followed by filling into capsules and the factors that affected drug release were addressed. Further, studies of the pharmacokinetics were performed in beagle dogs. Finally, *in vivo-in vitro* correlations of the sustained release systems and bioequivalence with conventional preparations were evaluated. The mini-tablet combination released the two drugs over 24 h *in vivo* with a steady plasma concentration, a markedly lower C_{max}, extended T_{max} and better bioavailability. In conclusion, sustained releases of the two drugs were obtained with this mini-tablet combination, which offers a feasible formulation and promising development value for hypertensive patients who need long-term therapy.

1. Introduction

Mini-tablets represent a new trend in tablet design with enormous potential for targeted populations, providing improved swallowing, flexible dosing and a combination of different release patterns and/or different active compounds (Aleksovski et al. 2015; Mohamed et al. 2013; Podczeck et al. 2007). Besides several common biopharmaceutical advantages of multiple-unit systems, such as broad gastro-intestinal distribution and less significant all-or-nothing effect, mini-tablets offer more uniform size, shape and porosity and a more reproducible manufacturing process than other multiple-unit dosage forms, like pellets, microspheres and granules (Abdul et al. 2010; Munday 1994). Therefore, mini-tablets are suitable as a combination therapy system for hypertension.

Hypertension management guidelines issued by American (James et al. 2014) and European (Mancia et al. 2013) professional organizations include combination of low dosage drugs, application of thiazide diuretic alone or in combination as an early treatment of hypertension, and utilization of sustained release or controlled release preparations to keep blood pressure smooth.

Clonidine hydrochloride (CLDH) is a centrally acting antihypertensive drug, treating high blood pressure by stimulating α_2-receptors in the brain, which decreases peripheral vascular resistance, lowers blood pressure, and inhibits motility and secretion of the gastrointestinal (GI) tract (Shen 2008). CLDH is one of the most commonly used drugs for treatment of medium and severe hypertension, especially suitable for hypertension patients with gastric ulcer disease in clinical application, but long-term use of CLDH leads to water and sodium retention which can be alleviated by combining with diuretics in patients (Wu and Huang 2005). Hydrochlorothiazide (HCTZ) is a thiazide diuretic used to deal with edema and lower the blood pressure. It inhibits sodium reabsorption in the distal tubules causing increased excretion of water and sodium as well as potassium and hydrogen ions, which can attenuate the symptoms of water and sodium retention caused by CLDH (Taddei 2010). Thus, the combined use of CLDH and HCTZ has a synergistic action improving therapeutic effect and reducing adverse effects.

In addition to drug combination, sustained released preparations have also shown great advantages for hypertension treatment, as they can reduce the fluctuations in blood concentration and smoothly control blood pressure with slowly released drugs. Compared with common drugs, sustained released preparations can reduce dosing frequency and improve the compliance of hypertension patients, who often need lifelong medication (Patra et al. 2007; Shimamoto et al. 2015). Therefore, the development of once-daily sustained release preparation is necessary and will have important social value for hypertension therapy.

However, the retention time of solid preparations in the effective absorption zone of the GI tract is only 8-12 h (Hejazi and Amiji 2002), and rapid GI transit can lead to incomplete drug release from the device and diminish the efficacy of the administered dose. HCTZ has poor water solubility with plasma half-life

of 6-14 hours and is only absorbed from the duodenum and jejunum (Klausner et al. 2003). Therefore, the ideal mucoadhesive drug delivery system would keep the dosage forms in the proximal part of the small intestine against GI transit, hence improving their bioavailability and taking effect for 24 h *in vivo*. In this study, a combination of mini-tablets with different formulation components has been developed to load CLDH and HCTZ independently, in order to modulate the release patterns of two drugs with widely different properties.

2. Investigations, results and discussion

2.1. Preparation of sustained release mini-tablet combinations

In our study, HPMC K15 M and Carbopol 934 were used as the matrix-forming and bioadhesive agents in order to effectively control drug release and ensure valid bioavailability by extending the retention time of dosage forms in the GI tract. The main release mechanism of high water solubility drugs (CLDH) in matrix tablets is diffusion, and poor solubility drugs (HCTZ) are often released based on matrix erosion (Hamed and Sakr 2001). Combination of HPMC K15 M and Carbopol 934 with their different swelling ability, gel structure and erosion, can gain a range of release patterns by optimizing the composition of the matrix.

MCC PH102 was used as a filler of immediate tablets with its excellent fluidity and compressibility. In order to increase the fluidity of the powders and reduce the moisture adsorption of HPMC K15 M and Carbopol 934, the spherical lactose was applied as a filler or release rate modifier in the formulation of sustained released tablets.

The direct compression method was used to prepare the immediate release tablets and sustained release tablets due to its well-known benefits, including fewer processing stages, the elimination of heat and moisture effects, increased productivity and reduction of the final cost of the product (Martinello et al. 2006).

2.2. Effects of the type of HPMC

The influence of the type of HPMC, including HPMC K4 M, K15 M, K100 M, on CLDH (Table 5: C1, C2, C3) and HCTZ (Table 5: H1, H2, H3) release is depicted in Fig. 1. A.

As shown in Figure 1 (A1), the release of CLDH from the matrix with different types of HPMC showed no significant difference referring to the f_2 values [67.4 (C1, C2), 71.1 (C2, C3), 62.3 (C1, C3)] of each curve. K series of HPMC has the same hydration speed and different viscosity in the aqueous environment. Among three of them, HPMC K100 M had the highest viscosity and K4 M had the lowest viscosity. However, viscosity of the gel matrix did not remarkably affect the release behavior of CLDH, which may be attributed to the high water solubility of CLDH, which can diffuse easily from the hydrated gel layer formed by matrix materials once the matrix contacts with water.

The release profile of HCTZ is presented in Fig. 1 (A2) and f_2 values of each curve are 72.8 (H1, H2), 92.8 (H2, H3), 73.8 (H1, H3), respectively. This indicates that the type of HPMC also had not significant effect on the release of HCTZ. Compared with the release profile of CLDH, release rate of the HCTZ apparently decreased due to its poor solubility. As discussed elsewhere, the drug release from the formulation decreases as the viscosity of the polymer increases, especially for poorly soluble drugs (Maderuelo et al. 2011). However, in our study, HCTZ release from the highest viscosity gel barrier generated by HPMC K100 M was not the slowest. The reason might be that high mass fraction of HCTZ that dispersed in the matrix and on the surface of mini-tablets formed many pores and maintained more drug release when HCTZ dissolved in the medium, which mitigated the influence of matrix viscosity on HCTZ release.

2.3. Effects of the amount of HPMC K15 M

HPMC K15 M was chosen as a main matrix of sustained release mini-tablets, because it has the longest GI tract transit time among the three types of HPMC, and the type of HPMC had no obvious impact on the release of CLDH and HCTZ, according to the results described above. The effects of the amount of HPMC K15 M on the release of CLDH and HCTZ are shown in Fig. 1 (B1) (Table 5: C4, C2, C5) and Fig. 1 (B2) (Table 5: H4, H2, H5).

As expected, due to increased tortuosity of a thicker gel barrier and decreased porosity that reduced the water uptake (Reza et al. 2003), the increase in the amount of HPMC K15 M resulted in decreased drug release. When the weight ratio of matrix in HCTZ SR was lowered to 10%, HCTZ was released nearly completely in the first two hours with the matrix collapse, which indicates that the integrity of hydrophilic gel matrix was essential to control the drug release.

2.4. Effects of the amount of Carbopol 934

Carbopol 934 was used as a matrix-forming and bioadhesive agent in order to effectively control drug release and extend the retention time of CLDH SR and HCTZ SR in the GI tract. The releases of the drugs decreased as the amount of Carbopol 934 increased, and the influence of the amount of Carbopol 934 on the release rate of CLDH was less than on that of HCTZ, as shown in Fig. 1 (C1) (Table 5: C2, C6) and Fig. 1 (C2) (Table 5: H2, H6). Carbopol 934 formed hydrophilic gel layer after swelling rapidly in the dissolution medium, from which CLDH diffused easier and more quickly than HCTZ, which might be attributed to the difference in water solubility between CLDH and HCTZ.

2.5. Effects of the type of filler

The effects of the type of filler, including hydrophilic lactose and hydrophobic MCC are shown in Fig. 1 (D1) (Table 5: C6, C7) and Fig. 1 (D2) (Table 5: H2, H7). The release profile of CLDH SR with lactose as the filler was similar to that with MCC, but the release curves of HCTZ SR with these two fillers were different. The HCTZ release from the formulation with lactose as the filler was faster than that with MCC as the filler, because water-soluble fillers such as lactose can facilitate pore production via faster penetration of the release medium, which results in a more porous matrix and a decrease in the tortuosity of the diffusion path of the drug. In contrast, MCC in the matrix caused a delayed penetration of the release medium into the mini-tablets, which inhibited the hydration and swelling of the polymers, thus reducing drug release (Lotfipour et al. 2004). It also may be explained by the fact that MCC can facilitate the formation of the gel barrier and the maintenance of matrix integrity, which could retard the release of HCTZ. However, as a low-dose and water-soluble drug, the release of CLDH from the sustained release mini-tablets seemed unaffected by the porosity and tortuosity of the hydrophilic gel matrix, and only depended on the hydration speed and degree of the matrix.

2.6. Optimized formulation and drug release

The compositions of optimized formulations for CLDH SR and HCTZ SR obtained by the central composite design-response

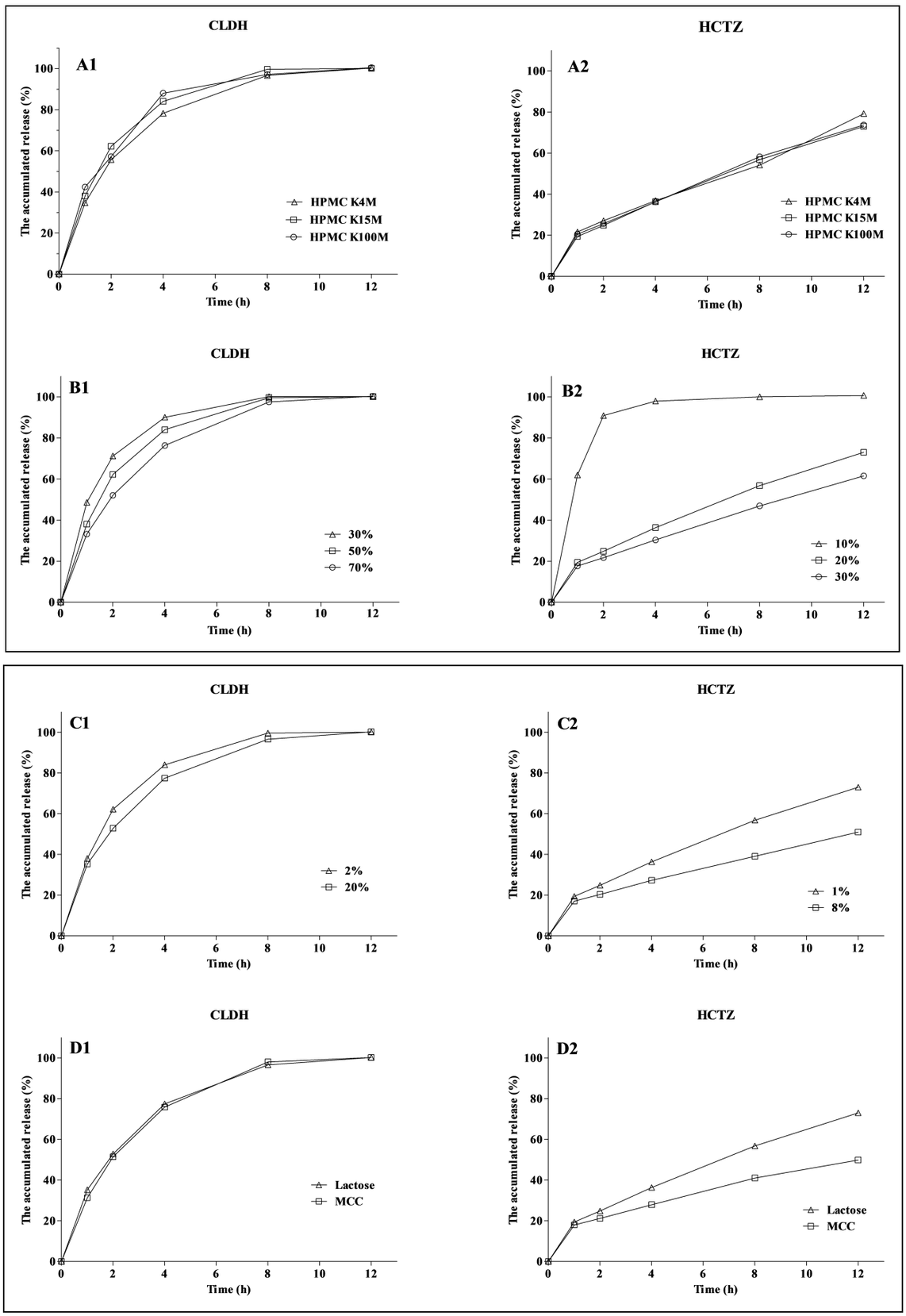

Fig. 1: Effects of (A) the type of HPMC, (B) the amount of HPMC K15 M, (C) the amount of Carbopol 934 and (D) the type of filler on CLDH and HCTZ release from sustained release capsules.

Table 1: Optimized formulations of CLDH SR, HCTZ IR and HCTZ SR (one mini tablet)

CLDH SR	mg	HCTZ IR	mg	HCTZ SR	mg
CLDH	0.1125	HCTZ	6	HCTZ	31.5
HPMC K15M	42	MCC	30	HPMC K15M	11.9
Carbopol 934	12.6	Kollidon CL	0.9	Carbopol 934	11.2
Lactose	14.9375	Lactose	32.95	Lactose	15.05
MS	0.35	MS	0.15	MS	0.35

Table 2: Pharmacokinetic parameters of CLDH and HCTZ after oral administration of sustained release capsules (SRC) or conventional tablets (CT) (n = 6)

Parameter		CLDH(Average ± SD)	HCTZ(Average ± SD)
C_{max} (ng/mL)	SRC	0.934 ± 0.186*	1514.35 ± 729.55*
	CT	1.413 ± 0.348	2387.48 ± 873.96
T_{max} (h)	SRC	2.83 ± 0.75*	4.83 ± 1.60*
	CT	0.95 ± 0.53	2.04 ± 1.00
$T_{1/2}$ (h)	SRC	2.41 ± 1.35	8.32 ± 0.53*
	CT	2.05 ± 1.22	4.29 ± 2.06
$AUC_{0\rightarrow\infty}$ ((ng/ml)*h)	SRC	4389.78 ± 1212.55	15426.47 ± 7169.87
	CT	4310.91 ± 1550.43	14605.51 ± 4416.00
MRT (h)	SRC	6.95 ± 2.31*	16.46 ± 2.72*
	CT	4.37 ± 2.43	7.43 ± 1.32

*$P < 0.05$

surface methodology (CCD-RSM) have been published (Xu et al., 2008), and are shown in Table 1. The releases of CLDH and HCTZ from mini-tablet combinations were detected in 0.1 N HCl, pH 6.8 PBS and deionized water. The results of the drug releases are depicted in Table 4.

The release rate of either CLDH or HCTZ in 0.1 N HCl from the sustained release mini-tablets combinations was faster than that in pH 6.8 PBS. This may be due to the ionization of Carbopol 934 at pH 6.8, a pH environment higher than its pKa of 6. Ionization of Carbopol 934 can lead to a development of negative charges along the backbone of the polymer, and repulsion of same charges uncoils the polymer into an extended structure to form denser gel layer that might have contributed to lower drug release from the matrix (Hassan et al. 2001). The release rate of CLDH and HCTZ in water was less than that in pH 6.8 PBS, because the ions in the release medium may hamper the hydration of the hydrophilic matrix and the formation of the gel barriers (Maderuelo et al. 2011).

2.7. Pharmacokinetics in dogs

The plasma concentration-time curves and pharmacokinetic parameters of CLDH and HCTZ following oral administration of conventional tablets or sustained release capsules in beagle dogs are shown in Fig. 2 and Table 2 . The plasma concentration of the drug in conventional tablets increased more rapidly and the drug was eliminated more quickly comparing to the sustained release capsules. The values of C_{max} and T_{max} of conventional tablets were about 1.413 ng/mL and 0.95 h for CLDH and 2387.48 ng/mL and 2.04 h for HCTZ. In contrast, the values of C_{max} and T_{max} of sustained release capsules were about 0.934 ng/mL and 2.83 h for CLDH and 1514.35 ng/mL and 4.83 h for HCTZ. The C_{max} decreased by about 34% for CLDH and 37% for HCTZ while the T_{max} was prolonged by about 2 times for both CLDH and HCTZ. Thus, the in-vivo releases of CLDH and HCTZ were simultaneously prolonged with sustained release capsules.

The AUC values of conventional tablets were 4310.91 ± 1550.43 (ng/mL) h for CLDH and 14605.51 ± 4416.00 (ng/mL) h for HCTZ, while the values of sustained release capsules were 4389.78 ± 1212.55 (ng/mL) h for CLDH and 15426.47 ± 7169.87 (ng/mL) h for HCTZ, suggesting that the absorption of the two drugs from the sustained release capsules was better than that from the conventional tablets. Absorption enhancement was obtained by increasing the retention time of mini-tablets at their optimal absorption site with bioadhesive polymers. In addition, it is worth noting that the values of AUC for HCTZ obtained from the sustained release capsules were higher than that from the conventional tablets, whereas the absorption of HCTZ was site-dependent and the drug was mainly absorbed in the upper GI tract (Klausner et al. 2003). Thus, it can be hypothesized that a complete release of HCTZ was achieved in the proximal GI tract, where the sustained release mini-tablets adhered for a longer time before they left this absorption site, which might be attributed to the bioadhesion of the tablets.

Compared to the conventional tablets, the values of F(%) were 101.8% for CLDH and 105.6% for HCTZ. The values of 90% confidence intervals (CI) of $AUC_{0\text{-}\infty}$ and C_{max} for both CLDH and HCTZ are described in Table 3, which indicated that $AUC_{0\text{-}\infty}$ was equivalent and C_{max} was non-equivalent for the sustained release capsule, according to the bioequivalence guidance of FDA (Bioequivalence Studies with Pharmacokinetic Endpoints for Drugs Submitted Under an ANDA).

2.8. In vivo-in vitro correlation

The *in vivo-in vitro* correlation (IVIVC) is recommended by the FDA as a predictive mathematical model that depicts the relationships between the *in vitro* properties of an oral formulation and the *in vivo* responses (Lu et al. 2011). A level A correlation, which represents a point-to-point relationship, was performed by comparing the fraction absorption *in vivo* to the accumulative release rate *in vitro*. The absorption fraction of CLDH was calculated with the Loo-Riegelman method because its in-vivo disposition was best fitted by a two-compartment

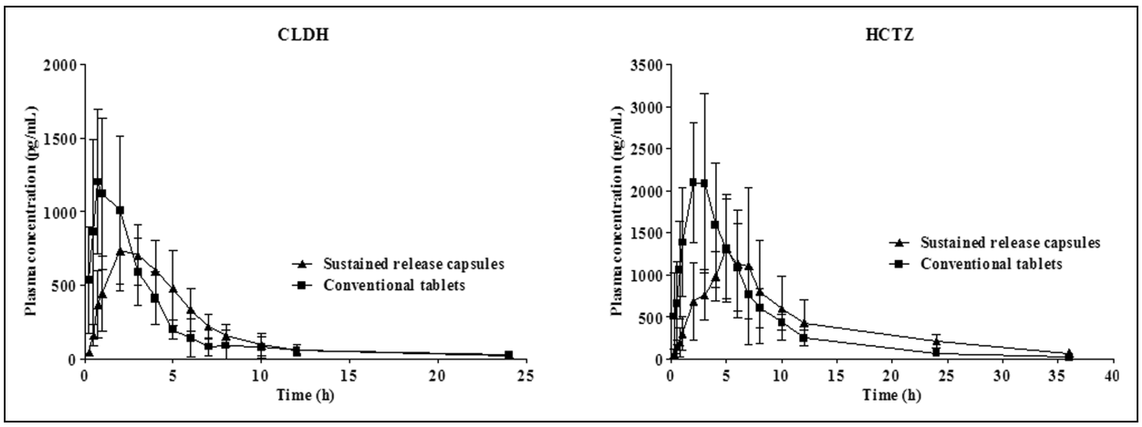

Fig. 2: Plasma concentration-time curves of CLDH and HCTZ after oral administration of sustained release capsules and conventional tablets (n = 6).

Table 3: The statistics analysis of bioequivalence of sustained release capsules and conventional tablets

Parameters	CLDH (%)	HCTZ (%)	Standard (%)	Conclusion
$AUC_{0-\infty}$	85.9-122.8	85.6-122.2	80-125	Bioequivalence
C_{max}	52.2-84.9	49.0-78.1	80-125	Non-bioequivalence

Table 4: In-vivo and *in-vitro* correlation of CLDH and HCTZ released from the sustained release capsules

CLDH	Time (h)	1	2	4	8	12	Correlation	R
In vitro (drug release, R, %)	0.1 N HCl	30.96	46.61	64.47	84.34	95.41	A = 0.015R-0.220	0.9696
	pH 6.8 PBS	24.19	39.31	58.66	79.32	91.03	A = 0.014R-0.115	0.9649
	H_2O	23.68	30.02	40.34	76.61	88.32	A = 0.017R-0.171	0.7595
In vivo (absorption fraction, A,%)	0.23	0.51	0.84	1.00	1.00			
HCTZ	Time (h)	1	2	4	8	12	Correlation	R
In vitro (drug release, R, %)	0.1 N HCl	25.12	36.82	54.11	72.79	81.23	A = 1.307R-19.573	0.9886
	pH 6.8 PBS	23.29	33.16	49.23	68.92	82.77	A = 1.346R-18.035	0.9669
	H_2O	18.91	23.27	35.94	63.51	81.86	A = 1.476R-14.179	0.8288
In vivo (absorption fraction, A,%)	13.19	31.95	54.66	76.69	77.83			

model, while the in-vivo course of HCTZ was best fitted by a one-compartment model and its fraction of absorption was calculated with the Wagner-Nelson method. The linear regression equations between the accumulative release rates of two drugs in 0.1 N HCl, pH 6.8 PBS and deionized water and absorption fraction were obtained by the least squares method. As shown in Table 4, the accumulative release rates of two drugs in 0.1 N HCl correlated well with the absorption fraction, which evidenced by the best linear correlation and the correlation coefficients. Therefore, the detection of *in-vitro* drug release was able to predict the drug disposition *in vivo*.

2.9. Conclusions

A combination of bioadhensive sustained release mini-tablets continuously and independently releasing CLDH and HCTZ was successfully developed. The optimized formulation not only prolonged drug release over 24 h *in vivo*, but also achieved better absorption and stable blood concentrations with a significant extension in T_{max} and reduction in C_{max} *in vivo*, which benefits the treatment of hypertension providing steady blood pressure. The mini-tablets were developed using direct compression technology before filled into capsules, which offered a flexible design to adjust the release courses of combination preparations with low costs and ease of manufacture. Therefore, the mini-tablet combination provides a feasible formulation and promising development value for hypertensive patients who need long-term treatment.

3. Experimental

3.1. Materials

CLDH and HCTZ were obtained from Changzhou Pharmaceutical Factory Co., Ltd. (Changzhou, China). The hydroxypropyl methylcelluloses (HPMC K4 M, K15 M, K100 M) were gifts from Colorcon Co., Ltd. (Shanghai, China). Carbopol 934 and MCC (VIVAPUR® PH 102) were supplied by Shanghai Chineway Pharmaceutical Technology Co., Ltd. (Shanghai, China). Kollidon CL was gifted by BASF (Germany). Magnesium stearate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Lactose (Flowlac® 100) was obtained from Meggle Pharmaceutical Co., Ltd. (Wasserburg, Germany). Methanol and formic acid were of chromatographic grade, and the other reagents were of analytical grade.

The animals were male beagle dogs (certificate no.SCXK (Jiangsu) 2012-0009) which were purchased from Suzhou Xishan Zhongke Laboratory Animal Co.,Ltd.

Table 5: Formulations of CLDH SR and HCTZ SR (all quantities are given in micrograms)

	CLDH SR						
	C1	C2	C3	C4	C5	C6	C7
CLDH	0.1125	0.1125	0.1125	0.1125	0.1125	0.1125	0.1125
HPMC K4M	35	-	-	-	-	-	-
HPMC K15M	-	35	-	21	49	35	35
HPMC K100M	-	-	35	-	-	-	-
Carbopol 934	1.4	1.4	1.4	1.4	1.4	14	14
Lactose	33.1375	33.1375	33.1375	47.1375	19.1375	20.5375	-
MCC	-	-	-	-	-	-	20.5375
MS	0.35	0.35	0.35	0.35	0.35	0.35	0.35
	HCTZ SR						
	H1	H2	H3	H4	H5	H6	H7
HCTZ	31.5	31.5	31.5	31.5	31.5	31.5	31.5
HPMC K4M	14	-	-	-	-	-	-
HPMC K15M	-	14	-	7	21	14	14
HPMC K100M	-	-	14	-	-	-	-
Carbopol 934	0.7	0.7	0.7	0.7	0.7	5.6	0.7
Lactose	23.45	23.45	23.45	30.45	16.45	18.55	-
MCC	-	-	-	-	-	-	23.45
MS	0.35	0.35	0.35	0.35	0.35	0.35	0.35

3.2. Preparation of the sustained release mini-tablet combinations

HCTZ is a poorly-soluble drug, which is released from sustained preparation too slowly to achieve an effective therapeutic concentration. Therefore we designed an HCTZ immediate-release part to replenish early release. Sustained release mini-tablets combination was composed of one CLDH sustained release mini-tablet (112.5 μg CLDH/tablet, CLDH SR), one HCTZ immediate-release mini-tablet (6 mg HCTZ/tablet, HCTZ IR) and one HCTZ sustained release mini-tablet (31.5 mg HCTZ/tablet, HCTZ SR). The filler of HCTZ IR was MCC PH102, and HPMC K15 M was chosen as the matrix material of CLDH SR and HCTZ SR.

Hydroxypropyl methylcellulose (HPMC), as hydrophilic gel matrix, is widely applied in the study of controlled release and sustained release preparations. HPMC K15 M has longer GI transit time than other types of HPMC, owing to its appropriate molecular weight and chain length of the polymer, which has the benefit of forming mutual cross-linked structures between the polymer chain and the mucous layer in the GI tract (Zhang et al. 2001). Carbopol 934 is a highly hydrophilic polyacrylic acid polymer which has been used to prepare gel formulations for topical administration due to its high viscosity and good bioadhension at low concentrations (Shin and Kim 2000). Carbopol 934 can form hydrogen bonds with the negatively charged mucus gel, following the formulation of physical entanglements, and adhere to the GI mucosa for a long time against GI movement (Mortazavi 1995). In order to prolong the residence time of the tablets in the GI tract, Carbopol 934 was added in sustained release mini-tablets to acquire bioadhesive ability. Because of the low dosage of CLDH, the ethanol solution of CLDH was uniformly dispersed in excipient powder, and then the ethanol was vaporized to obtain dry powder.

All the mini-tablets were prepared by a direct compression method. Briefly, the immediate-release mini-tablets were prepared with formulated amounts of HCTZ, MCC PH102 and aerosol as a glidant, while the prescription of the sustained release mini-tablets consisted of CLDH or HCTZ, HPMC K15 M, Carbopol 934 and spherical lactose. The powder mixture of each prescription was screened through an 80-mesh sieve and blended with magnesium stearate (MS) before tableting by a single-punch tablet machine (Shanghai Tianxiang and Chentai Pharmaceutical Machinery co., Ltd., Shanghai, China). The mixture powder was compressed directly in the tablet press die with the inner diameter of 4 mm under a pressure force of 6-8 kg/cm^2. HCTZ IR, HCTZ SR and CLDH SR were combined in one capsule to obtain sustained release mini-tablet combinations.

To optimize the formulation, factors of the matrix material and the filler were studied in terms of drug release in deionized water. The compositions for each formulation are presented in Table 5.

3.3. In-vitro drug release

Dissolution tests were conducted using small cup method, the third method of Chinese Pharmacopoeia 2010, edition 2, appendix XC, on a RCZ-8A dissolution tester (Tiandatianfa Tech., Ltd., Tianjin, China). The capsules were placed in 150 mL degassed water which was maintained at 37 ± 0.5°C with a rotation speed of 75 rpm. Three mL of samples was taken at predetermined time intervals (1, 4, 8, 12 h), filtered through 0.8 μm Millipore filters, and the concentration of the drugs was detected by HPLC then the same volume of fresh medium was added to the dissolution cups. The release profiles of the different formulations were obtained by plotting accumulated release rates against time.

In order to study the influence of various factors on the release, the similarity factor method that is advocated by the FDA was applied to assess the differences in release profiles. The similarity factor (f_2) is calculated as a logarithmic transformation of the sum squared error of the differences between the test preparation and the reference preparation according to Eq (1):

$$f_2 = 50 \times \log\left\{\left[1 + \frac{1}{n}\sum_{i=1}^{n}\left(\bar{R}_t - \bar{T}_t\right)^2\right]^{-0.5} \times 100\right\} \qquad (1)$$

where R_t and T_t are respectively the accumulated release rates of two preparations at each time point, and n is the number of the time points. The similarity factor is a number between 0 and 100, with 100 meaning that two profiles perfectly match, the more the values are close to 100, the more similar the profiles are. If the similarity factor is more than 50, release profiles are considered to be similar. If the f_2 value is lower than 50, there are significantly differences between two profiles (He et al. 2015).

3.4. Pharmacokinetics in beagle dogs

All the animal experiments were approved by the Nantong University Animal Management and Ethics Committee. A randomized, two-period cross-over design was used to study the pharmacokinetics in beagle dogs. Six male dogs were randomly assigned to two groups. The dogs in group A received two sustained release capsules (including 225 μg CLDH and 75 mg HCTZ/capsule) and the group B dogs were administered two CLDH and HCTZ conventional tablets with the same dosage. Both groups received the drugs after being fasted for 10 h. The washout period between consecutive administrations was one week. Four mL of blood was taken from the vein into tubes at 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24 and 36 h after administration. All blood samples were incubated for 30 min and centrifuged at 3000 rpm for 10 min to obtain serums. The serums were stored at –20°C and serum concentrations were detected by HPLC-MS/MS.

3.5. Sample preparation and analysis

The contents of CLDH and HCTZ in the release media were analyzed simultaneously using a LC-20AT HPLC system (Shimadzu, Japan) with a UV-Vis

detector (Shimadzu, Japan). The separation was conducted on a Synergi Fusion-RP C18 column (4.6 mm i.d. × 250 mm, 4 μm, Phenomenex, USA) at a column temperature of 25°C . The mobile phase was composed with methanol, water and phosphoric acid (20:80:0.04, v/v/v) that were pumped at a rate of 1.0 mL/min and monitored at 220 nm. Twenty μL of the supernatant was injected into the HPLC. The detective sensitivity program was as follows: 0-8 min, 0.005 AUFS; 8-13 min, 0.05 AUFS.

The extraction method of CLDH in plasma was as follows: 300 μL of plasma samples was mixed with 10 μL of internal standard (20 ng/mL huperzine A in methanol), followed by vortexing for 30 s. Then 1 mL ether was added to the last mixture, the sample was vortexed for 3 min and centrifuged at 13400 rpm for 10 min. Subsequently, 800 μL of organic solvent layer was dried by N_2, the sediment was dissolved by 80 μL of mobile phase and 20 μL of the supernatant was injected into the HPLC-MS/MS system. Extractions of HCTZ in plasma samples were made by the following steps. 100 μL of plasma samples and 10 μL of internal standard (50 ng/mL hdyroflumethiazide in methanol) were mixed by vortex for 30 s after adding 300 μL methanol, the mixture was vortexed for 3 min and then centrifuged at 134000 rpm for 10 min. Ten μL of the supernatant was injected into the HPLC-MS/MS system.

The HPLC-MS/MS system comprised Finnigan™ Surveyor HPLC equipment, Finnigan™ Surveyor autosampler and Finnigan™ TSQ Quantum Discovery MAX tandem mass spectrometer, fitted with an electrospray ionization (ESI) ion source (Thermo Electron, USA). Xcalibur 1.4 software (Thermo Electron, USA) was employed for data acquisition and analysis. HPLC isolation of CLDH was conducted on a Luna CN column (2.0 mm i.d. × 150 mm, 4 μm, Phenomenex, USA) at a column temperature of 25°C. The mobile phase was made up of methanol, water and formic acid (40:60:0.1, v/v/v) at the flow rate of 10 μL/min; HPLC isolation of HCTZ was conducted on a Synergi Fusion-RP C18 column (4.6 mm i.d. × 250 mm, 4 μm, Phenomenex, USA) at a column temperature of 25°C. The samples were eluted with a mixture of methanol and water (35:65, v/v) at a flow rate of 10 μL/min.

The HPLC eluent was diverted to a triple quadrupole tandem mass spectrometer equipped with an ESI ion source that operated in the positive ion mode for CLDH detection and the negative ion mode for HCTZ detection, and selected reaction monitoring (SRM) was used in drug quantification. The optimized parameters for mass spectrometric detection of CLDH were set as follows: spray voltage, 4.9 kV; capillary temperature, 340°C; sheath gas, N_2; pressure, 34 Arb; auxiliary gas, N_2; pressure, 2 Arb; collision gas, Ar; pressure, 1 v; precursor ion, m/z 229.9 - m/z 212.9 for CLDH, m/z 243.2 - m/z 210.9 for huperzine a; and collision energies, 32 V for CLDH and 30 V for huperzine A. The optimized parameters for mass spectrometric detection of HCTZ were set as follows: spray voltage, 4 kV; capillary temperature, 320°C; sheath gas, N_2; pressure, 42 Arb; auxiliary gas, N_2; pressure, 7 Arb; collision gas, Ar; pressure, 16 v; precursor ion, m/z 295.9 - m/z 268.9 for HCTZ, m/z 329.8 - m/z 239.8 for hydroflumethiazide; and collision energies, 25 V for HCTZ and hydroflumethiazide.

The endogenous substances in plasma did not interfere with the determination of the samples and internal standards. The standard curve of CLDH concentration in plasma was $A = 275.478C + 2.058$ ($R^2 = 0.998$) with linear concentration from 0.020-2 ng/mL. The absolute recoveries of low (0.0505 ng/mL), medium (0.201 ng/mL), and high (1.012 ng/mL) levels were $95.2 \pm 3.35\%$, $96.67 \pm 4.24\%$ and $99.72 \pm 1.62\%$ respectively, and the intra-day and inter-day precision were not greater than 5%. The standard curve of HCTZ concentration in plasma was $A = 50.537C + 0.393$ ($R^2 = 0.999$) with linear concentration from 2-5000 ng/mL. The absolute recoveries of low (50.5 ng/mL), medium (501.0 ng/mL), and high (2030 ng/mL) levels were 99.60 ± 5.92 %, 96.91 ± 4.06 % and 98.98 ± 2.04 % respectively, and the intra-day and inter-day precision were not greater than 10%. The stabilities of the samples under freezing and freeze-thaw cycles met the detection requirement of biological samples.

3.6. Data analysis and statistics

DAS 2.0 pharmacokinetics statistical software was used to fit the pharmacokinetics models and calculate pharmacokinetics parameters of the sustained release capsules and conventional tablets, including C_{max}, T_{max}, $T_{1/2}$, $AUC_{0\text{-}t}$ and MRT. Two-tailed *t*-tests were performed for C_{max} and $AUC_{0\text{-}t}$, and a non-parametric test was performed for T_{max}. The data were described by the mean ± standard deviation, and differences were considered statistically significant when P values were less than 0.05. The 90% confidence intervals were also calculated to evaluate bioequivalence of sustained release mini-tablet combinations and conventional tablets.

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