# Determination of Adriamycin Content in Pectin–Adriamycin Conjugate in a Two-Phase Reaction System by High-Performance Liquid Chromatography

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Received: 12 December 2016; accepted: 12 December 2016

A newly proposed method for detecting content of adriamycin in pectin–adriamycin conjugate has been developed and evaluated. The content of adriamycin was detected by selective degradation of adramycin to adriamycinone. It was realized by a two-phase reaction system (water–chloroform reaction system), in which adriamycin was quantitatively converted to adriamycinone. Therefore, the latter can be used to calculate the precise content of adramycin in the polymer drug. To develop the method, the catalyst for degradation, the extraction solvent for adriamycinone, the temperature and time of degradation, and the ratio of pectin–adriamycin conjugate were investigated. The optimal reaction condition was as follows: 30 mg of pectin–adriamycin conjugate dissolved in 25 mL of water was added to a mixture of 25 mL of hydrochloric acid (1.5 mol/L) and 50 mL of chloroform; the mixture was heated to 40 °C to react for 1.5 h; after that, the mixture was extracted with chloroform for three times, and then the organic layer was combined and, subsequently, evaporated to remove solvent. Under this condition, adriamycinone generation rate reached 99.87%. The quantitative method was evaluated for linearity, the limit of detection (LOD) and limit of quantitation (LOQ), recovery, accuracy, robustness, and precision. The recoveries were between 99.47% and 101.07% with relative standard deviation <1.23%. The LOD and LOQ were 0.06 and 0.17 μg/mL, respectively. Compared to the traditional ultraviolet (UV) detection, this method is considered to be more precise for detecting content of adriamycin in its polymer conjugate.

**Keywords:** Two-phase reaction system, pectin–adriamycin conjugate, high-performance liquid chromatography, adriamycinone generation rate, drug loading

## Introduction

Adriamycin (ADM) represents one of the most important anthraquinone antitumor drugs. To eradicate tumors, ADM intercalates between DNA base pairs and inhibits the synthesis of DNA with an aromatic tetracyclic ring [1]. ADM has been widely applied in treatment of various cancers, including acute lymphoblastic leukemia, myelogenous leukemia, breast cancer, lung cancer, ovarian cancer, stomach cancer, liver cancer, etc. [2–4]. However, severe dosing-limiting cardiotoxicity, myelosuppression, and multi-drug resistance are its undesirable toxic effects that limit its clinical application [5–8]. In recent years, polymer pro-drugs have emerged as one of the most interesting fields for targeted cancer therapy due to their ability to reduce toxicity, improve efficiency, accumulate in tumors by the enhanced permeability and retention effect (EPR), and prolong duration of activity by sustained release [9–11]. Pectin is a natural water-soluble polysaccharide with good biocompatibility and excellent biological stability. The pectin-backbone is comprised of D-galacturonic acid units by repeating α-(1-4)-linkage [12]. For decades, pectin has been studied as vehicle materials for drug delivery [13, 14]. Pectin–adriamycin conjugate (PAC), an antitumor pro-drug that is comprised of pectin and ADM through attaching amino group of ADM to carboxyl of pectin via amide bond, has been synthesized by our group in previous studies [15]. Compared with ADM, PAC has stronger targeting activity and lower side effects in pharmacological test in vitro and in vivo using cytotoxicity animal tumor models. Therefore, PAC may be developed as a new targeted anti-cancer drug.

The determination of active components in polymer drugs is generally performed using ultraviolet (UV) or high-performance liquid chromatography (HPLC) [16]. UV is regarded as a useful analysis method and has been successfully applied to a variety of polymer pro-drugs including polysaccharide components, antibody, and liposome [17, 18]. It has many advantages such as rapidity, simplicity, and lower cost, yet the unacceptable error and higher limit of detection (LOD) and limit of quantitation (LOQ) compared to HPLC are its disadvantage that is unfavorable to precisely detect active components in polymer pro-drugs. Furthermore, both polymer carriers and small molecular impurities with UV adsorption could interfere with the precision of UV. In our previous studies, drug loading of PAC was also detected by using UV and had a relatively large error because of the poor water solution of PAC and traces of impurities contamination [15]. HPLC cannot directly be used to detect content of polymer pro-drugs because all of analytes are polymer components that do not match with traditional chromatographic columns [19, 20]. Thus, the analytes needed to be degraded into small molecular components that can be detected by HPLC. Hydroxypropyl methacrylate copolymers pro-drugs (HPMA conjugates) including PK1 and PK2 were the known pro-drugs that have entered phase 2 trial. Determination of their drug loading was promoted in a homogeneous reaction system by hydrolysis PK1 or PK2 to be adriamycinone (ADM-ONE) in hydrochloric acid (0.5 mol/L)

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DOI: 10.1556/1326.2017.00201

and by using HPLC to quantitatively detect the content of ADM-ONE to extrapolate drug loading of PK1 or PK2 [21]. The generated hydrolyzate ADM-ONE, that is not stable in acid medium, is the part unit of ADM without the amino sugar ring and is the major metabolite of ADM in the body. The drawback of this method is that the reaction time needs to be accurately controlled because ADM-ONE is not stable when retained in acid medium for a long time. Thus, it is difficult using this method to precisely detect drug loading for PAC because, compared to water soluble components PK1 or PK2, PAC is the poor water soluble component that required more time to complete the degradation. A long reaction time may lead to continuous degradation of ADM-ONE, so the HPLC method described in PK1 cannot be directly applied in detection of the active component of PAC. A modified HPLC is proposed to try to overcome the instability of ADM-ONE in acidic medium in the previous reported method, and to establish a reliable measurement for detecting drug loading of PAC.

In this study, the modified HPLC method using a two-phase degradation system was designed to overcome the drawback of the abovementioned HPLC. The designed two-phase system allowed the degradation to be carried out in water, and subsequently, the generated ADM-ONE quickly entered an organic phase from water in terms of the difference of distribution coefficient of ADM-ONE between water and the organic phase. Three extraction solvents (chloroform, dichloromethane, and ethyl acetate) were selected to test the optimal extraction rate. To realize the aim of optimization of the modified HPLC method, the other degradation parameters including catalyst, reaction time, and temperature and ratio of PAC were investigated and optimized. In addition, appropriate chromatographic separation conditions and method validation were also thoroughly evaluated.

## Experimental

**Reagents and Materials.** Standard substance: ADM (99.5%), ADM-ONE (99.5%), and daunomycinone (internal standard, 99.5%) were purchased from the National Institute of Control of Pharmaceutical and Biological Products (Beijing, China). One batch of PAC (drug loading: 25.5%) and pectin were kindly gifted by Chongqing Lummy Pharmaceutical Co., Ltd. Hydrochloric acid (12.0 mol/L), dichloromethane, ethyl acetate, nitric acid, phosphoric acid, ammonium acetate, and chloroform, analysis of pure (AR), were purchased from Sichuan Kelong Co., Ltd. (Chengdu, China). Acetonitrile of LC grade was supplied by Merck (USA). Deionized water was supplied by our laboratory.

**Apparatus.** Model 1260 HPLC equipped with model G1315 diode array detector (DAD) as UV monitor and equipped with model G1329B autosampler was used for separation and detection (Agilent, USA). Model OPT-1-10T Youpu ultrapure water system was used for preparing deionized water (Chengdu Youpu Electronics Co., Ltd., China). One-hundred-thousandth gram of accuracy of scales was used for weighting samples (Mettler Toledo, Switzerland).

**Method of HPLC.** Agilent Zorbax SB-$C_{18}$ column (250 mm × 4.6 mm, 5 μm) was used for separation and maintained at 25 °C in column oven. The mobile phase was ammonium acetate solution (0.05 mol/L, pH = 4.5)–acetonitrile–water = 30:35:45 (v/v/v). The UV detection wavelength was set at 254 nm. The flow rate of the mobile phase was maintained at 1.0 mL/min, and the sample size was 20 μL.

**Preparation of Stock Standard Solutions.** One milligram of standard ADM-ONE or internal standard was weighted accurately and dissolved in 10 mL of mobile phase. The obtained solution was then eluted with the mobile phase to get concentrations of 1, 3, 5, 10, and 17 μg/mL, respectively. The standard stock solutions can be stored in the dark at about 4 to 8 °C for 7 days.

**Optimization of Parameters in Degradation.** In order to achieve optimal degradation conditions, a variety of parameters were investigated, including (1) catalyst: several catalysts including hydrochloric acid, sulfuric acid, nitrate acid, and perchloric acid were used for selection; (2) reaction time: several time points including 0.5, 1.0, 1.5, 2.0, and 2.5 h were used for optimization; (3) reaction temperature: several temperature points including 20, 30, 40, and 50 °C were used for optimization; (4) ratio of PAC: three gradient ratio of PAC controlled at 0.4, 1.2, and 2.0 mg/mL (the three gradient corresponded to 10, 30, and 50 mg of PAC dissolved in 25 mL of water, respectively) were used in the experiments; (5) extraction solvent selection: three solvents including dichloromethane, ethyl acetate, and chloroform were chosen for test. Finally, the best parameters were chosen in the experiment.

**Degradation Reaction for PAC.** In this process (the scheme was presented in Figure 1), 25 mL of hydrochloric acid (1.5 mol/L) and 50 mL of chloroform were mixed together at room temperature, and 30 mg of PAC dissolved in 25 mL of water was added to the mixture, and then the mixtures were reacted to 40 °C for 1.5 h with vigorous stirring. With completion of the degradation, the reaction mixture was cooled to room temperature for stratification. Subsequently, extraction was performed, and later, a further 100 mL (50 mL × 2) of chloroform was used for extraction twice. After extraction, all the organic layers were combined and removed. The obtained residue was used for further analysis.

**Preparation of Sample Solutions.** The residue which was prepared by the method of "section Degradation Reaction for PAC" was set to be 50 mL with the mobile phase. Accurate 1 mL of the solution was transferred to a volumetric flask via Transferpettor and later was eluted with mobile phase to obtain a solution with an appropriate concentration.

**The Formula for Extraction Rate, ADM-ONE Generation Rate, and Drug Loading of PAC.** Extraction rate is defined as that the extracted ADM-ONE is accounted for the percentage

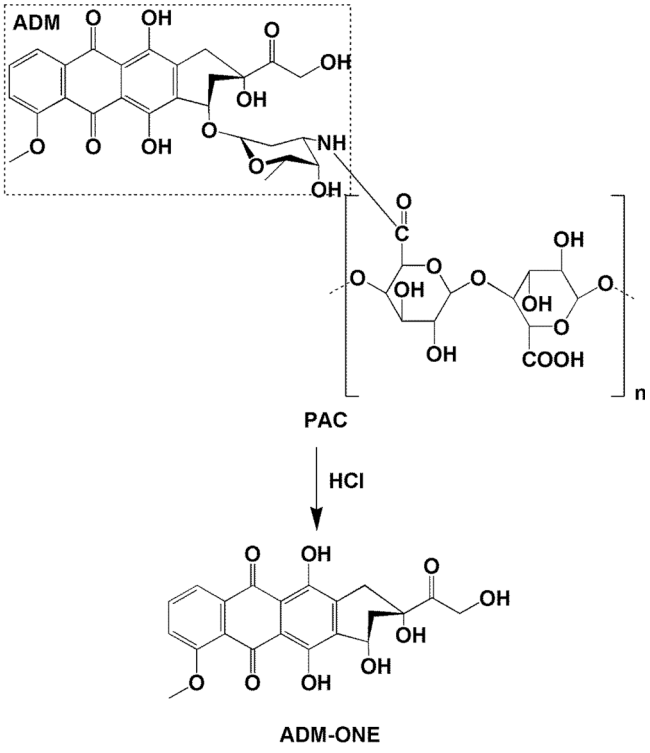


**Figure 1.** Scheme for degradation of PAC

of the total quality of ADM-ONE in PAC. ADM-ONE generation rate (AGR) is defined as that the quality of ADM-ONE converted in the degradation is accounted for the percentage of the total quality of ADM-ONE in PAC. Drug loading of PAC is defined as that the total quality of ADM in PAC is accounted for the percentage of the total quality of PAC. Extraction rate, AGR, and drug loading are calculated using the following equations:

$$\text{Extraction rate} = \frac{\text{the extracted amount of ADM} - \text{ONE}}{\text{total amount of ADM} - \text{ONE in PAC}} \times 100\%$$

where $C_{sample}$ is the extracted concentration of the internal standard and $C_{standard}$ is the added theoretical concentration of the internal standard.

In the education of "drug loading," the detected amount of ADM results from ADM-ONE.

## Results and Discussion

**Degradation Mechanism.** The mechanism of this modified HPLC method relies on the stability difference of glycosidic and amide bond of PAC in acidic environment, the former being more sensitive to the latter in acidic medium in terms of the reported literatures [22]. Therefore, it is feasible to selectively cleave glycosidic bond to qualitatively obtain ADM-ONE by control of the reaction conditions. Briefly, PAC was dispersed in water containing a certain concentration of hydrochloric acid, while the degradation was performed also in the same medium. With the proceeding of the degradation, ADM-ONE continuously generated. The obtained ADM-ONE subsequently transferred from water to chloroform, avoiding continuous degradation of ADM-ONE. During the extraction process, chloroform has the ability to fully extract ADM-ONE from water. The obtained ADM-ONE was collected to calculate drug loading of PAC.

**Method Validation.** Study of the method validation applied the optimal degradation conditions, which was demonstrated in Table 1. The used HPLC method was evaluated for linearity, LOD and LOQ, precision, accuracy, and robustness. Three typical chromatograms were shown in Figure 2. As shown form the results, the standard ADM-ONE (Figure 2A) and the degradation product (Figure 2B) have the same retention time (9.15 min), indicating that they are regarded as the same component. The retention time of the internal standard is 4.12 min (Figure 2C), indicating that it has a good resolution with ADM-ONE.

**Linearity.** Under the optimal experimental conditions, calibration curves were set up using a series of standard solutions of ADM-ONE or internal standard with concentrations varying from 1.0 to 17 μg/mL, respectively. The obtained curve for ADM-ONE was linear with $r^2$ = 0.9997, and the regression equation was $y = 69.2164x - 0.9223$. The obtained curve for internal standard was linear with $r^2$ = 0.9994, and the regression equation was $y = 17.8550x - 28.1616$.

**Limit of Detection and Limit of Quantitation.** LOD and LOQ were determined by serial dilution of standard ADM solutions to obtain signal-to-noise ratios of 3 and 10, respectively. The LOD was found to be 0.06 μg/mL (S/N = 4.1), and the LOQ was found to be 0.17 μg/mL (S/N = 16.2), respectively.

**Precision.** The precision was determined by detecting the repeatability (intra-day precision) and the intermediate (day to day) precision of peak areas at three different concentrations of standard ADM-ONE. For five consecutive injections, the repeatability of the peak areas of standard ADM-ONE was determined as the relative standard deviation (RSD %). Intermediate precision was evaluated over 3 days by analyzing the same sample with the same method. These results indicate that intra-day and inter-day RSD were <1.33% and <1.45%, respectively, showing that the method was sufficiently precise for qualitative and quantitative analysis.

**Accuracy.** Accuracy was evaluated by spiking PAC solutions (drug loading: 25.5%) at three levels in the range of 80–120% with respect to a specified level of 15.0 μg/mL to a standard ADM-ONE solution with concentration at 3.0 μg/mL, respectively. The optimal degradation conditions were used for each solution, and the analysis for spiking recoveries of each solution was repeated three times ($n$ = 3). The results were shown in Table 2, indicating that the recoveries were in the range of 99.47% to 101.07% with RSD <1.23%.

**Robustness.** Robustness was tested by small change of the parameters in the modified HPLC method. A variation of 0.5% acetonitrile in the mobile phase never affected the resolution against other impurities (if there were impurities in the samples) except that retention time was changed. By adjusting the flow rate at 0.8 and 1.2 mL/min and adjusting the column temperature at 25 ± 2 °C to study the effect on the resolution, the results indicated that, in all of the cases, the resolution against other impurities (if there were impurities in the samples) was above 2.0.

**Selection of Extraction Solvent.** Ideal extraction solvent is required to have the properties including immiscibility with water, good solubility for ADM-ONE, stable in acidic medium, a moderate polarity corresponding to the moderate polarity of the extracted component, and high extraction efficiency for ADM-ONE. Thus, three organic solvents including dichloromethane, ethyl acetate, and chloroform were used. PAC (drug loading: 25.5%) was used as the reactant to promote the experiment and the operation referred to section of Degradation Reaction for PAC. To improve the extraction rate and avoid continuous degradation of ADM-ONE, the reaction mixture was quickly cooled to room temperature for stratification when the degradation was finished. Subsequently, extraction was performed, and after each extraction, an equivalent volume of organic solvent was added to the reaction mixture. The results are shown in Figure 3. Compared to dichloromethane and ethyl acetate, chloroform has the highest extraction efficiency rate under the same extraction times. Especially, as the extraction times reached three, almost all of the ADM-ONE (99.5 ± 0.8%) in the degradation mixture was extracted, while, under the same condition, the extraction rate was 94.2 ± 0.7% for dichloromethane and 88.3 ± 1.2% for ethyl acetate, respectively. Meanwhile, further increasing extraction times using chloroform negatively affected the extraction rate, indicating that the extraction equilibrium appeared and the further extraction did not improve the extraction rate. On the other hand, with increasing the extraction times to extraction equilibrium for ethyl acetate (four times) and dichloromethane (three times), the extraction rate was still no more than 90.0% and 95.0%, respectively, indicating that, even at the extraction equilibrium, both ethyl acetate and dichloromethane had lower extraction rate compared to chloroform. Thus, chloroform was selected as the most suitable extraction solvent and the optimal extraction times were set at three.

**Optimization of Degradation Parameters.** In the degradation process, ADM-ONE was the degraded product that was collected to calculate AGR and extrapolate drug loading of PAC. Apart from extraction solvent, AGR is also affected by several factors, including catalyst, reaction temperature, and time and ratio of PAC. In order to evaluate these factors and get the best parameters, appropriate experiments were designed to evaluate the whole factors. For simplifying the operation and obtaining the results rapidly, single factor experiments

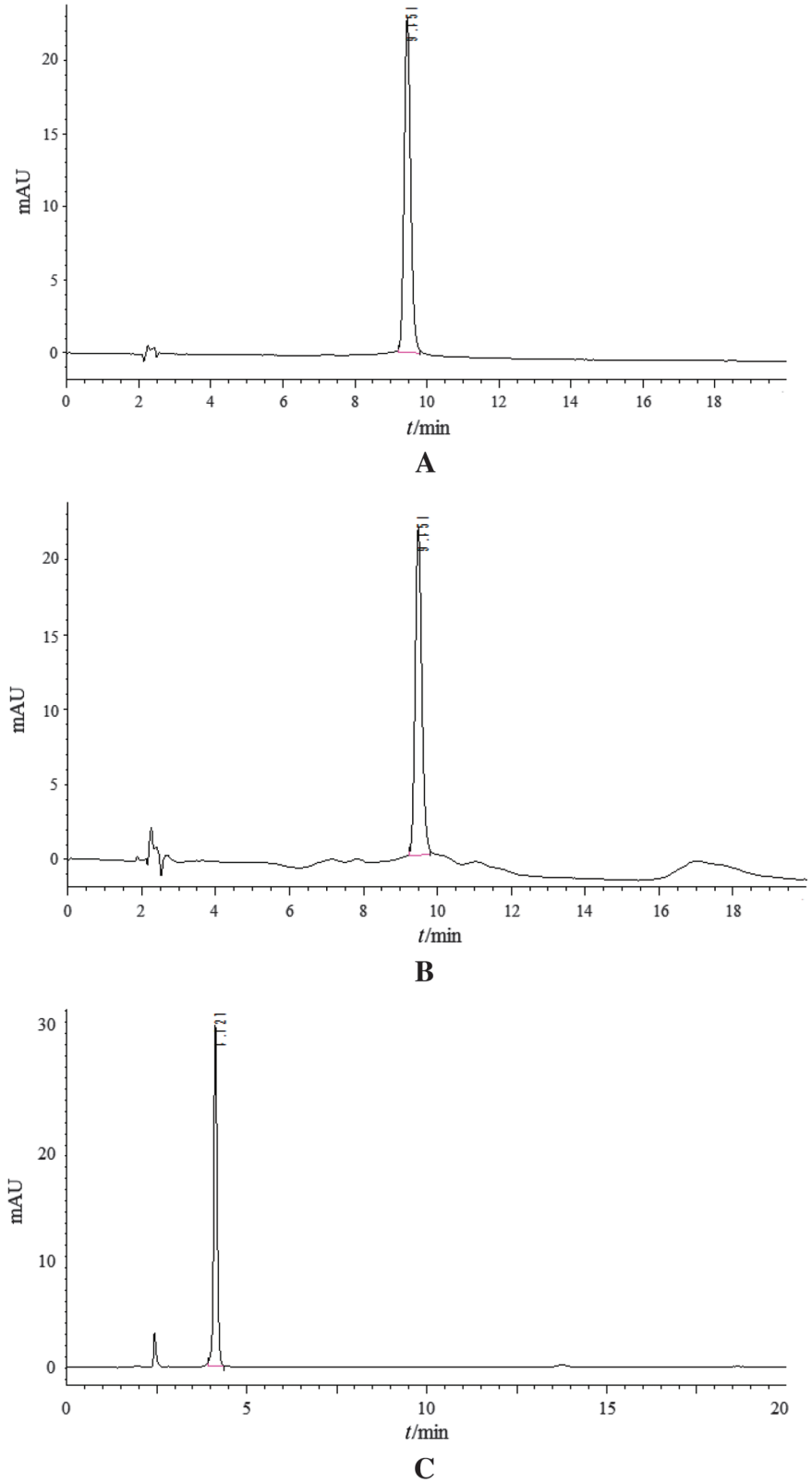


**Figure 2.** Typical chromatograms of (A) ADM-ONE standard, (B) degradation product, and (C) the internal standard

were performed to select catalysts and their best working concentrations and orthogonal experiments with four factors (ratio of PAC, volume of catalyst, reaction temperature, and time) and three level conditions were applied to select the other degradation parameters.

***Catalyst Selection and Its Optimal Working Concentration.*** A suitable catalyst is one of the most important factors to affect the degradation. Catalysts selected in the study need to fit the following conditions, including fast reaction rate, good selectivity, and high compatibility with the reaction system. Based on the acid-catalyzed principle, in the process, hydrion directly participates in cleavage of amino-glycoside bond in PAC and the reaction rate is directly proportional to the concentration of hydrion; thus, higher concentration of hydrion is more beneficial to accelerate the reaction rate of the degradation. However, appearance of continuous degradation product derived from ADM-ONE to a large extent could be caused by excess of hydrion. According to the abovementioned requirements, strong mineral acid such as

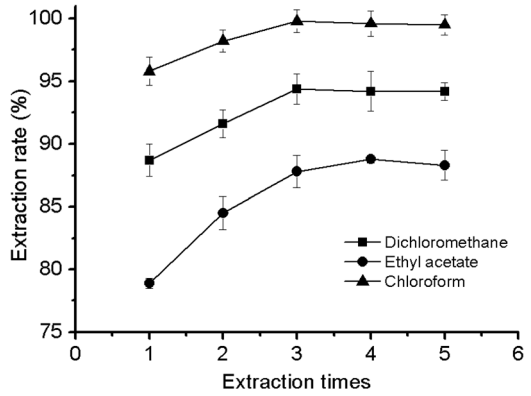


**Figure 3.** Evaluation of extraction rate for dichloromethane, ethyl acetate, and chloroform response to different extraction times

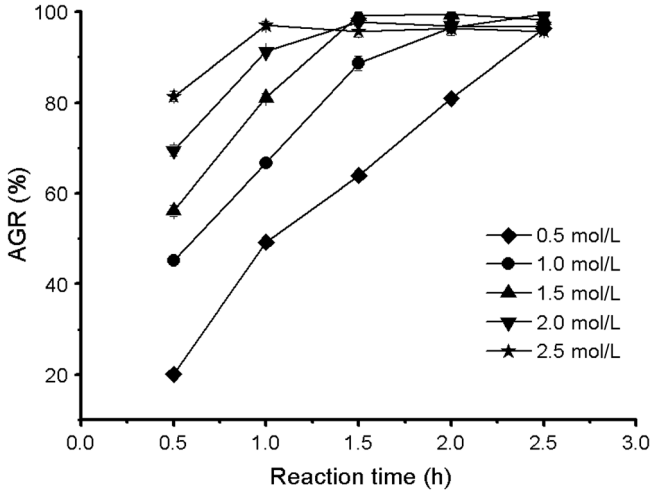


**Figure 4.** Effect of hydrochloric acid with different concentrations on AGR

hydrochloric acid, sulfuric acid, nitrate acid, and perchloric acid were the candidates. Nevertheless, nitrate acid and perchloric acid with strong oxidizability that was harmful to ADM-ONE were not selected in the experiment. In addition, taking into account the environmentally friendly reason, sulfuric acid that has similar catalytic ability compared to hydrochloric acid was also excluded. Thus, hydrochloric acid was selected as the optimal catalyst at last. Different concentrations of hydrochloric acid at 0.5, 1.0, 1.5, 2.0, and 2.5 mol/L were used for optimizing the concentration. In order to evaluate the efficiency of hydrochloric acid used in the degradation, the used PAC with drug loading of 25.5% was applied as the reactant to perform the experiment. Experiment procedures referred to section Degradation Reaction for PAC, and chloroform was used as the extraction solvent. Internal standard was added to the reaction system after the degradation. As shown from Figure 4, under the same reaction time, higher concentration of hydrochloric acid corresponds to higher AGR. Concentration at 2.5 mol/L had the fastest reaction rate compared to the other used concentrations, but some impurities quickly appeared within a short period of time, indicating that higher concentration could cause the continuous degradation of ADM-ONE. Concentrations at 2.0 mol/L and 1.5 mol/L also had good catalytic efficiency and reached the maximum AGR when reacting for 1.5 h. However, as the reaction time was over 1.5 h, there were a few impurities for concentration at 2.0 mol/L and there was no presence of impurities for concentration at 1.5 mol/L, indicating that 1.5 mol/L compared to 2.0 mol/L was a more appropriate concentration in the experiment. As the concentrations reduced to 1.0 mol/L or 0.5 mol/L, the reaction rate was slower and did not reach the maximum AGR if the reaction time was over 2.5 h. Hence, hydrochloric acid with concentration at 1.5 mol/L was selected as the optimal concentration.

***Orthogonal Experiments to Optimize the Degradation Conditions.*** Apart from catalyst and extraction solvent, the main factors affecting AGR include ratio of PAC, volume of hydrochloric acid, reaction temperature, and reaction time. Orthogonal experiments with four factors (reaction time, temperature, volume of hydrochloric acid, and ratio of PAC) and three levels conditions were applied to evaluate the influence of the factors for the degradation. Experiment procedures referred to section Degradation Reaction for PAC, and chloroform was used as the extraction solvent. Internal standard was added to the reaction system after the degradation. Table 1 shows the results of the orthogonal experiments. As seen from the results, volume of hydrochloric acid with the maximum range of 6.82 is the most important factor with highest response to affect AGR. That corresponds to the acid-catalyzed principle for the degradation mentioned in the above section. The larger volume of hydrochloric acid provides greater amount of hydrion that is beneficial to accelerate reaction rate, whereas the excessive hydrochloric acid could induce impurities to decrease AGR. Thus, according to the results, 25 mL of hydrochloric acid was regarded as the optimal volume. Reaction temperature is another important factor affecting the degradation. Higher temperature has a faster reaction rate but induces more impurities, whereas lower temperature significantly reduces reaction rate. Thus, 40 °C was selected as the optimal temperature for the degradation. In addition, longer reaction time or higher

**Table 1.** Results of orthogonal experiments

| Experiment number | Factor | | | | AGR[a] (%) |
|---|---|---|---|---|---|
| | A (Ratio of PAC, mg/mL) | B (Time, h) | C (Volume of hydrochloric acid, mL) | D (Temperature, °C) | |
| 1 | 1 (10) | 1 (1.5) | 1 (15) | 1 (20) | 89.32 |
| 2 | 1 (10) | 2 (2.0) | 2 (25) | 2 (30) | 95.69 |
| 3 | 1 (10) | 3 (2.5) | 3 (35) | 3 (40) | 93.21 |
| 4 | 2 (30) | 1 (1.5) | 2 (25) | 3 (40) | 99.87 |
| 5 | 2 (30) | 2 (2.0) | 3 (35) | 1 (20) | 92.65 |
| 6 | 2 (30) | 3 (2.5) | 1 (15) | 2 (30) | 90.13 |
| 7 | 3 (50) | 1 (1.5) | 3 (35) | 2 (30) | 94.07 |
| 8 | 3 (50) | 2 (2.0) | 1 (15) | 3 (40) | 91.96 |
| 9 | 3 (50) | 3 (2.5) | 2 (25) | 1 (20) | 96.31 |
| K1 | 278.22 | 283.26 | 271.41 | 278.28 | |
| K2 | 282.65 | 280.30 | 291.87 | 279.89 | |
| K3 | 282.34 | 279.65 | 279.93 | 285.04 | |
| k1 | 92.74 | 94.42 | 90.47 | 92.76 | |
| k2 | 94.22 | 93.43 | 97.29 | 93.30 | |
| k3 | 94.11 | 93.22 | 93.31 | 95.01 | |
| Range | 1.48 | 1.20 | 6.82 | 2.25 | |
| The optimal conditions | A2 | B1 | C2 | D3 | |

[a]AGR is the average of three experiments.

**Table 2.** Results of recoveries and RSD for PAC spiked at various levels

| Actual concentration[a] ($n$ = 3, μg/mL) | Measured concentration[a] ($n$ = 3, μg/mL) | Recovery ($n$ = 3) (%) | RSD ($n$ = 3) (%) |
|---|---|---|---|
| 3.110 | 3.090 | 99.47 | 1.23 |
| 4.150 | 4.170 | 100.60 | 0.81 |
| 4.820 | 4.870 | 101.07 | 0.86 |

[a]Actual and measured concentrations were the concentrations of ADM-ONE.

**Table 3.** Results for quantification of drug loading of PAC

| Samples | Drug loading (w/w, %) | RSD ($n$ = 3) (%) |
|---|---|---|
| Bulk-1 | 25.47 | 1.12 |
| Bulk-2[a] | 12.71 | 1.20 |
| Bulk-3[a] | 8.55 | 1.08 |
| Bulk-4[a] | 6.40 | 0.89 |

[a]Bulk-1 was the PAC with drug loading of 25.5%; bulk-2, bulk-3, and bulk-4 were prepared with pectin: bulk-1 in 1:1, 2:1, and 3:1 weight ratios, respectively.

(lower) ratio of PAC never significantly improved the degradation efficiency apparently. Therefore, according to the results of Table 1, the optimal condition was A2B1C2D3 and the optimal degradation reaction condition was as follows: 30 mg of PAC dissolved in 25 mL of water was added to the mixture of 25 mL of hydrochloric acid (1.5 mol/L) and 50 mL of chloroform; the mixture was heated to 40 °C for 1.5 h; after fulfillment of the degradation, the mixture was extracted with chloroform triple (50 mL × 3). Under this condition, AGR reached 99.87%.

***Assay of Bulk Drugs.*** Four batches of PAC were degraded under the optimal conditions. The obtained ADM-ONE in the degradation was analyzed with the optimal working conditions and drug loading was calculated using the formula mentioned in the above section. The results for drug loading are listed in Table 3, indicating that the newly proposed method is reliable for determination of drug loading of PAC.

## Conclusions

For determination of drug loading of PAC, a newly proposed method with high efficiency and precision was established in this study. This method was conducted by precisely detecting content of ADM-ONE to extrapolate drug loading of PAC. The optimal degradation procedures were carried out in a water–chloroform reaction system that allowed ADM-ONE generated in the degradation to be transferred from water to chloroform with an extraction rate up to 99% in a short period of time, avoiding continuous degradation of ADM-ONE in acidic aqueous medium. Through a series of experiments, chloroform was selected as the extraction solvent and hydrochloric acid was used as the optimal catalyst. By performing the orthogonal experiments the optimal degradation condition was confirmed, in which volume of hydrochloric acid was verified as the most important factor in response to AGR. This method is efficient, selective, accurate, and repeatable. The results show that, compared to UV and HPLC described in PK1 or PK2, this modified method is expected to be more precise for measurement of drug loading of PAC.

**Acknowledgments.** This study was supported by National Major Scientific and Technological Special Project for "Significant New Drugs Development" during the Twelfth Five-year Plan Period (No. 2011ZX09102-001).

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