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Simultaneous high-performance liquid chromatographic determination of a rhein-diclofenac prodrug and its active compounds

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In order to study the hydrolytic characterization of an anti-inflammatory prodrug (RD-1) *in vitro*, a simple, specific, precise and accurate method for the simultaneous determination of prodrug and its two hydrolytic active compounds was development using reverse phase high-performance liquid chromatography (RP-HPLC). The chromatographic separation was performed on an ODS-2 C_{18} column (250 mm × 4.6 mm, 5.0 μm particle size) with a simple elution programme. The mobile phase was methanol-0.1%phosphoric acid solution (adjusted pH to 2.3) (80:20, v/v); wavelength of 257 nm and mobile phase flow rate of 1.0 mL/min was utilized for the quantitative analysis. Excellent linear behaviors over the investigated concentration ranges were observed with the values of R^2 higher than 0.999 for all the analytes. The validated method was successfully applied to the simultaneous determination of prodrug and its active components.

1. Introduction

The worldwide increase of human life span is accompanied by a heavy socioeconomic burden of widespread musculoskeletal diseases and their substantial morbidity and mortality (Wang et al. 2006). Chemotherapeutic prodrugs with capability to target certain tissues or cell types may enhance the potency or eliminate the side effect of drugs (Dharap et al. 2005; Kumpulainen et al. 2008; Rotem et al. 2008). Although many prodrug approaches have been demonstrated, osseous tissues are considered to be limited as target sites due to their biological properties. In contrast to other tissues, the blood flow rate in bones is very low.

Many bioactive compounds have been discovered from natural sources. Active lead compounds can also be further modified to enhance the biological profiles and developed as clinical trial candidates (Itokawa et al. 2008). Pharmacological research revealed that rhein has anti-inflammatory activity and it is how widely used in compound anti-inflammatory preparations (Tamuraa et al. 2002; Gonnot et al. 2007). But its unfavorable physical characteristic and side effects has limited its more extensive clinic use. Therefore diacerein, the prodrug of rhein, was developed to treat osteoarthritis. Diacerein can be completely metabolized by animals and humans to rhein, an active metabolite of diacerein, and reduces the production of superoxide anion in human neutrophils (Debord et al. 1994; Tamura et al. 2001). It is known that tetracycline possesses bone affinity (Perrin 1965), and can thus be used as carrier of bone-targeted drugs. Rhein is one of the anthraquinone components isolated from *Rheum palmatum L.*, and has a structure similar to tetracycline. We have confirmed the bone affinity of rhein by hydroxyapative (HAP) affinity tests *in vitro* (Wang et al. 2007; Rotem et al. 2008). Consequently we choose rhein as remolding target and potentially active drug, linked with diclofenac (a nonsteroidal anti-inflammatory used to treat osteoarthritis) by ester bond as bone-targeting anti-inflammatory prodrug (RD-1). Synthesis details and analysis data (such as NMR, MS, IR and elemental analysis) have already been published (Duan et al. 2009). Rhein and diclofenac were expected to be released *in vivo* and exert synergistic effects against inflammation. The hydrolytic activation *in vitro* and HAP binding capabilities would be detected firstly. So it is very important to establish an effective, accurate and reliable method for the determination of prodrug and its two hydrolytic active compounds.

In this study, a simple, accurate and reliable analytical method for the simultaneous determination of prodrug and its active hydrolytic components (their chemical structures were shown in Figure 3) was developed using RP-HPLC. The baseline separation of the target components was achieved within 20 min. As a result, the RP-HPLC method is particularly suitable for the routine analysis of the prodrug RD-1 and its active compounds.

2. Investigations, results and discussion

2.1. Chromatographic separation

Different mobile phases and wavelengths were tested to identify the investigated components. Considering the total resolution of the chromatographic separation, the running time and solvent/reagent consumption, methanol-phosphoric acid solution

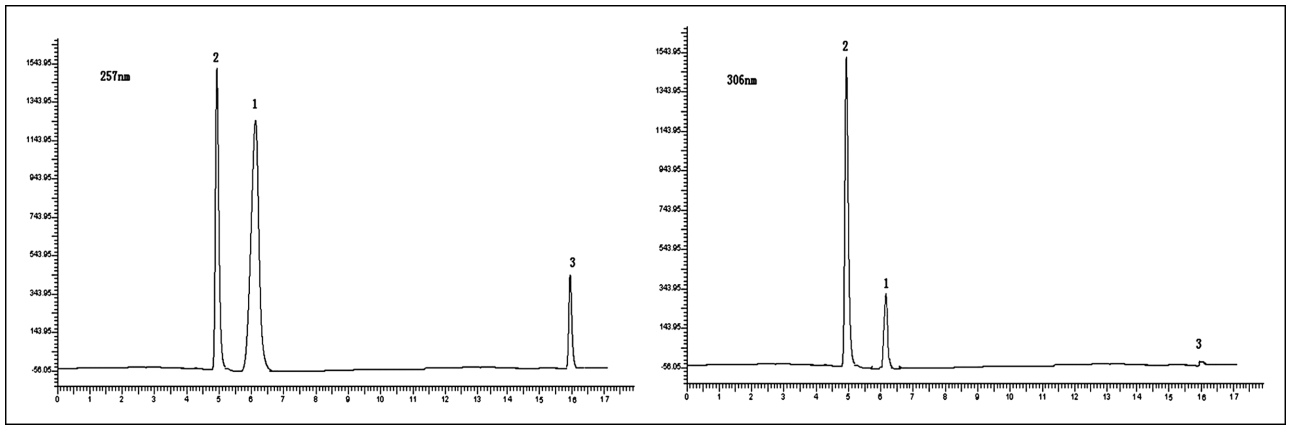

Fig. 1: The typical chromatographic profiles of the compounds at various detection wavelengths. (1, rhein; 2, diclofenac; 3, RD-1)

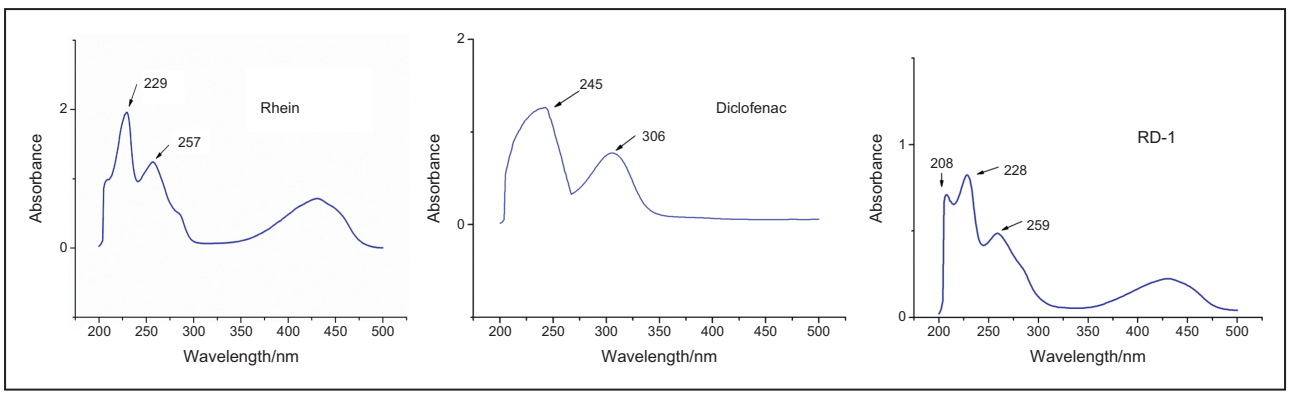

Fig. 2: The representative UV spectra of three compounds

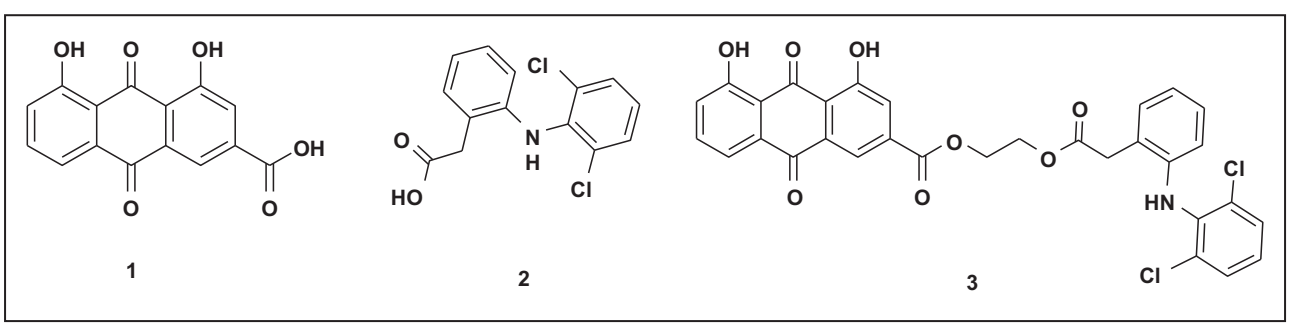

Fig. 3: Chemical structures of the three compounds (1, rhein; 2, diclofenac; 3, RD-1)

was chosen as mobile phase for the separation. The typical chromatographic profiles of the standard solution are shown in Fig. 1. Almost no interference was presented in the chromatographic separation, and each target peak had a good resolution. Because of the different UV characteristic of these three compounds investigated, two wavelengths (257 nm and 306 nm) were tested to evaluate the sensitivity and selectivity for the quantitative analysis and 257 nm was eventually chosen as detection wavelength. The chromatograms of the standard solution at two different detection wavelengths are shown in Fig. 1. The representative UV spectra of three compounds are shown in Fig. 2.

2.2. *Regression analysis on calibration curves*

Integrated chromatographic peak areas were plotted against the corresponding concentration of the injected standard solutions to obtain the calibration curves. The regression equations were established using eight concentration levels on the consecutive six days. The detailed descriptions of the regression curves are presented in Table 1. The good linearity (Coefficient of determination $R^2 > 0.999$) was achieved in the investigated ranges for all the analytes.

2.3. *Repeatability, precision and stability*

The mixture standard solutions were used for the test of repeatability by six continuous injections. The results of repeatability

Table 1: Regression analysis on calibration curves (n = 6)

Components	Regression equation (y = ax + b)	R^2	Linear range (mgL^{-1})
Rhein	y = 2568576.06x-35313.89	0.9999	3.1∼92.1
Diclofenac	y = 3782138.08x-44850.06	0.9998	3.2–96.1
RD-1	y = 1537694.47x-5404.05	0.9993	6.6–200.6

Table 2: Repeatability of the developed method (n = 6)

Components	Retention time (min)		Peak area	
	Average	R.S.D (%)	Average	R.S.D (%)
Rhein	6.13 ± 0.029	0.62	1735566.47 ± 34919.814	2.01
Diclofenac	4.91 ± 0.057	0.93	2454065.60 ± 32936.23	1.28
RD-1	16.08 ± 0.36	0.77	1537268.35 ± 30986.23	1.92

Table 3: Intra-assay and inter-assay precision of the developed method (n = 3)

Components	Retention time (min)		Peak area	
	Average	R.S.D (%)	Average	R.S.D (%)
Intra-assay				
Rhein	6.15 ± 0.033	0.77	1738866.61 ± 46465.96	2.67
Diclofenac	4,92 ± 0.064	1.06	2453960.06 ± 39622.45	1.52
RD-1	16.04 ± 0.72	2.03	1574729.27 ± 29929.46	1.90
Inter-assay				
Rhein	6.15 ± 0.043	0.97	1751428.18 ± 55306.40	3.16
Diclofenac	4.95 ± 0.094	1.69	2454283.73 ± 43857.28	1.96
RD-1	16.06 ± 0.75	2.95	1544566.62 ± 43133.11	2.79

of the solution are shown in Table 2, and all the R.S.D. values were lower than 2.0%.

The instrument precision was examined by performing the intra-day and inter-day assays by three replicate injections of the mixture standard solutions used above. The intra-assay precision was performed with the interval of 4 h within one day, while the inter-assay precision was performed over six days. The precision results for a solution at medium concentration is presented in Table 3, and it is shown that the R.S.D. values of retention times were lower than 3.0%, while the R.S.D. values of peak area were lower than 3.2%.

For the stability test, the same real sample was analyzed within 24 h at the room temperature, and the solution was found to be rather stable. The R.S.D. values of the retention time and peak area were both lower than 2.0%.

2.4. Recovery test

The recovery test for the standard from samples is generally used to evaluate the accuracy of the newly developed analytical method. The RD-1 we synthesized may be seen as crude drug, the analysis method we established above was not affected by praeparation, or method of extraction. Therefore, a recovery test was not performed.

3. Experimental

3.1. Chemicals and reagents

RD-1 was synthesized and purified. Rhein and diclofenac were refined from crude drugs. Their contents, which are detected by the area normalization method, are 99.4% and 99.7% respectively. Methanol (HPLC grade) was purchased from Hanbang Science&Technology (Jiangsu, China). Phosphoric acid was AR reagents, and was purchased from Sinopham Chemical Reagent Company (Shanghai, China). Other reagents were all of AR grade. Deionized water was used throughout the experiments.

3.2. Instrumentation and analytical conditions

The HPLC system PE 200 series (Perkin Elmer, U.S.A), equipped with the Totalchrom workstation software (Perkin Elmer) and comprised a binary pump, an online vacuum degasser, an autosampler, and a UV detector was used for the chromatographic analysis. All separations were carried out on an ODS-2 C_{18} column (250 mm × 4.6 mm, 5.0 μm particle size) from Hanbang Science&Technology (Jiangsu, China). The mobile phase was methanol-water-0.1%phosphoric acid solution(adjusted pH to 2.3) (80:20, v/v), the column temperature was maintained at 25 °C and wavelength was 257 nm. A constant mobile phase flow rate of 1.0 mL/min was employed throughout the analyses.

3.3. Standard solution preparation

The standard stock solutions of diclofenac (92.1 mg/l), rhein (96.1 mg/l) and RD-1 (200.6 mg/l) were prepared in methanol and stored away from light at 4 °C. Working solutions of the lower concentration were prepared by appropriate dilution of the stock solution. All solutions were filtered prior to analysis through a 0.45 μm syringe filter and determined by HPLC three times. The calibration curve for each compound was constructed by plotting the peak area as a function of the standard analyte concentration.

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