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Determination of anthraquinone prodrug and its hydrolytically active compounds using RP-HPLC

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Abstract: In order to study the hydrolytic characterization of an anti-inflammatory prodrug (RI-1) *in vitro*, an effective, accurate and reliable method for the simultaneous determination of the prodrug and its two hydrolytic active compounds is developed using reverse phase high-performance liquid chromatography (RP-HPLC). The chromatographic separation is performed on an ODS-2 C_{18} column (250 mm × 4.6 mm, 5.0 μm particle size) with a simple elution program. The mobile phase is V(methanol) : V(0.1% phosphoric acid solution) = 90∶10 (adjust pH to 2.3). A wavelength of 225 nm and a mobile phase flow rate of 1.0 mL/min are utilized for the quantitative analysis. Excellent linear behaviors over the investigated concentration ranges are observed with values of R^2 higher than 0.999 for all the analytes. The validated method is successfully applied to the simultaneous determination of the prodrug and its active components can be used to detect hydrolytic characterization *in vitro*.

Key words: high-performance liquid chromatography (HPLC); quantitative analysis; anthraquinone prodrug; active components

The worldwide prolongation of the human life span is accompanied by a heavy socioeconomic burden of widespread musculoskeletal diseases and their substantial morbidity and mortality[1]. Chemotherapeutic prodrugs with capabilities of targeting certain tissues or cell types may enhance the potency of drugs or eliminate their side-effects[2–4]. 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[5]. Pharmacological researches have revealed that rhein has anti-inflammatory effects and now it has been widely used in compound preparations as anti-inflammatory drugs[6–7]. But its unfavorable physical characteristics and side effects have limited its 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, which reduces the production of superoxide anions in human neutrophils[8–9]. As it is known that tetracycline possesses bone affinity[10], it can be used as a carrier of bone-targeting drugs. Rhein is one of the anthraquinone components isolated from *Rheum palmatum L*, and its structure is similar to tetracycline. We have confirmed the bone affinity of rhein by a hydroxyapative (HAP) affinity method *in vitro*[4,11]. Consequently, we choose rhein as a remolding target and a potentially active drug, linked with ibuprofen (a nonsteroidal anti-inflammatory used to treat osteoarthritis in clinics) by an ester bond as a bone-targeting anti-inflammatory prodrug. Rhein and ibuprofen are expected to be released *in vivo* and to exert synergistically effects on inflammation. The hydrolytic activation *in vitro* and the HAP binding capabilities will be detected first. So it is very important to establish an effective, accurate and reliable method for the determination of the prodrug and its two hydrolytically active compounds.

In this paper, a simple, accurate and reliable analytical method for the simultaneous determination of the prodrug and its active hydrolytic components (as shown in Fig. 1) is developed using RP-HPLC. The baseline separation of the target components is achieved within 15 min. As a result, the RP-HPLC method is particularly suitable for simultaneously analyzing the prodrug mentioned above and its hydrolytically active compounds.

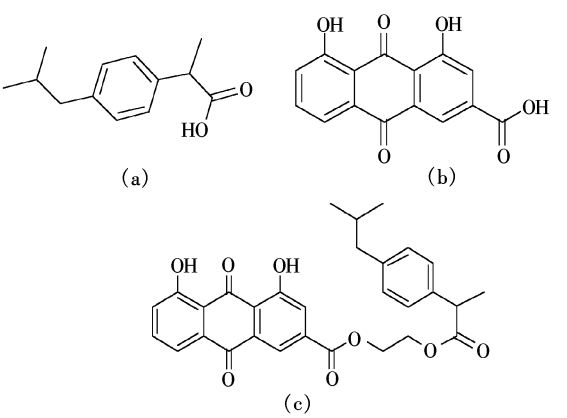

Fig. 1 Chemical structures of the three compounds. (a) Ibuprofen; (b) Rhein; (c) RI-1

1 Experimental

1.1 Chemicals and reagents

RI-1 is synthesized and purified. Rhein and ibuprofen are refined from crude drugs. Their contents, which are detected by the area normalization method, are 99.4% and 99.2%, respectively. Methanol (AR grade) is purchased from Hanbang Science and Technology (Jiangsu, China). Phosphoric acid is the analytical reagent, and is purchased from Sinopham Chemical Reagent Company (Shanghai, China). Other reagents are all of AR grade. Deionized water is used throughout the experiments.

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1.2 Instrumentation and analytical conditions

The HPLC system PE 200 series (Perkin Elmer, USA) equipped with the Totalchrom workstation software (Perkin Elmer) and comprised of a binary pump, an online vacuum degasser, an autosampler and a UV detector are used for the chromatographic analysis. All separations are carried out on an ODS-2 C_{18} column (250 mm × 4.6 mm, 5.0 μm particle size) from Hanbang Science and Technology (Jiangsu, China). The mobile phase is V(methanol) : V(0.1% phosphoric acid solution) = 90∶10 (adjust pH to 2.3). The column temperature is maintained at 25 ℃ and the wavelength is 225 nm. A constant mobile phase flow rate of 1.0 mL/min are employed throughout the analyses.

1.3 Standard solution preparation

The standard stock solutions of ibuprofen (20.1 mg/L), rhein (29.1 mg/L) and RI-1 (56.3 mg/L) are prepared in methanol and stored away from light at 4 ℃. Working solutions of the lower concentration are prepared by appropriate dilution of the stock solution. All solutions are filtered prior to analysis through a 0.45 μm syringe filter and determined by HPLC three times. The calibration curve for each compound is constructed by plotting the peak area as a function of the standard analyte concentration.

2 Results and Discussion

2.1 Chromatographic separation

Different mobile phases and wavelengths are tested to identify the investigated components. Considering the total resolution of the chromatographic separation, the running time and the solvent/reagent consumption, the mobile phase methanol-phosphoric acid solution is chosen for the separation. The typical chromatographic profiles of the standard solution are shown in Fig. 2. Almost no interference is present in the chromatographic separation, and each target peak has a good resolution. Because of the different UV characteristics of the three investigated compounds, the detections at two wavelengths (225 nm and 254 nm) are tested to evaluate the sensitivity and selectivity for the quantitative analysis and 225 nm is chosen as the detection wavelength. The chromatograms of the standard solution at three different detection wavelengths are shown in Fig. 2. The respective UV spectra of the three compounds are shown in Fig. 3.

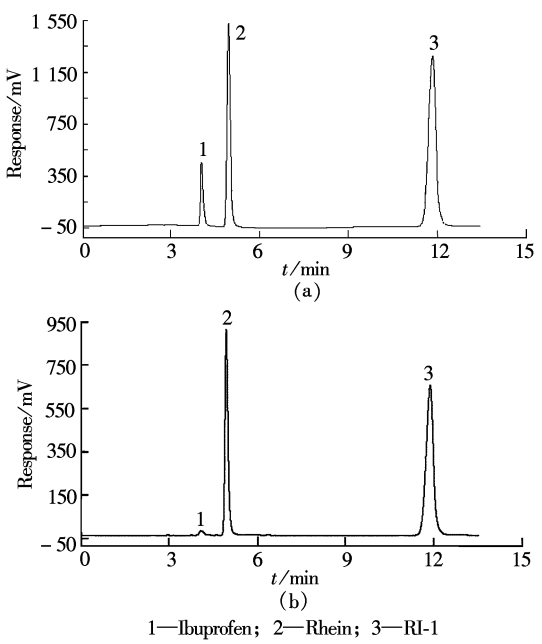

Fig. 2 The typical chromatographic profiles of the compounds at various detection wavelengths. (a) 225 nm; (b) 254 nm

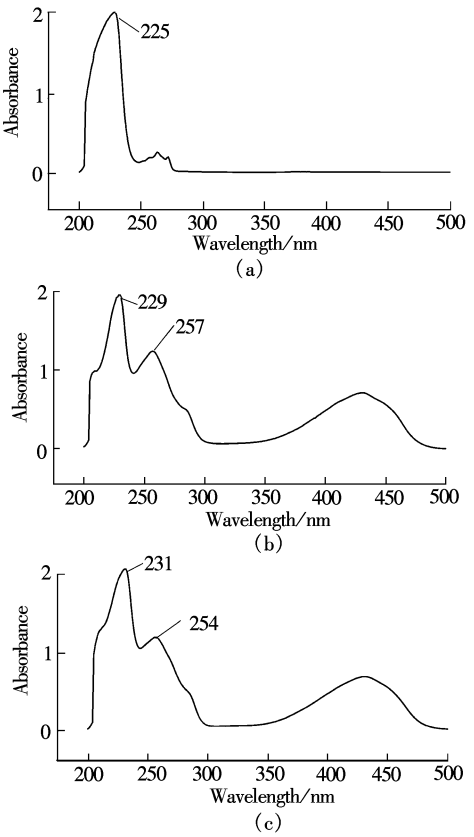

Fig. 3 Respective UV spectra of three compounds. (a) Ibuprofen; (b) Rhein; (c) RI-1

2.2 Regression analysis on calibration curves

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

Tab. 1 Results of regression analysis on calibration curves

Components	Regression equation ($y = ax + b$)	R^2	Linear range/(mg·L^{-1})
Ibuprofen	$y = 1\,649\,548x - 35\,313$	0.999 6	4.02 to 100.5
Rhein	$y = 3\,782\,138x - 44\,850$	0.999 8	2.91 to 145.5
RI-1	$y = 2\,192\,755x - 61\,244$	0.999 9	5.63 to 337.8

2.3 Repeatability, precision and stability

The mixture standard solutions are used for the test of repeatability by continuous injection six times. The results of the repeatability of the solutions are shown in Tab. 2, and all the R.S.D. (relative standard derivation) values are lower than 1.0%.

The instrument precision is examined through performing the intra-day and inter-day assays by three replicate injections of the standard mixture solutions used above. The intra-assay precision is performed with intervals of 4 h in one day, while the inter-assay precision is performed over a period of six days. The precision results of the solution at medium concentration are presented in Tab. 3. It is shown that the R. S. D. values of the retention times are lower than 1. 3%, while the R. S. D. values of the peak areas are lower than 4. 0%.

For the stability test, the same real sample is analyzed within 24 h at room temperature, and the solution is found to be rather stable. The R. S. D. values of the retention times and the peak areas are both lower than 2. 5%.

Tab. 2 Repeatability of the developed method($n = 6$)

Components	Retention		Peak area	
	Average/min	R. S. D. /%	Average	R. S. D. /%
Ibuprofen	4. 03 ± 0. 029	0. 73	454 065. 60 ± 2 936. 23	0. 65
Rhein	4. 94 ± 0. 050	1. 00	1 735 566. 47 ± 34 919. 814	2. 01
RI-1	11. 87 ± 0. 052	0. 44	1 178 016. 58 ± 16 059. 76	1. 36

Tab. 3 Intra-assay and inter-assay precision of the developed method($n = 3$)

Components		Retention time		Peak area	
		Average/min	R. S. D. /%	Average	R. S. D. /%
Intra-assay	Ibuprofen	4. 03 ± 0. 033	0. 81	453 960. 06 ± 3 622. 45	0. 80
	Rhein	4, 95 ± 0. 050	1. 00	1738 866. 61 ± 46 465. 96	2. 67
	RI-1	11. 85 ± 0. 050	0. 43	1160 214. 50 ± 28 505. 78	2. 46
Inter-assay	Ibuprofen	4. 05 ± 0. 043	1. 07	454 283. 73 ± 4 857. 28	1. 07
	Rhein	4. 94 ± 0. 064	1. 29	1 751 428. 18 ± 55 306. 40	3. 16
	RI-1	11. 86 ± 0. 080	0. 68	1 109 638. 67 ± 43 709. 41	3. 93

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 RI-1 we synthesize may be seen as a crude drug, and the analysis method we establish above is not affected by praeparatum and the method of extraction. Therefore, the recovery test is not performed.

3 Conclusion

An expected anti-inflammatory prodrug is synthesized and an accurate and reliable analytical method for the determination of the prodrug and its active drugs, rhein and ibuprofen is developed using RP-HPLC. High linearity, repeatability, intra-day and inter-day precision, accuracy and reliability are presented in the method validation procedure. The new compound is designed as a prodrug and is expected to release the active compounds under certain conditions. So the method we establish above is promising in the use of measuring prodrug hydrolytic experiments *in vitro*.

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RP-HPLC 法同时测定蒽醌类前药及其活性水解产物

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摘要:为了研究一个结构全新具有潜在抗炎活性蒽醌类前药的体外水解活性,利用反向高效液相色谱法(RP-HPLC)建立了专属而灵敏的同时测定抗炎前药及其活性水解产物的方法. 色谱条件如下:ODS-2 C_{18} 柱(250 mm ×4.6 mm,5.0 μm 填料),流动相 V(甲醇):V(0.1% 磷酸水溶液) =90∶10(调节 pH =2.3),检测波长 225 nm,流速 1.0 mL/min. 在此条件下,各化合物到达基线分离,3 个化合物的拟合标准曲线的 R^2 均大于 0.999. 该方法同时测定了前药及其活性代谢产物,可以用于前药体外水解的研究.

关键词:高效液相色谱法;定量分析;蒽醌类前药;活性化合物
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