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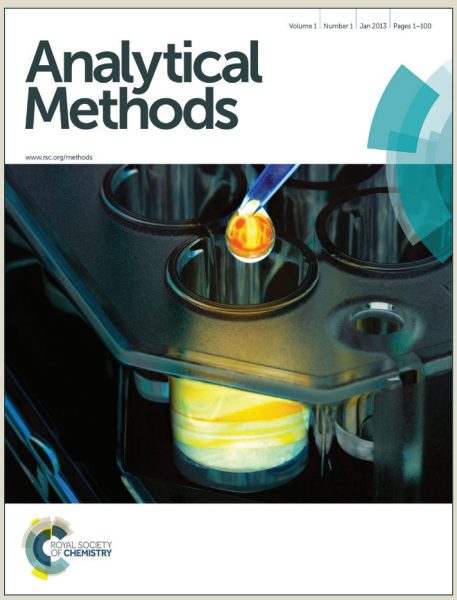

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Preparation, characterization and cytotoxicity activity of rhein argininate

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Abstract

Rhein is a natural anthraquinone, which can be isolated from the rhizomes of rhubarb, a traditional Chinese medicine herb showing antitumor activity. The poor aqueous solubility of rhein hampers its broader applications. Rhein argininate was prepared as a potential cytotoxicity formulation by co-grinding together 1:1 molar mixture of rhein and L-arginine for 8 hours. Rhein argininate was prepared by co-grinding together 1:1 molar mixture of rhein and L-arginine for 8 hours. Characterization of rhein argininate was achieved by nuclear magnetic resonance, mass spectrometry, X-ray powder diffraction, infrared spectroscopy, and differential scanning calorimetry techniques. HPLC method was used to study the solubilization of rhein argininate. The MTT method was used to assay the cytotoxicity activity of rhein argininate. The solubility of rhein argininate was enhances 8 folds as compared to rhein in water.. The cytotoxicity on cancer cell line activity of rhein argininate was 4 times that of rhein. The satisfactory water solubility and cytotoxicity on cancer cell line activity of rhein argininate will be potentially useful for its application as a new pharmaceutical formulation in cancer treatment in the future.

Keywords: Rhein argininate; preparation; characterization; cytotoxicity activity

Introduction

Over the past years, herbal medicines had been used worldwide to treat many conditions [1-3]. Plant origin drugs have served as the foundation for a large fraction of the current pharmacopoeia [4,5]. About half of the drugs in current clinical use are derived from natural sources [6].

Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is a natural anthraquinone, which can be isolated from the rhizomes of rhubarb, a traditional Chinese medicine herb showing antitumor activity. Recent researches have demonstrated that rhein can induce apoptosis of human cancer cells, including lung adenocarcinoma A549 [7], human promyelocytic leukemia cells (HL-60) [8], human colon adenocarcinoma cell line COLO 320 DM [9], human umbilical vein endothelial cells (HUVECs) [10], nasopharyngeal carcinoma-derived cell line NPC-039 [11], human tongue cancer SCC-4 [12], human breast cancer MCF-7 cells [13], human gastric cancer SGC-7901 cells [14], and human hepatocellular carcinoma BEL-7402 Cells [15]. The poor aqueous solubility of rhein hampers its broader applications. Therefore, further studies on rhein need to be done to improve its solubility properties. L-arginine has been found to be an effective enhancer to increase the solubility of drug substances [16-18].

The aim of the present study was to prepare and characterize rhein argininate, assay the solubilization and cytotoxicity activity of rhein argininate, and make some effort to shed light on the potential development of rhein in the treatment of cancer.

Materials and methods

Materials

Rhein raw material was purchased from the Shaanxi Sciphar Hi-tech Industry Co., Ltd., Xian, China. L-arginine was obtained from Zibo Qianhui Fine Chemical Co. Ltd., Shandong, China.

The reagents used to prepare mobile phases for HPLC analyses were all HPLC grades, and were obtained from Thermo Fisher Scientific (USA). All other chemicals and solvents were analytical grade.

The human lung adenocarcinoma A-549 cell line was obtained from the cancer institute & hospital Chinese Academy of Medical Science (Beijing, China).

Preparation of rhein argininate and physical mixture

Rhein argininate was prepared by co-grinding together 1:1 molar mixture of rhein and L-arginine for 8 hours. Rhein and L-arginine were separately pulverized in agate mortars for 3 min. The calculated amounts of both compounds were weighted out at a molar ratio of 1:1 and mixed together with a spatula until a homogeneous mixture was obtained.

Characterization of rhein argininate

Nuclear magnetic resonance (NMR)

The ^{1}H NMR and ^{13}C NMR were measured by Bruker AVIIHD 600MHz nuclear magnetic resonance instrument.

Mass spectrometry (MS)

The Mass spectrometry were measured by AB SCIEX QTOF mass spectrometry instrument (QSTAR Elite).

Powder X-ray diffraction (XRPD)

XRPD patterns were taken at ambient temperature and were obtained with a D/Max-2550 diffractometer with CuKα radiation (40 kV, 150 mA), at a scanning rate of 8° min $^{-1}$. Powder samples were mounted on a vitreous sample holder and scanned with a step size

of 2θ = 0.02 ° over the 3° - 80° 2θ range.

Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra was obtained by using a PerkinElmer Spectrμm 400 Nicolet Avatar 360 FTIR spectrometer with 4 cm^{-1} resolution and 16 scans between the wave number of 4000 cm^{-1} and 650 cm^{-1}. In order to avoid the polymorphic transformation by pressure, FTIR-ATR attenuated total reflection technology was employed.

Differential scanning calorimetry (DSC)

DSC analyses were carried out using a DSC1 system (Mettler-Toledo Inc, Switzerland). Samples (2 mg to 5 mg) were hermetically sealed in aluminum crucibles to prevent any mass loss of moisture. All sample measurements (in triplicate) were performed at a heating rate of 10 °C min^{-1} from 30 °C to 350 °C.

Solubilization test

An excess amount of rhein argininate was suspended in 20 mL of water (ca. pH 7.83), sheltered from light and stirred for 0 - 10 h at 25 ± 2 °C. The solution was then filtered on a 0.45 μm cellulose acetate membrane. After removing the insoluble substance by filtration, the analyte was checked as follows: HPLC analysis of the samples was conducted using an Agilent 1200 HPLC system (Agilent Technologies, Inc, USA) equipped with an Agilent Eclipse XDB-C18 (150 × 4.6 mm, 5 μm) column and a diode array UV detector. The injection volume was 10 μL. The oven temperature was 35 °C. The mobile phases were composed of acetonitrile and 0.1 % phosphoric acid, at a ratio of 42 to 58. The eluent was filtered using a 0.22 μm pore filters, with the flow rate was 1mL min^{-1}. The solubility was determined by the measurement at 254 nm.

Assay of cytotoxicity activity

The human lung adenocarcinoma A-549 cells were cultured at 37 °C under a humidified

5 % CO_2 and 95 % air atmosphere in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10 % fetal calf serum in 25 cm^2 tissue culture flasks, 1 % penicillin-streptomycin (100 Units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin) and 2 mM L-glutamine.

Cytotoxicity on cancer cell line were assessed using the MTT assay [19,20]. Briefly, the tetrazolium salt MTT solution was prepared freshly as 0.5 mg mL^{-1} in phosphate-buffered saline just before use. Cells were seeded in 96- well plates (6000 cells $well^{-1}$). Twenty-four hours later, the cells were treated with rhein and rhein argininate (0, 10, 20, 40, 80, or 160 µM) for 48 h. The positive control is 5-fluorouracil. Control groups were maintained with the same amount of DMSO. Then 20 uL of MTT dye (5 mg mL^{-1}) was added to each well. The plates were incubated in a CO_2 incubator for 4 h. After 4 h incubation period, the inhibition of cell growth induced by the tested compound was detected by eluting the dye with DMSO and optical density (OD) was reordered by using a 96 well micro plate reader (BIO-RAD, model 680, USA) at 492 nm. All assays were done in triplicate. The IC_{50} was defined as the drug concentration that resulted in a 50 % reduction in the number of cells compared to the untreated control.

Results and discussion

The ^{13}C-NMR (DMSO-d6) of Rhein arginate showed 15 resonances attributable to rhein 191.81, 181.74, 166.69, 162.30, 161.43, 161.29, 137.33, 133.67, 132.75, 124.26, 123.82, 120.11, 119.30, 116.17, 116.05 and 6 resonances attributable to arginine: 170.64, 53.29, 27.84, 24.87, 39.92, 156.95. The ^{1}H-NMR (DMSO-d6) of Rhein arginate showed: 7.69(s, Ar-H_2), 8.17(s, Ar-H_4), 7.75(d, Ar-H_5), 7.83(t, Ar-H_6), 7.39(d, Ar-H_7) for rhein, and 3.53(t, H-2'), 2.21(t, H-5'), 1.60(m, CH_2), 1.68(m, CH_2) for arginine.

The +TOF MS shows *m/z* 175.1([Margine+1], 100%), the –TOF MS shows *m/z* 283.2([Mrhein-1], 61%) and *m/z* 239.2([Mrhein-1-CO_2], 100%). This means the rhein argininate easy break as the fragment of rhein positive ions and argine anion.

The XRPD patterns of rhein, L-arginine, their physical mixture and rhein argininate are illustrated in Figure 1. As indicated in Fig. 1a and 1b, rhein an L-argine are all in crystalline form. The XRPD pattern of the physical mixture (Fig. 1c) confirmed the presence of both species as isolated solids, as the diffractogram showed both rhein peaks and the L-arginine peaks. Rhein argininate has an amorphous pattern (Fig. 1d), in which no diffraction peaks of rhein or L-arginine can be found from the diffractogram. This phenomenon verifies the formation of new phase between rhein and L-arginine.

Figure 1.

The FT-IR spectra of rhein, L-arginine, their physical mixture and rhein argininate are shown in Figure 2. The spectrum of rhein shows strong absorption bands in the range of 3180-2554, 1690–1570, 1488–1156 and 938–703 cm^{-1} (Fig. 2 (2a)). The IR spectra of L-arginine can be characterized by the intense band at 3358–3258 cm^{-1} corresponding to vibration of the hydrogen-bonded NH groups as well as the band at 3053–2862 cm^{-1} assigned to absorption by the CH_2 and CH groups (Fig. 2 (2b)). The spectra of the physical mixture corresponds simply to the superposition of the spectra of the individual components, and the spectra of the physical mixture and that of rhein are very similar (Fig. 2 (2c)). Due to the influence of L-arginine, the characteristic bands of rhein can not all exist in the spectra of rhein argininate, the spectra of the rhein argininate manifests an intensity modify and a certain shift, and the range from 3358 to 2864 cm^{-1} was shown as

a brand absorption peak (Fig. 2 (2d)). The most intense absorptions in the rhein argininate are located at 1666, 1624, 1548, 1370 and 1266 cm^{-1}, corresponding to the carbonyl stretching mode, aromatic C=C stretch vibrations and C-O stretching modes, respectively. All these phenomena jointly indicate that some of the existing hydrogen bonds formed between NH_2 groups of L-arginine are broken and new bonds are formed between rhein and L-arginine in rhein argininate.

Figure 2.

The DSC diagram of rhein, L-arginine, their physical mixture and rhein argininate are presented in Figure 3. The thermogram of the rhein illustrates a sharp endothermic peak at 329.6 °C (Fig. 3 (3a)). The thermogram of L-arginine shows two endothermic peak at 98.3 and 216.3 °C (Fig. 3 (3b)), There is a crystal water in the arginine. The peak 98.3 °C indicating dehydration process. In the DSC curves of the rhein and L-arginine physical mixture, two endothermic peaks at 97.5 and 216.3 °C (Fig. 3 (3c)) were found. Corresponding to the free L-arginine, the L-arginine was melting and decomposed when the temperature higher than 240 °C. By contrast, the research found that a very board endothermic band, between 40 and 140 °C, which gains a maximum at 74.3 °C (Fig. 3 (3d)) in the DSC curves of rhein argininate. The disappearance of the L-arginine fusion peak can be related the formation of rhein argininate.

Figure 3.

The solubilization of rhein argininate was tested by HPLC. The content of the rhein in the

rhein argininate was 61.8% (62.0%, theory value). The concentration profiles versus time of rhein, physical mixture and rhein argininate were shown in Figure 4. The assay exhibited good linearity over the concentrations ranging from 2.5 to 50.0 ug mL $^{-1}$. A linear equation of the curve was: Y = 33.267 x - 70.974 (r^2 = 0.9996, n = 6). The maximum concentration (C_{max}) of the rhein, physical mixture and rhein argininate were 5.2, 26.4, 42.0 ug mL^{-1}, respectively. The times to reach the maximum concentration (T_{max}) of rhein, the physical mixture and rhein argininate were 120, 600, 45 min, respectively. It is interesting to compare the solubilization of rhein, the physical mixture and rhein argininate. The T_{max} of rhein argininate was one third that of rhein, the C_{max} of rhein argininate (measured as rhein) was about 8 folds than that of rhein, which means the rhein argininate can obviously improve the water solubility of rhein. The physical mixture needs a long time to reach equilibrium, and the C_{max} was increased which means that L-arginine has the solubilization ability.

Figure 4.

The cytotoxicity on cancer cell line results indicate that the increase of the dose of rhein or rhein argininate leads to a significant decrease in the percentage of viable cells. The IC_{50} of rhein and rhein argininate were 73 uM mL^{-1} and 19 uM mL^{-1}, respectively, which means rhein argininate has the better cytotoxicity than rhein.

Conclusions

In this research, rhein argininate was prepared as potential cytotoxicity on cancer cell line formulation by co-grinding method. The NMR, MS, XRPD, FT-IR and DSC were used to

characterize the rhein argininate, all the analytical methods confirmed the formation of rhein argininate. Solubilization test results showed that rhein argininate could enhances the solubility of rhein by 8 folds as compared to that in water.

Determined by MTT experiment in vitro showed that rhein argininate has a significant inhibition to human lung adenocarcinoma A549 cells, following the increase of concentration, the cell inhibitory rate increased, and the cytotoxicity on cancer cell line is about 4 times than that of rhein.

In summary, this research demonstrated that rhein argininate is novel formulation of rhein and is expected to be effective and useful as a potential cytotoxicity formulation in cancer treatment in the future.

Acknowledgments

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Figure 1

XRPD pattern of rhein (1a), L-arginine (1b), rhein and L-arginine physical mixture (1c) and rhein argininate (1d).

Figure 2

FT-IR spectra of rhein (2a), L-arginine (2b), rhein and L-arginine physical mixture (2c) and rhein argininate (2d).

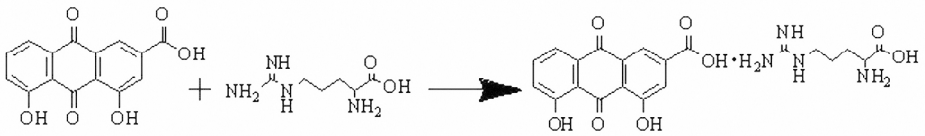
Figure 3

DSC curves of rhein (3a), L-arginine (3b), rhein and L-arginine physical mixture (3c) and rhein argininate (3d).

Figure 4

Concentration–time profile of rhein, physical mixture and rhein argininate determined by HPLC–DAD detector:

♦ rhein; ▲ rhein and L-arginine physical mixture; ■ rhein argininate .



	solubilization	antitumor activity
	C_{max}	IC_{50} (A549)
Rhein	5.2 ug / mL	73 uM / mL
Rhein argininate	42.0 ug / mL	19 uM / mL

237x126mm (300 x 300 DPI)

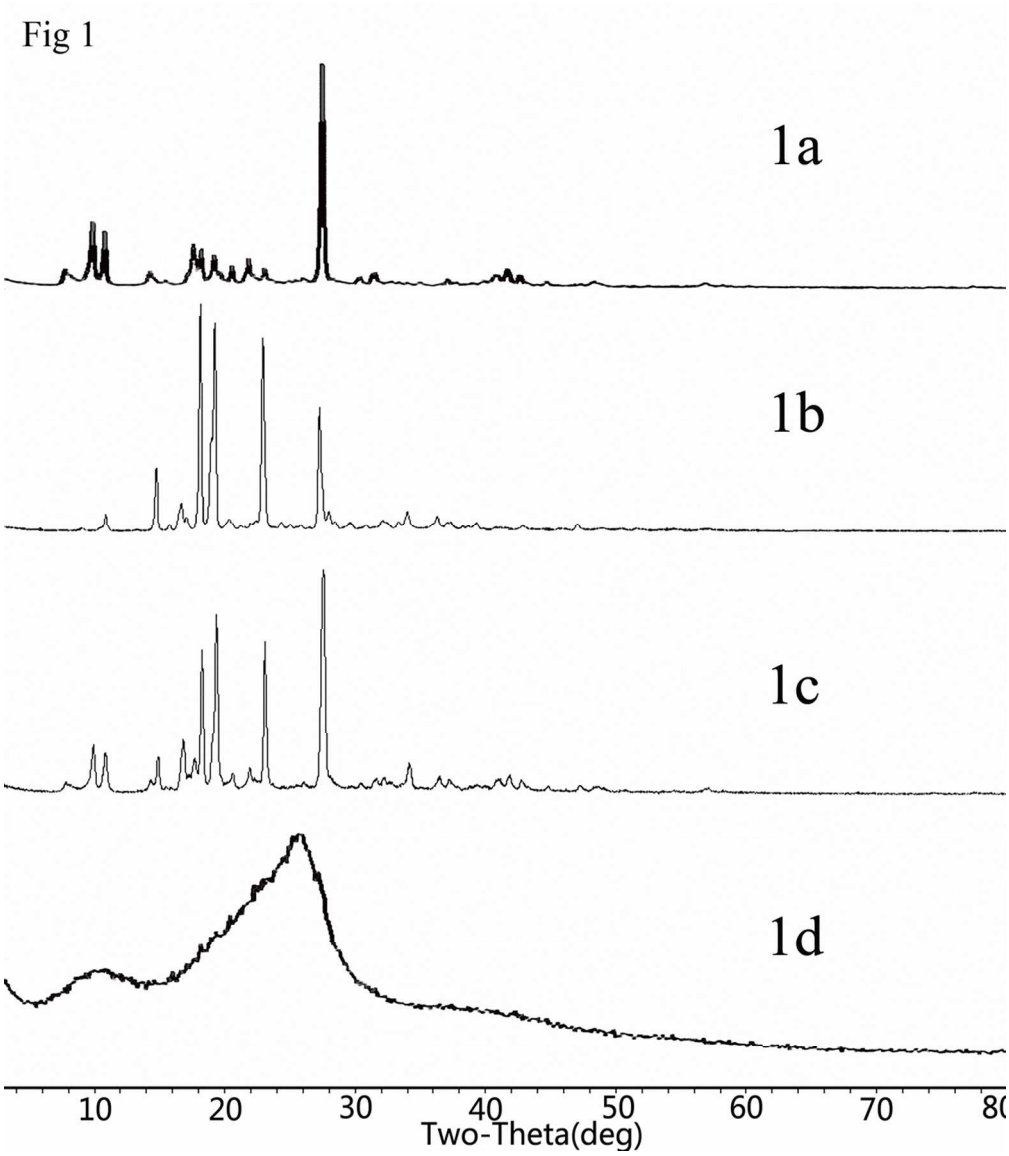


Fig 1
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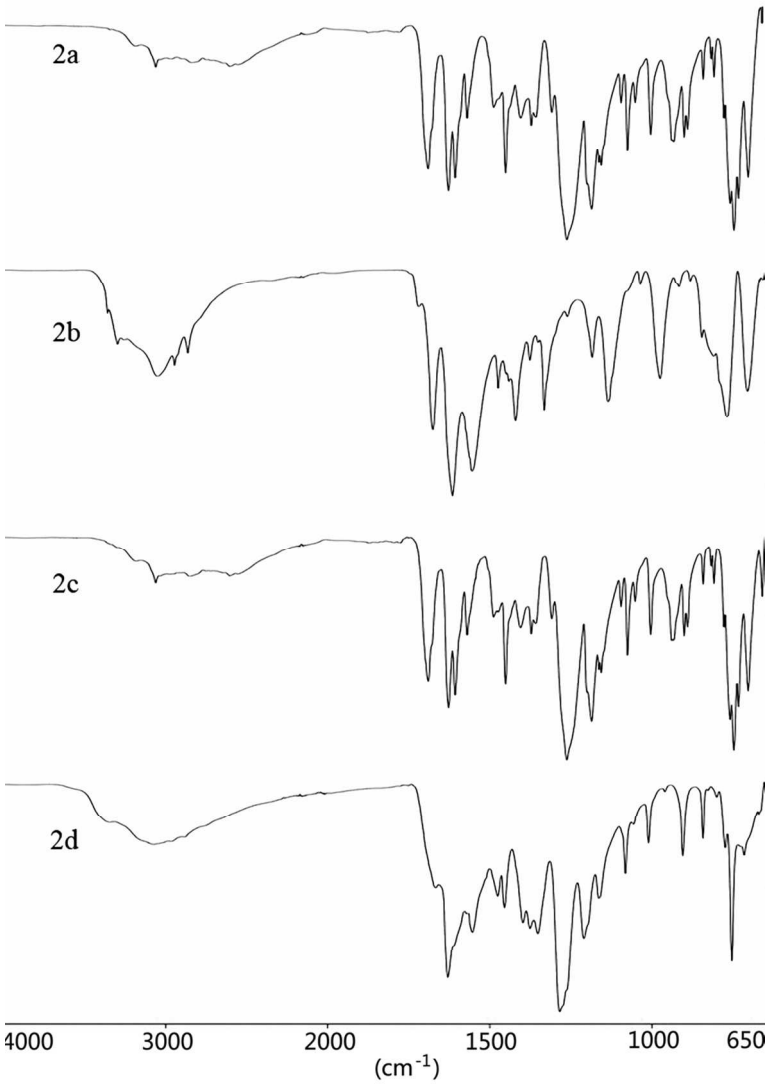
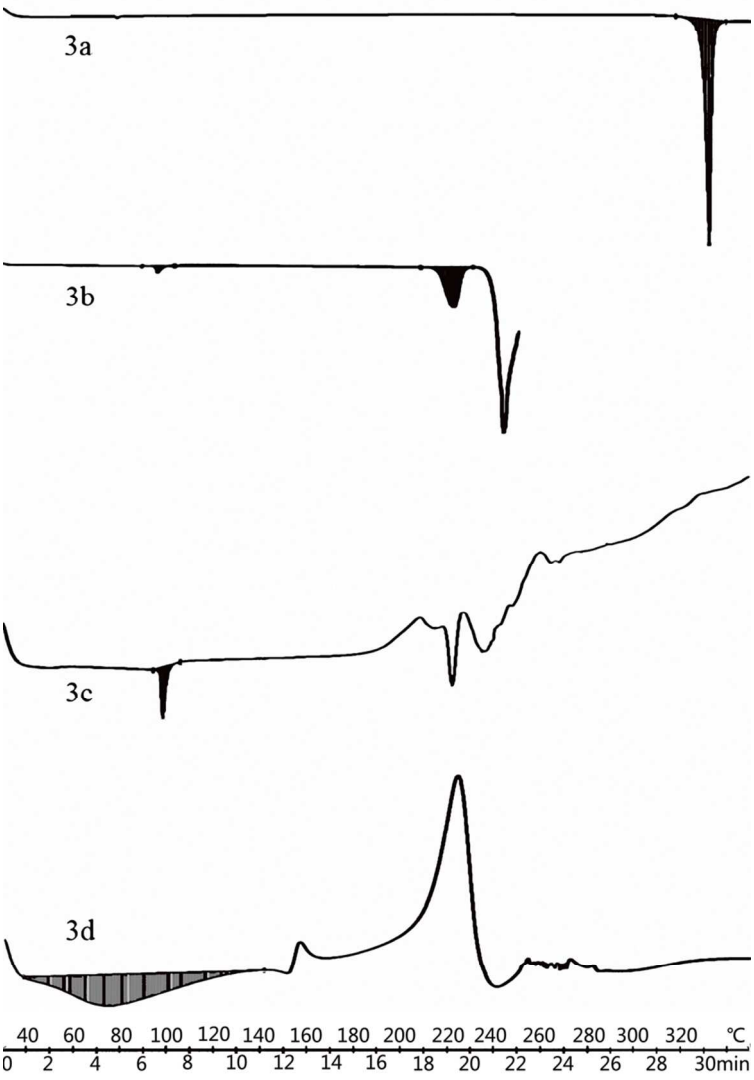
Fig 2



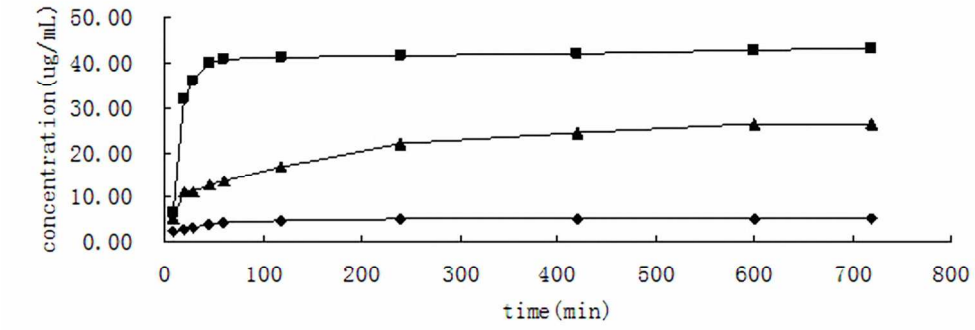
FT-IR spectra of rhein (2a), L-arginine (2b), rhein and L-arginine physical mixture (2c) and rhein argininate (2d).
84x133mm (300 x 300 DPI)

Fig 3



DSC curves of rhein (3a), L-arginine (3b), rhein and L-arginine physical mixture (3c) and rhein argininate (3d).
116x187mm (300 x 300 DPI)

Fig 4



Concentration–time profile of rhein, physical mixture and rhein argininate determined by HPLC–DAD detector
127x58mm (300 x 300 DPI)