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Simultaneous determination and pharmacokinetics of protein unbound aspirin and salicylic acid in rat blood and brain by microdialysis: An application to herbal–drug interaction

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ABSTRACT

Aspirin is commonly used for the prevention of myocardial infarction and ischemic stroke; whereas the Chinese people employ the bu-yang-huan-wu-tang (BYHWT) as a routine herbal formulation for the treatment and prevention of transient ischemic stroke. The current study develops a microdialysis technique coupled to a validated liquid chromatography system to measure free-form aspirin and salicylic acid for herbal–drug interaction in rat blood and brain. The intra- and inter-day precisions in biological dialysates were within 0.1–9.4% in the concentration ranges of 0.1–50 μg/mL and the accuracies ranged from −4.7 to 6.1%. The pharmacokinetic data demonstrate that the area under the concentration time curve (AUC) of the aspirin was 2031 ± 266 min μg/mL after aspirin administration (100 mg/kg, i.v.). The AUC of salicylic acid was 12660 ± 1799 min μg/mL, which suggests that aspirin is quickly hydrolyzed to salicylic acid in blood and the metabolite can also be detected within 15 min in brain dialysate. The herbal–drug pharmacokinetic interaction showed no significant effect in blood and brain. The results of pharmacodynamics for the bleeding time suggested that there were no significant differences between the aspirin alone group and the BYHWT pretreated group. However, the bleeding time has been prolonged when compared aspirin alone or the group pretreated with BYHWT to the blank control. The conclusion provides practical information for clinical practice for the herbal formulation BYHWT and aspirin used concurrently.

1. Introduction

Aspirin is used for the prevention of myocardial infarction and ischemic stroke because of its relative safety and low cost. However, a collaborative meta-analysis of randomized trials showed that aspirin was only 22% more effective for reducing ischemic vascular events than a placebo [1]. Moreover, a small-scale pilot study on Australian patients with acute ischemic stroke demonstrated that aspirin resistance tended to be more prevalent in patients at increased risk of cerebral ischemic events [2]. A similar study on Korean patients found similar results [3]. Therefore, the complementary and alternative medicine has been suggested as ways to improve the efficacy and diminish the side effects for the prevention of ischemic stroke [4].

Traditional Chinese medicine and other herbal medicines have been used for thousands of years in Asia and are now increasingly being popular with Western consumers [4]. However, the purity, efficacy, and safety of herbal products are still controversial. Since unanticipated herbal–drug interactions are still little understood, collecting such information is important, for the elder patients with high risk of adverse effects. Thus, we have designed the pharmacokinetic and pharmacodynamic studies to examine the interaction of popular herbal formulations with aspirin. The herbal formulation, bu-yang-huan-wu-tang (BYHWT), originally appeared in the traditional herbal text Yi-lin-gai-guo (Chinese meaning: corrections of errors among physicians), written in 1830, and has been used for the treatment of ischemic stroke. The herbal formulation comprises seven herbs: *Astragalus mongholicus* Bunge, *Angelica polymorpha* Maxim. var *sinensis Olive*, *Paeonia lactiflora* Pall, *Ligusticum chuanxiong* Hort.; *Ligusticum wallichii* Franch, Prunus Persica (L.) Batsch, *Carthamus tinctorius* L., and *Allolobophora calinosa* (Savigny) trapezoides (Ant. Duges).

Considerable evidence has implicated that administration of bu-yang-huan-wu-tang may reduce spinal ischemia/reperfusion damage. The neuroprotective effect may be mediated, in part,

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by an increase in the transcription of thioredoxin [5]. In addition, accumulating evidence has suggested that the ameliorative effects of bu-yang-huan-wu-tang on Coronary heart disease with Qi deficiency and blood stasis syndrome in rats are mediated by the improvement of hemorheological disorders and energy metabolism [6]. Moreover, it associated with the inhibition of C-reactive protein and CD40 and the regulation of endothelium-derived vasoactive factors [7]. A recent study has found that the bu-yang-huan-wu-tang is able to protect mice against stroke and extend lifespan through the down-regulation of genes involved in inflammation, apoptosis, angiogenesis and blood coagulation, and an up-regulation of genes mediating neurogenesis and nervous system development. The changes in expression after treatment with bu-yang-huan-wu-tang are beneficial after ischemic stroke [8].

Aspirin and salicylic acid in biological samples have been detected by several methods, including fluorescence spectroscopic detection [9], gas chromatography coupled with mass spectrometry [10], liquid chromatography hyphenated with electrochemical detection [11], ultraviolet detection [12–14], and tandem mass spectrometry [15,16]. However, these methods measure the total form of aspirin and salicylic acid, but not the protein-unbound form. Microdialysis is a useful sampling technique for measuring protein-unbound endogenous and exogenous substances in vivo. The protein-unbound drug levels reflect its biological activity and so this form is considered as the therapeutic portion [17,18].

The primary objective of this study is to develop an optimized microdialysis sampling and assay system to simultaneously monitor the protein-unbound forms of aspirin and salicylic acid in the rat blood and brain. The secondary objective is to examine the pharmacokinetic and pharmacodynamic interactions of the herbal formula BYHWT and aspirin in the peripheral circulation and central nervous system. To our knowledge, this is the first study to investigate the herbal–drug interaction between BYHWT and aspirin.

2. Experimental

2.1. Chemicals and reagents

Aspirin, salicylic acid, chloralose and urethane were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). The herbal formulation of the pharmaceutical product BYHWT was purchased from Sun Ten Pharmaceutical Co. LTD and was of high quality according to the GMP for Chinese Crude Drugs. It contained the following composition: *A. mongholicus* Bunge (120 g), *A. polymorpha* Maxim. var *sinensis Olive* (6 g), *P. lactiflora* Pall (6 g), *L. chuanxiong* Hort., *L. wallichii* Franch (3 g), Prunus Persica (L.) Batsch (3 g), *C. tinctorius* L. (3 g), and *A. calinosa* (Savigny) trapezoides (Ant. Duges) (3 g). The above herb extraction yield of the decoction was 25%. Each 10.5 g of BYHWT extract contained starch:concentrate herbal decoction (4.5:6.0, w/w). The herbal formulation of concentrated herbal extracts (Powdered) has been used medicinally for patients. Acetic acid, sodium citrate, dextrose, sodium chloride, potassium dihyhrogen phosphate (KH_2PO_4) and orthophosphoric acid (H_3PO_4, 85%) were acquired from E. Merck (Darmstadt, Germany). Acetonitrile of analytical grade was purchased from ECHO Chemical Co. (Taiwan). Deionized water from Millipore (Milford, MA, USA) was used for all aqueous solutions in this study.

2.2. Experimental animals

Male Sprague Dawley rats (230–270 g) were provided by the Laboratory Animal Center at National Yang-Ming University (Taipei, Taiwan) and housed in a cage with free access to food (Laboratory Rodent Diet 5001, PMI Feeds Inc., Richmond, IN, USA) and water were available ad libitum. The animals were received at 6–7 weeks of age and acclimated for at least one week. The rats were maintained on a 12 h light-dark cycle. All experimental animal surgery procedures were approved by the institutional animal experimentation committee of National Yang-Ming University.

2.3. Drug administration

BYHWT (100 mg/mL) was dissolved in 2% carboxymethyl cellulose. Although the dosing of BYHWT depends on the indications, the formulation of concentrated herbal extracts (Powdered) for clinical directions was 3.5 g/time 3–4 times daily in adults. According to the dose translation from animal to human studies [19], we used the body surface area normalization method to convert the dose for translation from humans to rat. In our study, the dose of BYHWT (100 mg/kg, p.o.) was appropriate for the treatment of ischemic stroke in rats.

The parallel study design was divided into the groups of aspirin alone (10, 30 or 100 mg/kg, i.v.) and the experimental group was pretreated with BYHWT (100 mg/kg, p.o.) for 5 consecutive days, and on the sixth day, aspirin (10, 30 or 100 mg/kg) was injected into the femoral vein.

2.4. Microdialysis experiments

The microdialysis system (Supplementary data Fig. A) was composed of a CMA/100 microinjection pump, a CMA/140 microfraction collector (CMA, Stockholm, Sweden) and microdialysis probes. The microdialysis probes for blood and brain sampling were home-made devices. The silica capillary had a concentric design and the active length of 10 mm and 3 mm for blood and brain, respectively [13]. The tip of the probe was covered with a dialysis membrane (molecular weight cut-off of 13,000 Da, Spectrum, Languna Hills, CA, USA) (Supplementary data Fig. B). After rats were anesthetized by the anesthetic mixture (1 mL/kg, i.p.) of urethane (1 g/mL) and chloralose (0.1 g/mL), a polyethylene tube (PE-50; Clay Adams, NJ, USA) was implanted in the femoral vein for drug administration. The blood microdialysis probe was positioned within the jugular vein toward the right atrium and then perfused with anti-coagulant citrate dextrose (ACD) solution consisting of citric acid 3.5 mM, sodium citrate 7.5 mM, and dextrose 13.6 mM. The brain microdialysis probe was implanted in the striatum zone and perfused with Ringer's solution (consisting of NaCl 8.6 g; KCl 0.3 g; $CaCl_2$ 0.33 g in 1000 mL H_2O; pH 7.0). The flow-rates of ACD and Ringer's solution were set at 2.0 μL/min by a microinjection pump for blood and brain microdialysis. The dialysates were collected every 15 min for 8 h and preserved at −20 °C refrigerator. The concentrations of aspirin and salicylic acid in the dialysates of blood and brain were determined by a validated HPLC-UV system.

2.5. HPLC instrumentation

HPLC-UV instrumentation was performed with a Shimadzu chromatographic pump (LC-20AT), a DGU-20A5 degasser, an autosampler (SIL-20AC) and a photo-diode array detector (SPD-M20A) (Shimadzu, Kyoto, Japan). The analytes were separated by reverse-phase C18 column (Merck, Purospher STAR, 250 mm × 4 mm i.d.; particle size 5 μm, Darmstadt, Germany). The UV absorbance wavelength for detection of aspirin and salicylic acid was set at 240 nm. The mobile phase for analysis of blood and brain microdialysates consisted of acetonitrile – 10 mM KH_2PO_4 (29:71, v/v, pH 2.5 adjusted by H_3PO_4). The mobile phase was filtered through a Millipore 0.22 μm filter (Bedford, MA, USA) and degassed by a sonicator (Branson, CT, USA) for 5 min prior to use, and the flow rate was set at 1 mL/min.

2.6. Method validation

The stock solution of aspirin and salicylic acid was prepared with 50% acetonitrile (v/v) at a concentration of 1 mg/mL and stored at −20 °C refrigerator. The stock solution was diluted with 50% acetonitrile (v/v) to prepare a series of working standard solutions. The calibration standards were prepared by mixing different concentrations of the working standard solutions (5 μL) into the blank blood or brain dialysates (45 μL). The spike samples were directly injected into HPLC-UV without prior sample purification. The external standard method was used for the calibration curves. The intra-day and inter-day variabilities for aspirin and salicylic acid were assayed (six replicates) at concentrations of 0.1, 0.5, 1, 5, 10 and 50 μg/mL on the same day and on six consecutive days, respectively. The calibration curves for brain dialysates, ranging from 0.1 to 10 μg/mL, were prepared using the same procedure. The method was validated to examine its selectivity, linearity, accuracy, precision, and lower limit of quantification (LLOQ). The selectivity was examined to determine whether aspirin and salicylic acid could be separated from the biological matrix. Linearity of the calibration curve was corroborated when the coefficient of determination (r^2) was greater than 0.995. The accuracy was estimated by the nominal concentration (C_{nom}) and the mean value of the observed concentrations (C_{obs}) as follows: accuracy (%) = (C_{obs}/C_{nom}) × 100. The precision, relative standard deviation (RSD), was calculated from the observed concentrations as follows: RSD (%) = (standard deviation (SD)/C_{obs}) × 100. Accuracy and precision values within ±15% were considered acceptable in the experimental concentration range. The LLOQ was defined as the lowest concentration of the calibration curve with precision less than 20% and accuracy within 80–120%.

To investigate the stability of aspirin and its metabolite in blood and brain dialysates, spiked samples with nominal concentrations of 0.1, 1, and 10 μg/mL were stored under different conditions which may be encountered during handling process and sample storage. Freeze–thaw stability was assessed over three freeze and thaw cycles. Short-term stability was determined by keeping the samples at room temperature for 6 h. Long-term stability was evaluated by analyzing samples kept at −20 °C for 14 days. Autosampler stability was determinate kept at the autosampler temperature (8 °C) for 12 h. All stabilities were calculated as the ratio of average concentration and freshly prepared samples (n = 3). The stability (%) was calculated as follows: stability (%) = [($C_{obs} - C_{nom}$)/(C_{nom})] × 100.

2.7. Recovery of microdialysis probes

A retrodialysis method was used to estimate in vivo recovery. Three different concentrations (0.1, 1 and 10 μg/mL) of aspirin were prepared in ACD and Ringer solution for blood and brain microdialysis probes, respectively. After a 2 h stabilization period, the ACD and Ringer's solution containing aspirin and salicylic acid were perfused through the microdialysis probes at a constant flow rate of 2.0 μL/min by the microinjection pump. The analytes of the perfusate (C_{perf}) and the collected dialysate (C_{dial}) were measured by HPLC-UV detection. The in vivo recovery (R_{dial}) of aspirin across the dialysis membrane was calculated as $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$.

2.8. Bleeding time method (BT)

Bleeding time was evaluated according to a previous report [20] and measured at 2 h after the intravenous injection of aspirin. The rats' tails were warmed for 5 min in warm water bath at 37 °C and then dried, after which a standard incision 5 mm long and 1 mm deep vertical to the skin was made with a sharp scalpel. This experiment was tested at different sites on the tail of 7, 8, 9, 10, or 11 cm from the tip. Bleeding time was measured from the first drop of blood and it was checked at 10 s interval until bleeding stopped. Bleeding time was the average of the five sites on each rat.

2.9. Pharmacokinetic data and statistical analysis

Concentrations of the analytes in the dialysate (C_m) were converted to protein-unbound concentrations (C_u) by the following equation: $C_u = C_m/R_{dial}$. Each individual set of data was calculated for pharmacokinetic parameters by the pharmacokinetic program, WinNonlin Standard Edition Version 1.0 (Scientific Consulting, Apex, NC, USA). Pharmacokinetic parameters of maximum

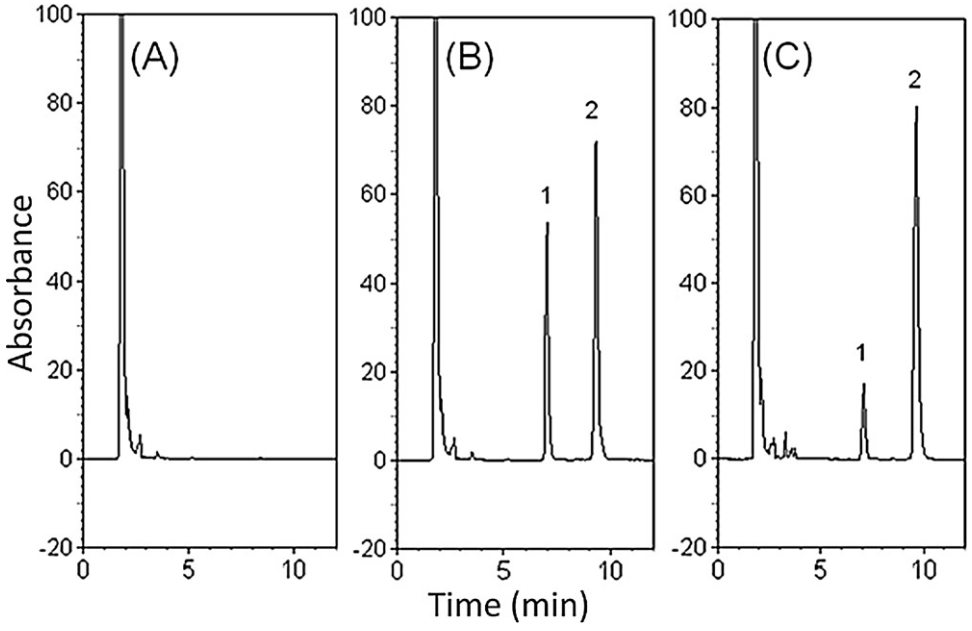

Fig. 1. Typical chromatograms of (A) blank blood dialysate; (B) blank blood dialysate spiked with ASA (20 μg/mL) and SA (20 μg/mL); (C) blood sample containing ASA (6.3 μg/mL) and SA (23 μg/mL) collected at 15–30 min after administration of aspirin (100 mg/kg, i.v.). 1: acetylsalicylic acid (ASA); 2: salicylic acid (SA).

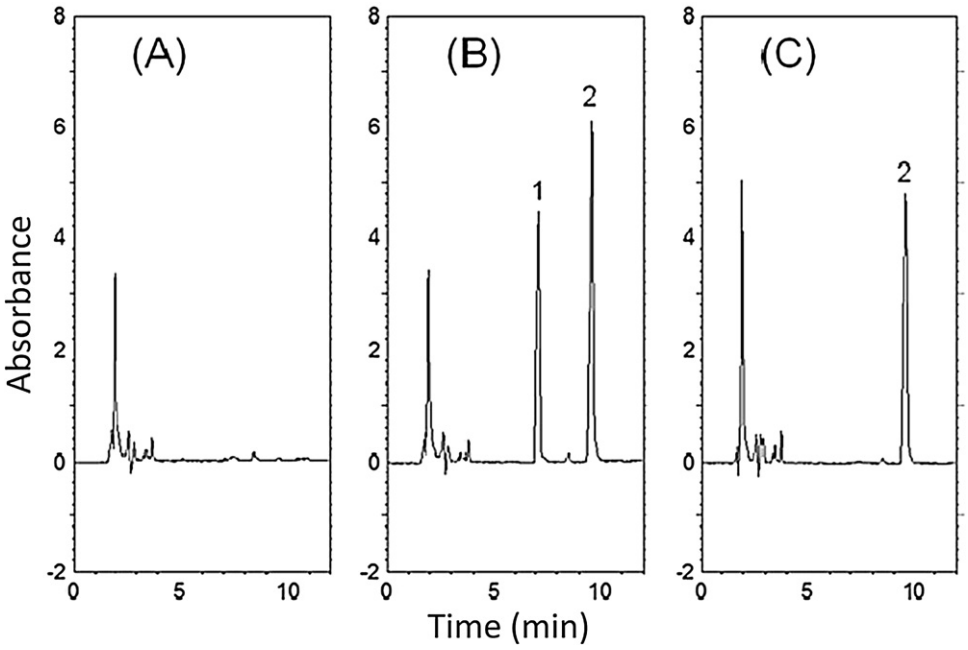

Fig. 2. Typical chromatograms of (A) blank brain dialysate; (B) blank brain dialysate spiked with ASA (1.5 μg/mL) and SA (1.5 μg/mL) and; (C) brain sample containing SA (1.3 μg/mL) collected at 105–120 min after administration of aspirin (100 mg/kg, i.v.). 1: acetylsalicylic acid (ASA); 2: salicylic acid (SA).

concentration of drug (C_{max}), elimination half-life ($t_{1/2}$), area under the concentration–time curve (AUC), clearance (Cl), time of occurrence for maximum (T_{max}), and apparent volume of distribution (V_d) were used in this study. All data are presented as mean ± standard error of mean (S.E.M.). Student's *t*-test with $\alpha < 0.05$ was used as the level of significance.

3. Results and discussion

3.1. Analytical consideration and HPLC optimization

The HPLC-UV method for separating analytes from the endogenous interferences was evaluated by analyzing the drug-free (blank) blood and brain dialysates. Since aspirin and salicylic acid are all hydrophilic compounds, microdialysis was an appropriate way to obtain the analytes from the endogenous matrix [21]. Both aspirin and salicylic acid have been reported to have appreciable blood–brain barrier penetrability. Thus for more complete analysis, multiple microdialysis probes were simultaneously inserted into single animal at multiple sites to monitor blood and brain levels in real time.

To optimize the separation of analytes from the dialysates of the acidic analytes, the mobile phases were adjusted to acidity with 10 mM KH_2PO_4/acetonitrile (71:29, v/v, pH 2.5 adjusted by orthophosphoric acid) for strengthening the analytes to bind with the stationary phase. However, in a basic mobile phase, the performance of peak separation became worse, broad tailing and overlapping analytes. Compared with previous reports [12–14], our method provide good sensitivity, selectivity and symmetry for the peaks. Typical chromatograms of aspirin and salicylic acid in rat blood and brain dialysates were shown in Figs. 1 and 2. The retention times of aspirin and salicylic acid were 7 and 9.8 min, respectively. None of the observed peaks interfered with the analytes within the retention times of the aspirin and salicylic acid. Fig. 1A showed the chromatogram of a blank blood dialysate. Fig. 1B showed the chromatogram of standard aspirin (20 μg/mL) and salicylic acid (20 μg/mL). Fig. 1C showed the real blood sample containing aspirin (6.3 μg/mL) and salicylic acid (23 μg/mL) collected at 15–30 min dialysate after aspirin administration (100 mg/kg, i.v.). Fig. 2A showed the chromatogram of a blank brain dialysate. Fig. 2B showed the chromatogram of standard aspirin (1.5 μg/mL) and salicylic acid (1.5 μg/mL). Fig. 2C showed the real brain dialysate containing only salicylic acid (1.3 μg/mL) collected at 105–120 min after aspirin administration (100 mg/kg, i.v.).

3.2. Method validation for linearity, precision, accuracy and stability

The coefficient of determination (r^2) for the calibration curve covering the linear range of 0.1–50 μg/mL is greater than 0.995. The lower limit of quantitation (LLOQ) was at the lowest concentration of aspirin and salicylic acid in blood and brain dialysate that was under the acceptable criteria; the accuracy and precision were within ±20%. The LLOQ of aspirin and salicylic acid was determined to 0.05 μg/mL. The precision and accuracy for inter-day and intra-day precision in blood and brain are presented in Tables 1 and 2. Accuracy and precision values were within ±15% covering the actual range, and the experimental concentrations were considered acceptable. These results suggest that the method was reliable and valid for the analysis of aspirin and salicylic acid in the dialysates of blood and brain for pharmacokinetic study.

The results of stability were summarized in Table 3. Aspirin and salicylic acid were generally stable under the storage and analytical process conditions used throughout the study, and the concentrations were maintained within 15% deviation of the initial values.

3.3. In vivo recovery of aspirin and salicylic acid from microdialysis probe

To estimate the in vivo recovery, a retro-dialysis calibration technique was utilized. The concentrations range of aspirin and salicylic acid were 0.1, 1 and 10 μg/mL with three individual experiments for each concentration. Average recoveries of aspirin and salicylic acid were 36.9 ± 0.5% and 45.4 ± 2.9% in blood, 14.2 ± 3.2% and 22.4 ± 2.19% in brain, respectively (Table 4). Three distinct

Table 1
Intra-day and inter-day precision and accuracy for the determination of aspirin and salicylic acid in blood microdialysis samples.

Nominal concentration (μg/mL)	Observed concentration (μg/mL)	Precision (%)	Accuracy (%)
Intra-day			
Aspirin			
0.05	0.05 ± 0.01	15.2	−11.6
0.1	0.10 ± 0.01	7.9	−2.5
0.5	0.52 ± 0.01	1.3	3.0
1	1.02 ± 0.01	0.6	1.8
5	4.94 ± 0.01	0.2	−1.3
10	10.0 ± 0.01	0.1	0.3
50	48.7 ± 1.69	3.5	−2.6
Salicylic acid			
0.05	0.05 ± 0.01	17.6	−14.7
0.1	0.10 ± 0.01	5.3	−1.1
0.5	0.51 ± 0.01	1.1	2.8
1	1.02 ± 0.01	0.7	2.0
5	4.95 ± 0.02	0.3	−1.1
10	10.0 ± 0.04	0.4	0.4
50	47.7 ± 0.84	1.8	−4.7
Inter-day			
Aspirin			
0.05	0.05 ± 0.01	16.8	−13.9
0.1	0.10 ± 0.01	9.1	−0.1
0.5	0.52 ± 0.01	1.2	3.2
1	1.01 ± 0.002	0.3	1.4
5	4.93 ± 0.01	0.2	−1.4
10	10.03 ± 0.01	0.1	0.3
50	49.79 ± 0.27	0.5	−0.4
Salicylic acid			
0.05	0.05 ± 0.01	18.1	−14.7
0.1	0.10 ± 0.004	3.2	1.9
0.5	0.52 ± 0.002	0.2	3.3
1	1.02 ± 0.01	0.5	1.5
5	4.94 ± 0.01	0.1	−1.3
10	10.03 ± 0.002	0.0	0.3
50	10.03 ± 0.004	0.4	−4.2

Data expressed as mean ± S.D. ($n = 6$)

Table 2
Intra-day and inter-day precision and accuracy for the determination of aspirin and salicylic acid in brain microdialysis samples.

Nominal concentration (μg/mL)	Observed concentration (μg/mL)	Precision (%)	Accuracy (%)
Intra-day			
Aspirin			
0.05	0.05 ± 0.01	16.4	−13.9
0.1	0.11 ± 0.002	4.6	5.1
0.5	0.49 ± 0.01	2.9	−1.1
1	0.99 ± 0.02	1.5	−0.8
5	5.01 ± 0.02	0.3	0.2
10	10.0 ± 0.01	0.1	0.0
50	49.6 ± 1.50	3.0	−0.8
Salicylic acid			
0.05	0.05 ± 0.01	16.4	−14.5
0.1	0.10 ± 0.01	8.2	−1.8
0.5	0.50 ± 0.01	2.4	0.2
1	1.01 ± 0.02	2.1	1.0
5	5.03 ± 0.04	0.8	0.7
10	9.98 ± 0.02	0.2	−0.2
50	48.1 ± 0.88	1.8	−3.8
Inter-day			
Aspirin			
0.05	0.05 ± 0.01	18.6	−15.9
0.1	0.11 ± 0.01	5.5	6.1
0.5	0.50 ± 0.02	4.4	−0.7
1	0.98 ± 0.02	1.6	−1.7
5	5.02 ± 0.02	0.3	0.3
10	9.99 ± 0.01	0.1	−0.1
50	50.37 ± 0.57	1.1	0.7
Salicylic acid			
0.05	0.05 ± 0.01	17.4	−13.1
0.1	0.10 ± 0.01	9.4	0.4
0.5	0.50 ± 0.02	3.7	0.3
1	1.00 ± 0.03	3.1	0.3
5	5.06 ± 0.05	1.0	1.1
10	9.97 ± 0.03	0.3	−0.3
50	48.2 ± 0.38	0.8	−3.6

Data expressed as mean ±S.D. ($n = 6$).

factors have to be considered during the process of microdialysis membrane permeation, including concentration of drug, oil/water partition coefficient of drug, and surface area of the dialysis membrane [17]. Estimation of the in vivo recovery in tissues by the dialysis probe is dependent upon the microdialysis membrane permeation. In our study, the length of blood membrane was longer than brain membrane (Section 2.4). Hence, the surface area of the dialysis membrane may affect the extraction efficiency or the term of recovery directly. The data demonstrate that the recovery for microdialysis probes in blood or brain showed no significant differences in the concentration ranges of 0.1, 1 and 10 μg/mL. This result suggested that the recovery for microdialysis was concentration-independent, which can be applied to pharmacokinetic analysis.

Table 3
Stability of aspirin and salicylic acid in the dialysate of blood and brain ($n = 3$).

Concentration (μg/mL)	Stability (%)			
	Freeze–thaw stability	Short-term stability	Long-term stability	Autosampler stability
Blood				
Aspirin				
0.1	−2.2 ± 8.0	4.6 ± 4.9	−6.3 ± 4.6	3.4 ± 5.6
1	−2.2 ± 8.9	−0.2 ± 1.2	−7.7 ± 8.3	−1.3 ± 0.7
10	1.1 ± 0.3	0.7 ± 0.1	−9.3 ± 1.7	0.3 ± 0.2
Salicylic acid				
0.1	2.9 ± 7.4	7.7 ± 5.6	−0.3 ± 3.6	9.7 ± 6.9
1	−3.5 ± 10.3	1.1 ± 2.8	−6.6 ± 4.1	3.7 ± 3.2
10	3.2 ± 0.3	4.9 ± 0.3	−7.3 ± 0.9	7.5 ± 0.4
Brain				
Aspirin				
0.1	2.0 ± 5.4	−3.4 ± 3.5	−4.3 ± 3.6	−4.1 ± 3.9
1	6.2 ± 2.3	−1.0 ± 1.7	−4.5 ± 2.2	−2.3 ± 2.6
10	0.1 ± 4.1	0.4 ± 0.4	−1.1 ± 5.5	−0.5 ± 0.4
Salicylic acid				
0.1	9.5 ± 5.7	2.8 ± 2.9	−3.9 ± 2.8	5.7 ± 4.1
1	10.6 ± 2.8	3.4 ± 1.9	−6.8 ± 12.4	5.9 ± 2.5
10	3.9 ± 4.5	4.0 ± 0.1	−1.4 ± 5.0	6.0 ± 0.2

Data are expressed as means ± S.D. ($n = 3$).

Table 4
In vivo recovery (%) of microdialysis probe on aspirin and salicylic acid in rat blood and brain.

Concentration (μg/mL)	Recovery (%)	
Blood	Aspirin	Salicylic acid
0.1	36.9 ± 2.4	45.3 ± 3.2
1	36.3 ± 1.3	45.6 ± 2.7
10	37.4 ± 3.8	45.3 ± 1.7
Average	36.9 ± 0.5	45.4 ± 2.9
Brain		
0.1	14.7 ± 2.8	20.9 ± 1.65
1	14.4 ± 2.6	21.4 ± 3.04
10	13.9 ± 1.7	24.9 ± 0.74
Average	14.2 ± 3.2	22.4 ± 2.19

Data are expressed as means ± S.D. ($n = 3$).

3.4. Pharmacokinetics of aspirin and salicylic acid in blood

Fig. 3A and B showed the blood concentration–time curves of aspirin and salicylic acid, respectively, after the doses of aspirin administration (30 and 100 mg/kg, i.v.) for the groups of aspirin alone and pretreated with BYHWT. With the highest dose of aspirin (100 mg/kg), the AUC of the aspirin was 2031 ± 266 min μg/mL, and $t_{1/2}$ was 13.0 ± 1.27 min. However, the AUC of salicylic acid was 12660 ± 1799 min μg/mL, and $t_{1/2}$ was 269 ± 115 min (Table 5). The data demonstrated that aspirin is quickly hydrolyzed to salicylic acid within 15 min of blood dialysate. The elimination half-life of aspirin (13.0 ± 1.27 min) was shorter than that of salicylic acid (269 ± 115 min), which is in agreement with a previous report [22].

For the group pretreated with BYHWT, the AUC of aspirin was 2243 ± 459 min μg/mL, and $t_{1/2}$ was 12.5 ± 0.21 min (Table 5). These pharmacokinetic data demonstrated that pretreated with BYHWT group only slightly prolong elimination half-life and did not statistically alter the pharmacokinetics of aspirin. This phenomenon also occurs in the metabolite. These results illustrated that aspirin and salicylic acid were not significantly affected when pretreated with BYHWT (100 mg/kg, p.o.) for 5 consecutive days.

3.5. Pharmacokinetics of aspirin and salicylic acid in the brain

Fig. 4 showed the brain concentration of salicylic acid after the groups of aspirin alone (30 and 100 mg/kg) and pretreated with BYHWT (100 mg/kg, p.o. for 5 consecutive days). Rapid distribution of aspirin into the brain was followed by fast elimination to undetectable within 1 hr. Thus, we did not show the pharmacokinetic curve of aspirin. At the lower dosage of aspirin administration (10 mg/kg), neither aspirin nor salicylic acid could be observed in the brain. The results suggested that aspirin level was too low to be detected, which may be due to the short elimination half-life [23]. Comparing the salicylic acid levels in blood and in brain after aspirin administration alone (100 mg/kg, i.v.), the AUC of salicylic acid in blood was 12660 ± 1799 min μg/mL, which was much higher than the AUC of salicylic acid in brain (874 ± 120 min μg/mL). It is suggested that the levels of aspirin and salicylic acid may attribute to the permeability of the blood–brain barrier [24]. The pharmacokinetic interaction data (Table 6) demonstrate that BYHWT (100 mg/kg, p.o. for 5 consecutive days) did not significantly influence the concentration of salicylic acid in the brain after aspirin administration (30 and 100 mg/kg, p.o.).

3.6. Bleeding time

The bleeding time was performed by the parallel three groups of blank control, aspirin alone (30 and 100 mg/kg, i.v.) and pretreated with BYHWT (100 mg/kg, p.o.) for 5 consecutive days and on the sixth day aspirin was administered (30 and 100 mg/kg, i.v.). At the highest dosage of aspirin alone (100 mg/kg), the bleeding

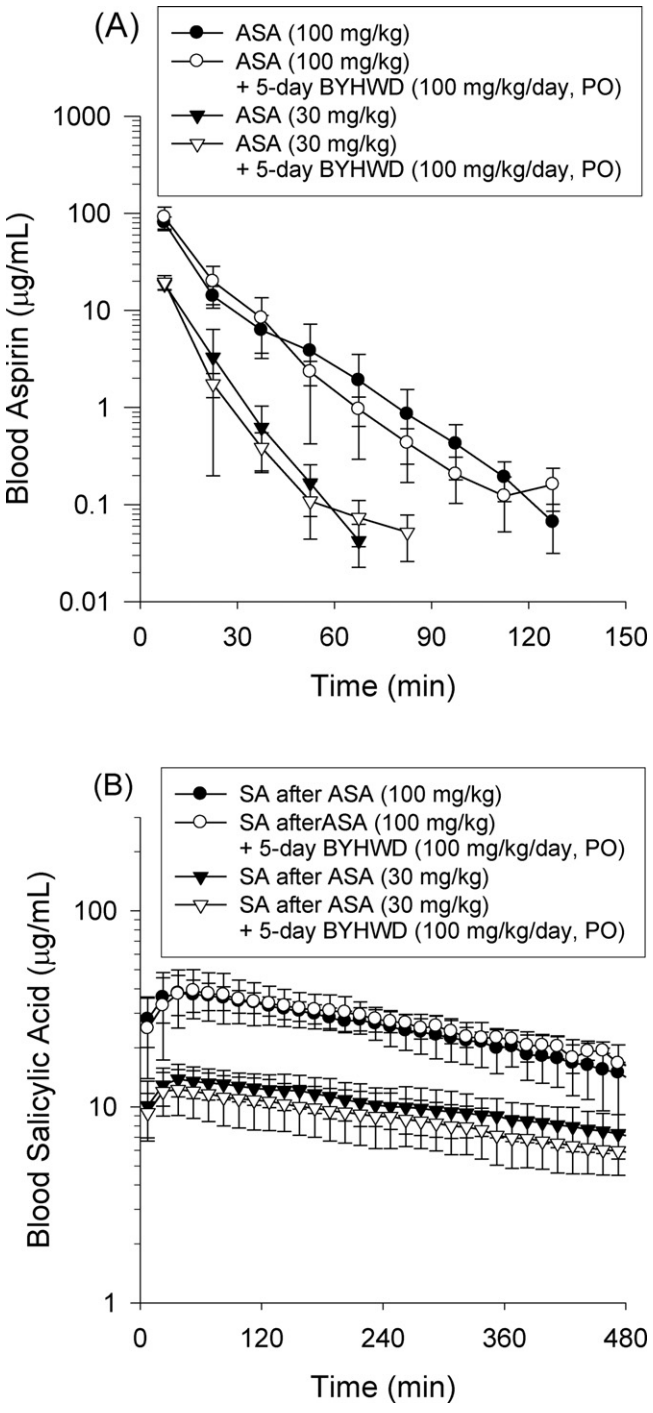

Fig. 3. Concentration–time curve of protein-unbound aspirin (A) and salicylic acid (B) in rat blood for the groups after aspirin administration 30 mg/kg (▼) and 100 mg/kg (●). The experimental groups were pretreated with bu-yang-huan-wu-tang (BYHWT, 100 mg/kg, p.o.) for 5 consecutive days and on the sixth day, aspirin (30 mg/kg) (▽) and 100 mg/kg (○) were injected into the femoral vein. ASA: acetylsalicylic acid; SA: salicylic acid.

times were 130 ± 11.7 and 136 ± 7.02 s for the group of aspirin alone and the group pretreated with BYHWT, respectively. At the medium dose of aspirin (30 mg/kg), the bleeding times were 127 ± 1.15 and 147 ± 17.0 s for the aspirin alone group and the group pretreated with BYHWT, respectively. Comparing the bleeding time, there was no significant difference between the groups of aspirin alone and pretreated with BYHWT. However, statistical increase was found when comparing blank control (99.5 ± 8.4 s) to aspirin alone or the group pretreated with BYHWT. These data demonstrated that the

Table 5
The pharmacokinetic parameters of aspirin and salicylic acid (SA) for the rat blood of the groups of aspirin alone (30 and 100 mg/kg, i.v.) and pretreated with bu-yang-huan-wu-tang (BYHWT, 100 mg/kg, p.o. for 5 consecutive days).

Parameter	Aspirin (100 mg/kg)	Aspirin (100 mg/kg) + BYHWT	Aspirin (30 mg/kg)	Aspirin (30 mg/kg) + BYHWT
Aspirin				
C_0 (μg/mL)	80.1 ± 8.14	91.1 ± 17.7	18.7 ± 1.63	19.5 ± 2.35
$t_{1/2}$ (min)	13.0 ± 1.27	12.5 ± 0.21	7.67 ± 0.41	10.8 ± 5.83
AUC (min μg/mL)	2031 ± 266	2243 ± 459	451.5 ± 15.8	499.3 ± 54.6
AUC/Dose	20.3 ± 2.66	22.4 ± 4.59	15.1 ± 0.53	16.64 ± 0.04
Cl (mL/min/kg)	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.002	0.06 ± 0.01
V_d (mL/kg)	0.53 ± 0.02	0.47 ± 0.06	0.47 ± 0.13	0.35 ± 0.01
Salicylic acid				
C_{max} (μg/mL)	38.2 ± 5.95	39.1 ± 7.79	13.8 ± 1.86	12.3 ± 2.28
$t_{1/2}$ (min)	269 ± 115	369 ± 65.4	337 ± 141	415 ± 68.6
AUC (min μg/mL)	12660 ± 1799	13343 ± 1886	5047 ± 796	4312 ± 862
AUC/Dose	127 ± 180	133 ± 18.9	168 ± 26.5	143 ± 28.7
Cl (mL/min/kg)	0.006 ± 0.002	0.005 ± 0.001	0.004 ± 0.001	0.04 ± 0.001
V_d (mL/kg)	2.24 ± 0.03	2.44 ± 0.52	1.85 ± 0.05	2.37 ± 0.48

Data expressed as mean ± S.E.M. (n = 6). C_{max}: the maximum plasma concentration, $t_{1/2}$: elimination half-life, AUC: area under the concentration–time curve, Cl: clearance, V_d: volume of distribution.

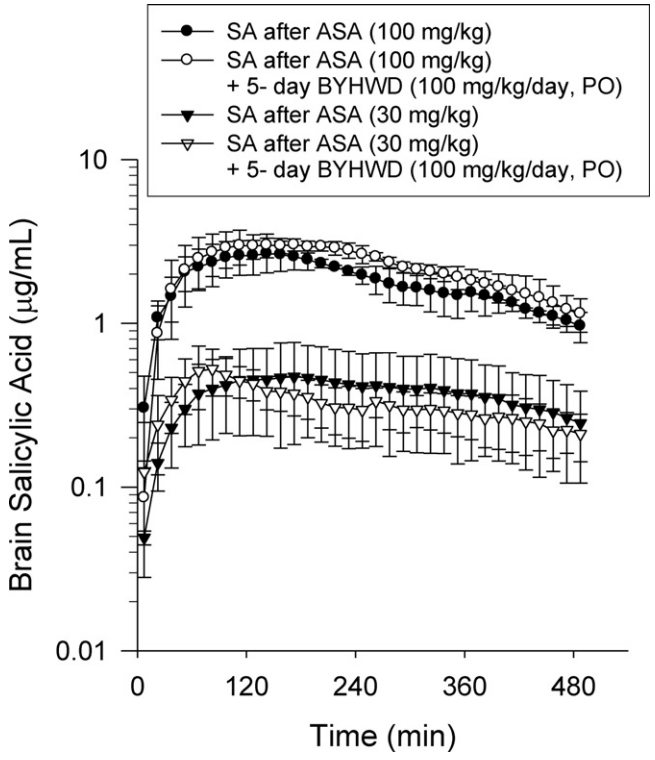

Fig. 4. Concentration–time curve of protein-unbound salicylic acid in rat brain for the groups after aspirin administration 30 mg/kg (▼) and 100 mg/kg (●). The experimental groups were pretreated with bu-yang-huan-wu-tang (BYHWT, 100 mg/kg, p.o.) for 5 consecutive days and on the sixth day, aspirin (30 mg/kg) (▽) and 100 mg/kg (○) were injected into the femoral vein. ASA: acetylsalicylic acid; SA: salicylic acid.

bleeding time was prolonged by the groups of aspirin alone and pretreated with BYHWT. The results reach agreement with the previous report that the bleeding time has been expanded after administration of aspirin [25].

3.7. Herbal–drug interaction

The interaction of herbal medicines and western medicines is an increasingly important issue, and the drugs with anti-coagulant or anti-platelet activities have frequently been implicated in herbal–drug interactions [18]. In a previous study, Ginkgo biloba in combination with aspirin increased the risk of spontaneous hyphema [26]. Similarly, *Tamarindus indica* L. fruit extract significantly increased the bioavailability of aspirin [27]. Our data demonstrated that the herbal formula of BYHWT (100 mg/kg, p.o. for 5 consecutive days) had no significant effect on the pharmacokinetics of aspirin (10, 30 and 100 mg/kg, i.v.) in blood and brain.

The traditional herbal literature from the Yi-lin-gai-guo of 1830, has been adjusted and confirmed by contemporary doctors of traditional medicine, based on trial and error. Each drug may have its own function in the formula for traditional Chinese medicine, and BYHWT contains 7 herbal medicines, which have been used for the treatment of circulation diseases of the peripheral and central nervous systems for an extended period of time without reports of serious drug–drug interaction.

In a previous clinical report, a combination therapy with extract of Astragalus extract and aspirin was found to be effective and safe for the treatment of acute cerebral infarction [28]. The current study, based on pharmacokinetic evaluation, indicates that BYHWT did not have direct effect with aspirin on the pharmacokinetics and pharmacodynamics of bleeding time. Although the results lack of significant herbal–drug interaction between the groups of aspirin and pretreated with BYHWT, the detailed pharmacological mechanism should be further investigated.

Table 6
The pharmacokinetic parameters of aspirin and salicylic acid (SA) for the rat brain of the groups of aspirin alone (30 and 100 mg/kg, i.v.) and pretreated with bu-yang-huan-wu-tang (BYHWT, 100 mg/kg, p.o. for 5 consecutive days).

Parameter	Aspirin (100 mg/kg)	Aspirin (100 mg/kg) + BYHWT	Aspirin (30 mg/kg)	Aspirin (30 mg/kg) + BYHWT
Salicylic acid				
C_{max} (μg/mL)	2.67 ± 0.45	3.11 ± 0.32	0.48 ± 0.2	0.53 ± 0.2
$t_{1/2}$ (min)	157 ± 45.2	211 ± 56.5	169 ± 44.7	265 ± 70.8
AUC (min μg/mL)	874 ± 120	1050 ± 132	178 ± 71.1	158 ± 36.4
AUC/Dose	8.74 ± 1.20	10.5 ± 1.3	5.93 ± 2.37	5.27 ± 1.21
Cl (mL/min/kg)	0.09 ± 0.02	0.07 ± 0.01	0.14 ± 0.05	0.12 ± 0.02
V_d (mL/kg)	29.7 ± 6.75	26.6 ± 9.79	50.8 ± 22.0	57.5 ± 16.9

Data expressed as mean ± S.E.M. (n = 6). C_{max}: the maximum plasma concentration, $t_{1/2}$: elimination half-life, AUC: area under the concentration–time curve, Cl: clearance, V_d: volume distribution.

4. Conclusion

Using an in vivo microdialysis technique in rats, this study reveals that the herbal formulation BYHWT has no significant effect on the pharmacokinetics of aspirin in blood and brain at the dosage regiment. A rapid and validated HPLC-UV detection for microdialysis has also been developed to simultaneously monitor protein-unbound form aspirin and salicylic acid in the blood and brain for pharmacokinetic studies. We hope that this study can contribute to the understanding of the herbal–drug interactions between Oriental herbal formation and common Western drug in clinical practice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.03.010.

References

[1] P.M. Ridker, N.R. Cook, I.M. Lee, D. Gordon, J.M. Gaziano, J.E. Manson, C.H. Hennekens, J.E. Buring, N. Engl. J. Med. 352 (2005) 1293.
[2] D. Bennett, B. Yan, L. Macgregor, D. Eccleston, S.M. Davis, J. Clin. Neurosci. 15 (2008) 1204.
[3] J.I. Seok, I.S. Joo, J.H. Yoon, Y.J. Choi, P.H. Lee, K. Huh, O.Y. Bang, Clin. Neurol. Neurosurg. 110 (2008) 110.
[4] A. Tachjian, V. Maria, A. Jahangir, J. Am. Coll. Cardiol. 55 (2010) 515.
[5] L. Wang, D.M. Jiang, J. Ethnopharmacol. 124 (2009) 219.
[6] W.R. Wang, R. Lin, H. Zhang, Q.Q. Lin, L.N. Yang, K.F. Zhang, F. Ren, J. Ethnopharmacol. 137 (2011) 214.
[7] H. Zhang, W.R. Wang, R. Lin, J.Y. Zhang, Q.L. Ji, Q.Q. Lin, L.N. Yang, J. Ethnopharmacol. 130 (2010) 98.
[8] H.W. Wang, K.T. Liou, Y.H. Wang, C.K. Lu, Y.L. Lin, I.J. Lee, S.T. Huang, Y.H. Tsai, Y.C. Cheng, H.J. Lin, Y.C. Shen, J. Ethnopharmacol. 138 (2011) 22.
[9] S. Torrado, R. Cadorniga, J. Pharm. Biomed. Anal. 12 (1994) 383.
[10] A. Battezzati, G. Fiorillo, A. Spadafranca, S. Bertoli, G. Testolin, Anal. Biochem. 354 (2006) 274.
[11] R.I. Catarino, M.B. Garcia, R.A. Lapa, J.L. Lima, E. Barrado, J. AOAC Int. 85 (2002) 1253.
[12] A. Chaudhary, J. Wang, S. Prabhu, Biomed. Chromatogr. 24 (2010) 919.
[13] R. Pirola, S.R. Bareggi, G. De Benedittis, J. Chromatogr. B: Biomed. Sci. Appl. 705 (1998) 309.
[14] E. Yamamoto, S. Takakuwa, T. Kato, N. Asakawa, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 846 (2007) 132.
[15] G.E. Kuehl, J. Bigler, J.D. Potter, J.W. Lampe, Drug Metab. Dispos. 34 (2006) 199.
[16] X. Xu, L. Koetzner, J. Boulet, H. Maselli, J. Beyenhof, G. Grover, Biomed. Chromatogr. 23 (2009) 973.
[17] T.H. Tsai, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 797 (2003) 161.
[18] C. Ulbricht, W. Chao, D. Costa, E. Rusie-Seamon, W. Weissner, J. Woods, Curr. Drug Metab. 9 (2008) 1063.
[19] S. Reagan-Shaw, M. Nihal, N. Ahmad, FASEB J. 22 (2008) 659.
[20] A. Garcia-Manzano, J. Gonzalez-Llaven, C. Lemini, C. Rubio-Poo, Proc. West Pharmacol. Soc. 44 (2001) 153.
[21] V. Di Matteo, M. Pierucci, G. Di Giovanni, A. Di Santo, A. Poggi, A. Benigno, E. Esposito, Brain Res. 1095 (2006) 167.
[22] I.H. Benedek, A.S. Joshi, H.J. Pieniaszek, S.Y. King, D.M. Kornhauser, J. Clin. Pharmacol. 35 (1995) 1181.
[23] M.G. Wientjes, G. Levy, J. Pharmacol. Exp. Ther. 245 (1988) 809.
[24] B. Bannwarth, F. Demotes-Mainard, T. Schaeverbeke, L. Labat, J. Dehais, Fundam. Clin. Pharmacol. 9 (1995) 1.
[25] C.H. Mielke Jr., M.M. Kaneshiro, I.A. Maher, J.M. Weiner, S.I. Rapaport, Blood 34 (1969) 204.
[26] M. Rosenblatt, J. Mindel, N. Engl. J. Med. 336 (1997) 1108.
[27] A. Mustapha, I.A. Yakasai, I.A. Aguye, Eur. J. Drug Metab. Pharmacokinet. 21 (1996) 223.
[28] H. Wu, J. Deng, Chi. Pharm. 19 (2009) 50.