—Regular Articles—

Sensitive Liquid Chromatographic Assay for the Simultaneous Determination of Ibuprofen and Its Prodrug, Ibuprofen Eugenol Ester, in Rat Plasma

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A rapid, sensitive, and specific reverse-phase high-performance liquid chromatography (HPLC) method was developed to quantitate ibuprofen (IBU) and its prodrug, ibuprofen eugenol ester (IEE), simultaneously in rat plasma. IBU, IEE, and the internal standard glycyrrhetic acid (GA) were detected by UV absorption at 230 nm. Extraction recoveries for all compounds ranged between 82.6% and 96.2%. Retention times of IBU, IEE, and GA were 5.62, 15.98, and 18.05 min, respectively. Calibration plots were linear over the range of 0.64 to 64 μg/ml for IBU, and 0.16 to 80 μg/ml for IEE. The limit of quantitation was 0.64 μg/ml for IBU and 0.16 μg/ml for IEE. The intra- and interday variations were less than 10% and accuracy was greater than 90%. The results showed that the established method is reproducible and sensitive and applicable to plasma samples collected from rats administered IBU and IEE intravenously.

Key words——Ibuprofen; Ibuprofen eugenol ester; prodrug; determination

INTRODUCTION

Ibuprofen is a well-known nonsteroidal antiinflammatory drug (NSAID) and has been widely used to treat inflammatory diseases.[1] However, the main disadvantages of ibuprofen, and of other NSAIDs of similar structure, are a relatively short plasma half-life, resulting in short activity duration, and pronounced upper gastrointestinal (GI) irritation and bleeding.[2,3] To overcome these disadvantages, many derivatives and related compounds have been synthesized. The use of prodrugs to mask temporarily mask the acidic group of NSAIDs has been postulated as an approach to decrease their GI toxicity. In addition, the biotransformation of the prodrugs to the parent compounds at target sites or sites of activity may be used to achieve rate- and time- controlled drug delivery of the active entities.[4—6] To obtain an ibuprofen prodrug with reduced GI toxicity, we synthesized ibuprofen eugenol ester (IEE). A preliminary pharmacologic study of IEE indicated that it retained the antiinflammatory and analgesic activity of the parent drug and reduced gastrointestinal irritation. Further pharmacologic and pharmacodynamic studies are ongoing. The chemical structures of IBU and IEE are depicted in Fig. 1.

To study the preclinical *in vivo* distribution and biotransformation of the prodrug, a method for simultaneous analysis of the prodrug and the parent compound is essential. Although several methods of

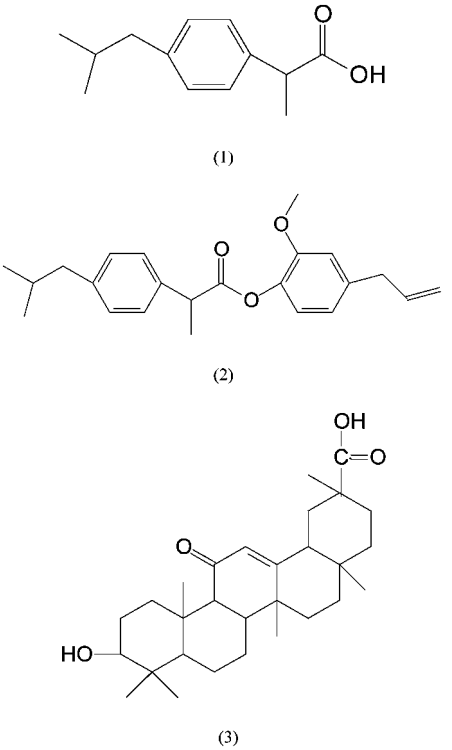

Fig. 1. Molecular Structures of IBU (1), IEE (2), and GA (3)

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high-performance liquid chromatographic (HPLC) analysis have been reported for IBU,[7—9] there are few HPLC methods to detect IBU and its prodrug simultaneously. The objective of this study was to develop a single HPLC analytical method for the determination of IBU and its prodrug IEE in rat plasma. This new method was applied to plasma samples collected from rats that were administered IBU and IEE intravenously.

EXPERIMENTAL

1. Chemicals and Reagents IBU was kindly provided by Xinhua Pharmaceutical Co. (Shandong, China). IEE (purity >99%, HPLC) was synthesized in Shenyang Pharmaceutical University and its structure was confirmed with IR, MS, and ^{1}H–NMR. Glycyrretic acid (GA) was ordered from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile, tetrahydrofuran, and methanol (HPLC grade) were obtained from Concord Tech. Co. (Tianjin, China). Trifluoroacetic acid was ordered from Beijing University (Beijing, China). All other reagents were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

2. Standard Solutions Six standard solutions containing binary mixtures of IBU and IEE were prepared in methanol. Concentrations of IBU and IEE were 2.56, 5.12, 25.6, 51.2, 128, and 256 μg/ml and 0.64, 3.2, 6.4, 32, 64, and 320 μg/ml, respectively. A solution of GA (40 μg/ml) was also prepared in methanol. Aliquots of the standard solutions (50 μl) were added to samples of rat plasma (200 μl) to provide six calibration standards containing the IBU and IEE concentrations in plasma of 0.64, 1.28, 2.56, 12.8, 32, and 64 μg/ml and 0.16, 0.8, 1.6, 8, 16, and 80 μg/ml, respectively. Low, medium, and high quality control (QC) samples were similarly prepared containing IBU and IEE concentrations in plasma of 0.64, 6.4, and 64 μg/ml and 0.8, 8.0, and 80 μg/ml, respectively. All the solutions were stored at 4°C and were warmed to room temperature before use.

3. Extraction Procedure Volumes of 50 μl of internal standard solution were added to 1.5-ml polypropylene centrifuge tubes, and the solutions were dried in a stream of nitrogen. To this, 200 μl of plasma standards, unknown samples, or QC samples were added and vortexed. Ice-cold methanol (1 ml) was added to precipitate plasma proteins. The tubes were vigorously mixed for 30 s and centrifuged for 10 min at 9000 g. The supernatant was transferred to a clean polypropylene centrifuge tube and evaporated to dryness under a stream of nitrogen gas at ambient temperature. The extraction residue was reconstituted with 100 μl of methanol by vortexing and a 20-μl volume was injected for analysis.

4. Chromatographic Conditions The HPLC system consisted of a Shimadzu LC-10AD pump (Kyoto, Japan), a Rhenodyne model 7725i injector with a 20-μl loop (Rhenodyne Inc., CA, USA), an SPD-10A ultraviolet (UV)-visible detector set at 230 nm, and a LC workstation for data collection. Samples were analyzed on a Lichrospher C18 column (200 mm×4.6 mm i.d., 5-μm particle size, Hanbon Science & Technology Co. Litd., Jiangsu, China), protected by a guard column (4 mm×3.0 mm i.d.) of the same material. The mobile phase consisted of acetonitrile-methanol-0.2% trifluoroacetic acid-tetrahydrofuran (50 : 10 : 18 : 2, v/v/v/v) and was filtered through a 0.45-μm filter and degassed before use. HPLC was run at the flow rate of 1.0 ml/min.

5. Assay Specifications

5-1 Selectivity HPLC peaks of IBU, IEE, and GA were identified on the basis of their retention times and UV spectra obtained using a stop-flow ''scan spectra''. Plasma samples from 6 drug-free rats were tested for the presence of endogenous compounds coeluting with IBU, IEE, and GA.

5-2 Recovery, Stability, and Limit of Quantitation Recovery rates of IBU and IEE were determined by comparison of peak heights obtained after injection of extracted QC samples and corresponding standard solutions (n=6) at the same concentration. The stability of IBU and IEE in the mobile phase was investigated by comparing duplicate low, medium, and high QC samples stored for 12 h at room temperature with freshly prepared QC samples. The limit of quantitation of IBU and IEE was calculated as the minimum concentration that could be quantified with no more than 15% relative standard deviation [R.S.D. (%)].

5-3 Linearity Calibration curve data were generated by spiking a series of plasma standards with 50 μl of the internal standard working solution, after which the extraction procedure and HPLC analysis were performed as described above. Quantitation of IEE or IBU was based on calibration curves of the peak height ratio (drug/internal standard) versus

concentration. Linearity was assessed in linear regression analysis with a weighting factor of $1/\lambda^2$.[10]

5-4 Precision and Accuracy The intra- and interday accuracy and precision of the analytical method were based on analysis of six replicates of the low, medium, and high QC samples on 3 different days. Assay precision was determined by calculating the R.S.D. (%) for each drug concentration. Accuracy was calculated by comparing measured concentrations with the known values. Accuracy and precision values within 15% covering the range of actual experimental concentrations were considered acceptable.[11,12]

6. Animal Studies Wistar rats (male and female, 12 weeks old, 230±30 g) were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (the experimental protocol was proved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University). Before the day of administration, the rats were fasted for 12 h but were allowed water ad libitum. Rats were administered 20 mg/kg (56.8 μmol /kg) of IEE or an equimolar dose of IBU (11.7 mg/kg) intravenously. Blood samples (approximately 0.5 ml) were drawn by puncture of the retroorbital sinus at specified times. Plasma was obtained by centrifugation and stored at −20°C. Samples were thawed and allowed to reach room temperature before analysis.

RESULTS AND DISCUSSION

The objective of this study was to develop an HPLC assay for the simultaneous determination of IBU and its prodrug IEE in rat plasma. The composition of the chromatographic mobile phase is a critical factor for the separation of monitored compounds from endogenous ones. When 0.2% trifluoroacetic acid was used in mobile phase, the peaks became sharper and more symmetric. It was also reported that trifluoroacetic acid enhances the resolution of catechins and eliminates their peak tailing.[13] Tetrahydrofuran in the mobile phase eliminated disturbing peaks in the vicinity of the peaks on the chromatogram of plasma. Methanol and acetonitrile were used together as organic modifiers, which made the analyte and internal standard separate well. A solvent mixture comprising methanol, acetonitrile, trifluoroacetic acid, and tetrahydrofuran provided more efficient HPLC separation than a methanol-trifluoroacetic acid system. The mobile phase also produces low column pressure as compared with methanol-trifluorlacetic acid.

Chromatograms corresponding to extracts of blank rat plasma (Fig. 2 A), a plasma sample spiked with IBU, IEE and GA (Fig. 2 B) and a rat plasma sample rat taken 40 min after intravenous administration of IEE (Fig. 2 C) are illustrated in Fig. 2. Each compound eluted with a sharp peak and distinct separation at baseline. The retention times of IBU, GA, and IEE were 5.62, 15.98, and 18.05 min, respectively. Drug-free plasma samples were consistently free of interference at the retention times corresponding to the compounds of interest.

The recovery, precision, and accuracy of the assay

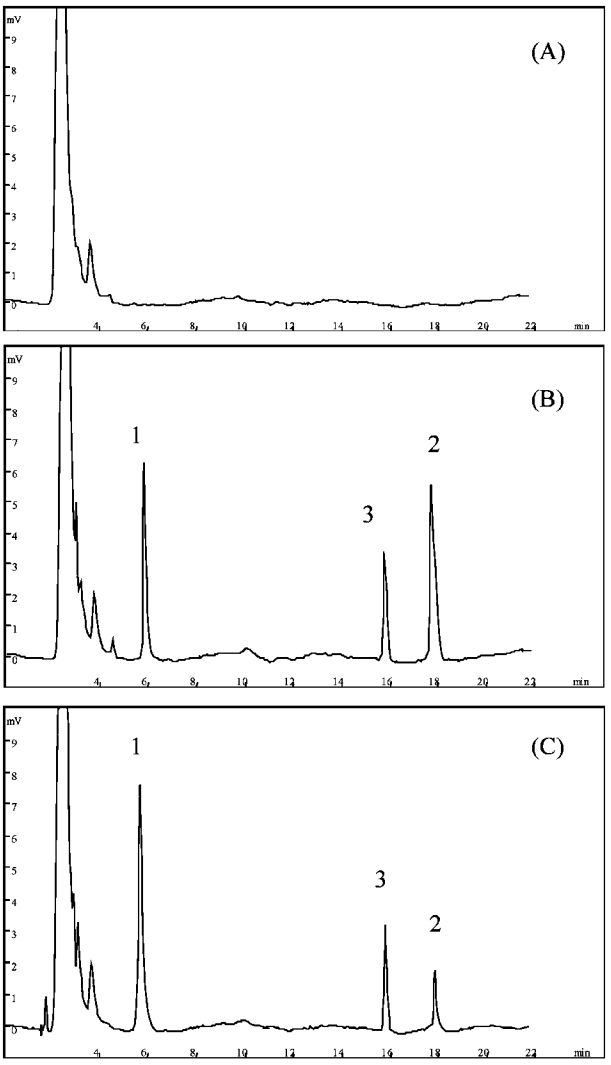

Fig. 2. Typical Chromatograms for Determination of Eugenol-ibuprofen Ester in Plasma Samples

(A) Chromatogram of a blank plasma sample, (B) chromatogram of a plasma sample spiked with IBU, GA, and IEE, (C) chromatogram of a rat plasma sample taken 40 min after intravenous administration of IEE to rats at the dose of 20 mg/kg. 1: IBU, 2: IEE, 3: GA.

Table 1. Recovery, Precision, and Accuracy of the Simultaneous of IBU and IEE in Rat Plasma

Compound	Concentration (μg/ml)	Recovery (%)	Measured conc. (mean±S.D.)	Precision (%)		Accuracy (%)	
				Intraday	Interday	Intraday	Interday
IBU	0.64	90.1±5.1	0.64±0.01	6.2	7.1	95.60	94.34
	6.4	89.7±4.2	6.37±0.42	6.6	4.7	98.64	93.23
	64	92.3±3.9	64.3 ±2.34	3.8	5.1	100.06	95.48
IEE	0.8	88.9±6.3	0.80±0.03	7.5	7.5	96.34	98.21
	8.0	86.7±4.1	8.32±1.25	5.4	3.9	97.60	102.53
	80	90.6±3.8	78.54±1.86	8.1	4.5	94.79	90.76

Data are based on analysis of six replicates on 3 separate days.

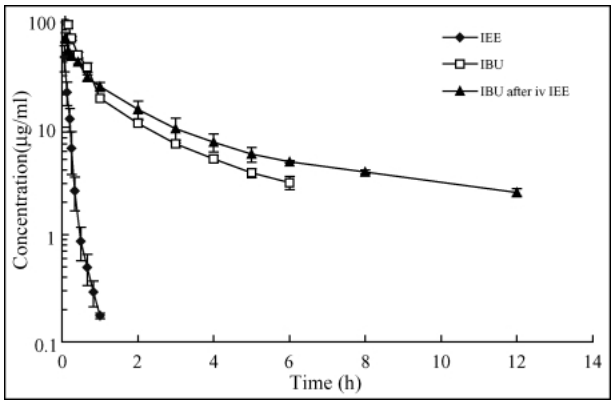

Fig. 3. Plasma Concentration-versus-Time Profiles

are summarized in Table 1. The limit of quantitation of the assay was 0.64 μg/ml for IBU and 0.16 μg/ml for the prodrug at a signal-to-noise ratio of 3. Calibration plots were linear over the range 0.64 to 64 μg/ml for IBU and 0.16 to 80 μg/ml for IEE, with satisfactory correlation coefficients ($>$0.996). IBU and IEE were found to be stable in the mobile phase at ambient temperature for up to 12 h, allowing a large number of samples to be processed in each analytical run.

Plasma concentrations of IBU or/and IEE as a function of time after intravenous administration of IBU or IEE are shown in Fig. 3. The prodrug was readily hydrolyzed *in vivo* resulting in relatively high plasma concentrations of IBU after the administration of IEE. The rapid *in vivo* hydrolysis might be attributed to the rapid capture of the prodrug by RES (Reticuloendothelial system)-rich organs after intravenous administration[14,15] and subsequent hydrolysis there. If the hydrolysis there was immediate, IBU would appear in the plasma more rapidly than expected. More studies are necessary to verify the above speculation and to elucidate the actual mechanism of the rapid *in vivo* release of IBU.

The determination of IBU and its prodrug IEE in rat plasma using this HPLC method is rapid, sensitive, and reproducible. The limit of quantitation of this method was sufficient to characterize the disposition of the prodrug and its bioconversion to IBU. The method can be applied to studies on the pharmacokinetics of IBU and IEE in rats.

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