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Short Communication

Simultaneous determination of bendamustine and its active metabolite, gamma-hydroxy-bendamustine in human plasma and urine using HPLC-fluorescence detector: Application to a pharmacokinetic study in Chinese cancer patients

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ABSTRACT

A simple, sensitive and cost-effective assay based on reversed phase high performance liquid chromatography (RP-HPLC) with isocratic mode for simultaneous determination of bendamustine (BM) and its active metabolite, gamma-hydroxy-bendamustine (γ-OH-BM) in human plasma and urine was developed and validated. Sample preparation involved protein precipitation by 10% perchloric acid-methanol solution. The peaks were recorded by using fluorescence detector (excitation wavelength 328 nm and emission wavelength 420 nm). The calibration curves were linear over concentration ranges of 8.192–10,000 ng mL^{-1} and 5–1000 ng mL^{-1} for BM in human plasma and urine as well as 10–1000 ng mL^{-1} and 5–1000 ng mL^{-1} for γ-OH-BM in human plasma and urine, respectively. Intra- and inter-run precisions of BM and γ-OH-BM were less than 15% and the bias were within ±15% for both plasma and urine. This validated method was successfully applied to a pharmacokinetic study enrolling 10 Chinese patients with indolent B-cell non-Hodgkin lymphoma and chronic lymphocytic leukemia administered a single intravenous infusion of 100 mg m^2 bendamustine hydrochloride.

1. Introduction

Bendamustine (BM), chemically, (4-{5-[bis-(2-chloroethyl) amino]-1-methyl-1H-benzimidazole-2-yl} butanoic acid) is a chemotherapeutic agent comprised a bifunctional mechlorethamine alkylating group, a purine like benzimidazole ring, and a butyric acid side chain [1] (Fig. 1A). This agent has shown clinical activity against various hematologic malignancies and has been commercially available in Germany for many years on the basis of a so-called 'fictitious' registration [2,3]. As a result of a re-registration procedure in Germany, the first full registration was granted in 2005. In 2008, BM was approved for marketing by the US Food and Drug Administration for chronic lymphocytic leukemia (CLL) and indolent B-cell non-Hodgkin's lymphoma (B-NHL) that has progressed during or within 6 months of treatment with rituximab or a rituximab-containing regimen [4,5]. To date, more than 100 clinical trials with BM have been initiated in America to assess its activity in CLL, B-NHL, multiple myeloma, solid tumors and lymphoma, etc. [6], showing that there is a great new interest in this very old drug.

It is known that BM metabolism consists of either N-dealkylation or hydroxylation of the methylene carbon at the C4 position of the butyric acid side chain [7]. The primary route of BM metabolism in humans is hydrolysis to the inactive metabolites, namely monohydroxy and dihydroxy BM. Two phase I metabolites of BM (gamma-hydroxy-bendamustine (γ-OH-BM, Fig. 1B) and N-desmethyl-bendamustine (M4)) which are formed via the minor CYP1A2 oxidative pathway have cytotoxic activity, and the relative potency was considered 1 for γ-OH-BM and 1/5 for M4, compared to bendamustine [8].

Recently, BM was approved by State Food and Drug Administration (SFDA) of China to enter phase I clinical trial. To perform the pharmacokinetic study, a method for the determination of BM and its major active metabolites should be developed and validated. However, few analytical methods have been developed and validated for the simultaneous determination of BM and its metabolites in biological fluids [9,10]. To our knowledge, Teichert et al. [9] reported an HPLC assay with fluorescence detector (FLD) for simultaneous determination of BM and its 7 metabolites in human bile, urine and plasma, but this method run over 70 min for each sample

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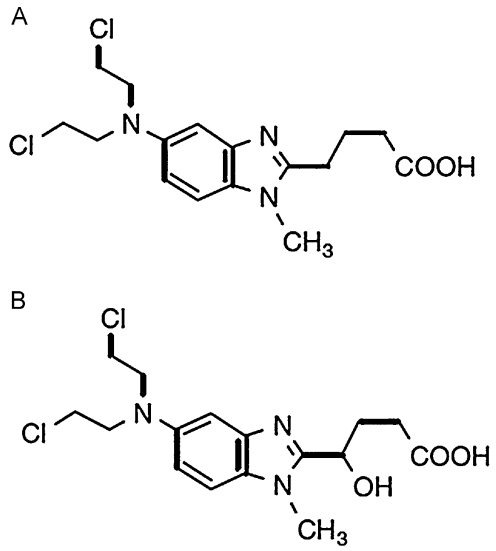

Fig. 1. Chemical structures of BM (A) and γ-OH-BM (B).

and the validation information was lacking; thus it was too time-costing to be applied to clinical study involving large sample size. An HPLC-MS/MS method was established to simultaneously determine BM, γ-OH-BM and M4 in human plasma and urine [10], but it employed tedious and costly sample preparation technique (solid-phase extraction) and time-consuming evaporating procedure. Also, the HPLC-MS/MS method requires relatively expensive instrumentation and highly skilled technical expertise, thus not always available to analytical laboratories for clinical study, and the reported method was partially validated for human urine samples. In such settings, selective and sensitive HPLC methods are preferable to more expensive LC-MS techniques.

Up to date, the following validation parameters: linearity, precision, accuracy, recovery and stability have not been reported combined in an HPLC-fluorescence method for BM and γ-OH-BM with its pharmacokinetic application. Therefore we developed and fully validated a simple, cost-effective and sensitive enough HPLC-FLD method for the simultaneous quantification of BM and its active metabolite (γ-OH-BM) in human plasma and urine. Compared with previously reported methods, our study focused on HPLC-FLD method involving simple protein precipitation procedure because of its wide availability in ordinary laboratories as well as its good specificity and reproducibility, satisfactory sensitivity, wide linear range, and simple sample preparation procedure. This method has been successfully applied to a clinical pharmacokinetic study involving 10 Chinese patients with B-NHL and CLL. The main pharmacokinetic characteristics of BM and γ-OH-BM were obtained in Chinese subjects for the first time, which can be helpful for its clinical use in cancer patients.

2. Experimental

2.1. Chemicals and reagents

Reference standards of bendamustine (purity 99.5%), γ-hydroxy-bendamustine (purity 99.2%) and 5-{5-[bis-(2-chloroethyl) amino]-1-methyl-1H-benzoimidazol-2-yl} valeric acid (internal standard, IS, purity 99.8%) were provided by Jiangsu Simcere Pharmaceutical R&D Co., Ltd (Nanjing, China). HPLC grade acetonitrile and methanol were purchased from Tedia Company, Inc. (Fairfield, USA), and deionized water was purified in a Purelab classic system ELGA Labwater (Shanghai, China). All other chemicals were of analytical grade from commercial sources.

2.2. Chromatographic conditions

The analysis was performed on an Agilent Technologies (Waldbronn, Germany) 1200 series HPLC system comprising degasser, binary pump, autosampler, thermostatted column compartment and fluorescence detector. Separation was achieved on an Agilent TC-C_{18} column (4.6 mm × 250 mm, 5 μm, Walbdbrom, Germany) with a Gemini C_{18} column (4.0 mm × 3.0 mm, 5 μm, Phenomenex, Torrance, USA) employed as a security guard column. The mobile phase was acetonitrile-10 mM potassium dihydrogen phosphate solution in a ratio 32:68 (*v/v*, pH 2.5). The flow rate was 1.0 mL min^{-1} and the column temperature was 30 °C. The injection volume was 20 μL, and the fluorescence detector was set at a combination of an excitation wavelength of 328 nm and emission wavelength of 420 nm.

2.3. Preparation of stock solutions, calibration standards (CS) and quality control (QC) samples

One mg mL^{-1} stock solutions for CS and QC samples were prepared separately in methanol for BM and γ-OH-BM. They were further diluted with methanol to obtain working solutions at several concentrations. CS and QC samples in plasma and urine were prepared by spiking drug-free human plasma and urine with the corresponding working solutions, respectively. The final calibration curve ranges of BM and γ-OH-BM were as follows: 8.192–10,000 ng mL^{-1} and 10–1000 ng mL^{-1} in plasma, respectively; 5–1000 ng mL^{-1} in urine.

The concentrations of QC samples of BM and γ-OH-BM were as follows: 20.48, 320 and 8000 ng mL^{-1} and 25, 250 and 800 ng mL^{-1} in plasma, respectively; 10, 100 and 800 ng mL^{-1} in urine. Stock solution of IS (1 mg mL^{-1}) was prepared in methanol, and was further diluted with methanol to prepare the IS working solution at the concentration of 10 μg mL^{-1}. The stock and working solutions were all stored at 4 °C, and the IS working solution and the CS were freshly prepared for each analytical run.

2.4. Sample preparation

Each 1000 μL thawed human plasma/urine sample was added with 100 μL of 6 M HCl and vortex-mixed for 30 s. Subsequently, 250 μL of acid-treated human plasma/urine sample was added with 25 μL IS (10 μg mL^{-1}) and vortex-mixed for 30 s, then the plasma/urine mixture was precipitated with 80 μL 10% perchloric acid-methanol solution, vortex-mixed for 3 min. All the previously treated plasma and urine samples were centrifuged at 15700 × *g* for 10 min, then 20 μL of supernatant was injected into the chromatographic system for analysis.

2.5. Method validation

The method was validated according to the FDA guidance of bioanalytical method validation [11] with emphasis on selectivity, linearity, sensitivity, intra- and inter-batch precision and accuracy, extraction recovery, and stability.

The selectivity of the method was measured by analysis of six blank plasma/urine samples of different origins for interference at the retention times of the BM, γ-OH-BM and IS. Calibration was performed by a least-squares linear regression of the peak area ratios of the analytes to the IS versus the respective standard concentration. The lower limit of quantification (LLOQ) was defined as the concentration of the lowest concentration standard in the calibration curve that was analyzed with accuracy within ±20% and a precision ≤20%. In order to assess the intra- and inter-day precision and accuracy, parallel analytical runs were performed on the same day and on three consecutive days. Each