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Short communication

Liquid chromatography method for determination of bivalirudin in human plasma and urine using automated *ortho*-phthalaldehyde derivatization and fluorescence detection

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed using solid-phase extraction, *o*-phthalaldehyde (OPA) derivatization and fluorescence detection for the determination of the direct thrombin inhibitor bivalirudin in human plasma and urine. The use of this assay will facilitate the study of the pharmacodynamics of bivalirudin in studies of special patient populations. A C_{18} bioanalytical column at a flow rate of 1 ml/min with an aqueous trifluoroacetic acid (0.1% TFA in deionized water, pH 2.2, v/v) mobile phase and methanol gradient was used. The assay demonstrated linearity from 3 to 20 μg/ml bivalirudin in plasma, with a detection limit of 1 μg/ml. The method was utilized in a study evaluating the pharmacokinetic and pharmacodynamic effects of bivalirudin in patients undergoing percutaneous coronary interventions (PCIs).

Keywords: Derivatization, LC; Bivalirudin

1. Introduction

Bivalirudin (Angiomax,® The Medicines Company, Parsippany, NJ) is a synthetic 20 amino acid peptide currently indicated as an anticoagulant for patients with unstable angina undergoing coronary angioplasty and is under investigation in other indications. Bivalirudin (D-phenylalanyl-L-prolyl-L-arginyl-L-prolyl-gly-cyl-glycyl-glycyl-glycyl-L-asparagyl-glycyl-L-aspartyl-L-phenylalanyl-L-glutamyl-L-glutamyl-L-isoleucyl-L-prolyl-L-glutamyl-L-glutamyl-L-tyrosyl-L-leucine trifluoroacetate (salt) hydrate), interferes with coagulation by binding both at the catalytic site and to the anion-binding site of thrombin. The preclinical and clinical pharmacokinetic and pharmacodynamic characteristics of bivalirudin have been studied in normal volunteers, patients with varying degrees of renal impairment [1], and in patients with acute coronary syndromes [2]. The relationship between dose and plasma concentration of bivalirudin is direct and linear, and the therapeutic range of bivalirudin plasma levels is approximately 1–15 μg/ml, depending on the dose used in specific applications. A highly specific LC–MS assay was developed and validated for clinical studies (same as [1]); however, this method is not readily accessible to provide pharmacokinetic data on bivalirudin in support of smaller on-going studies. The LC–MS assay is not currently published in the scientific literature as an analytical method and the reference does not report out validation criteria such as accuracy and precision; however it does report a linear range for plasma of 0.5–25 μg/ml, which is comparable to this method. The method detailed in the present communication utilizes C_{18} solid-phase extraction for plasma and urine samples and does not require the use of an internal standard. In addition, this method employs current high-performance liquid chromatographic (HPLC) bioanalytical column technology, automated sample derivatization with OPA and provides sufficient component resolution and sensitivity for measurement of bivalirudin in human plasma and urine. This method will facilitate further evaluation of bivalirudin in special patient populations and indications. For example, since bivalirudin has demonstrated clinical

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efficacy and significantly less bleeding in PCI compared to heparin or heparin with glycoprotein IIb/IIIa receptor inhibitors [3], including in patients with renal impairment [4], expanded data on the pharmacological profile of bivalirudin in special populations will provide information that can be correlated with clinical patient safety data.

2. Experimental

2.1. Chemicals

Bivalirudin was kindly donated by The Medicines Company (Parsippany, NJ, USA). Trifluoroacetic acid was reagent grade and methanol was Optima HPLC grade and both were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The *o*-phthalaldehyde (OPA), 800 μg/ml reagent solution was purchased from Pierce Chemical (Rockford, IL, USA) and used as received. Ultrapure distilled and deionized water was prepared in-house and filtered prior to use.

2.2. HPLC equipment and mobile phase

The HPLC equipment consisted of a Varian pump Model 9010 solvent delivery system (Walnut Creek, CA, USA). The analytical column was a Supelco Discovery Bio Wide-Pore C_{18}, 250 mm × 4.6 mm i.d., 5 μm packing, 300 Å (Supelco, Bellefonte, PA, USA). The C_{18} guard column, 30 mm × 4.6 mm i.d., 40–50 μm pellicular packing (Alltech, Deerfield, IL, USA) was replaced prior to each analytical run, which typically consisted of approximately 50 samples. The mobile phase consisted of an aqueous trifluoroacetic acid (0.1% TFA in deionized water, pH 2.2, v/v) and methanol gradient. The mobile phase gradient (linear) and time course was as follows (50:50 0.1% TFA in deionized water/methanol (v/v) at 0 min, 10:90 (v/v) at 15 min and hold 10 min, and 50:50 (v/v) at 26 min). The mobile phase was degassed using helium sparging and used at a flow-rate of 1.0 ml/min. Typical HPLC operating pressure was approximately 170 bar at ambient temperature. An injection volume of 100 μl of the OPA derivatized sample was accomplished using a Shimadzu SIL 6B autosampler (Tokyo, Japan). Component detection was achieved using a Shimadzu RF-535 Fluorescence detector (Tokyo, Japan) with excitation wavelength of 330 nm and emission wavelength of 445 nm. The detector operated at high sensitivity setting with a 1.5 s response time. A 345 kPa back-pressure regulator (SSI, State College, PA, USA) was coupled to the detector outlet to prevent mobile phase outgassing. Data acquisition and component computations were performed using Turbochrom4 chromatography software (PE Nelson, Norwalk, CT, USA).

2.3. Standard and control preparation

A stock standard of bivalirudin (50 mg/ml) was prepared in deionized water and stored at 4 °C. Working plasma standards of 3, 7.5, 10 and 20 μg/ml bivalirudin was prepared using blank human plasma as the diluent. A working urine standard containing 10 μg/ml bivalirudin was prepared using human blank urine as the diluent. Control plasma samples of 5 and 15 μg/ml were fortified with bivalirudin and was prepared using blank human plasma as the diluent. All working standards and controls were stored and maintained at −20 °C with the patient samples.

2.4. Sample conditions

Blood samples from patients undergoing PCI were collected at predetermined timepoints according to study protocols in tubes containing buffered sodium citrate. Heparin, EDTA or other anticoagulants were not allowed. Sample tubes were centrifuged at 3500 rpm for 15 min with plasma drawn off and frozen at −20 °C or below until analysis. Prior to analysis, plasma samples were thawed to ambient temperature, mixed thoroughly by inversion and centrifuged at 800 g for 5 min to eliminate fibrinous material. Urine samples were thawed to ambient temperature, mixed thoroughly by inversion and allowed to sit 15 min for particulate matter to settle out.

2.5. Sample preparation

Plasma and urine samples were extracted using Oasis® C_{18} HLB solid-phase cartridges (30 mg, 1 cc) (Waters, Milford, MA, USA) with each step using vacuum (5 in Hg) for sample processing. Prior to sample application, the cartridges were conditioned using 1 ml methanol followed by 1 ml 0.1% TFA in deionized water. Sample application consisted of 250 μl plasma or urine and 500 μl 0.1% TFA in deionized water. The sample wash consisted of using 1 ml methanol/0.1% TFA in deionized water (50:50, v/v). Sample elution consisted of using 500 μl methanol to elute bivalirudin into 12 × 77 polypropylene collection tubes. The sample eluant was vortex mixed for 15 s and transferred to a Shimadzu 4 ml autosampler vial. The Shimadzu SIL 6B autosampler automatically derivatized the bivalirudin by adding 500 μl OPA reagent to the autosampler vial, mixing three times and waiting 5 min for completion of derivatization prior to injection. For plasma and urine preparations, 100 μl of the derivatized solution was injected into the HPLC system.

3. Results and discussion

3.1. Method optimization

The C_{18} solid-phase extraction was optimized using various combinations of solvents such as acetonitrile, methanol and 0.1% TFA in deionized water. It was determined that 1 ml methanol/0.1% TFA in deionized water (50:50, v/v) was the most effective solvent mixture, amount used and

optimum ratio to eliminate bivalirudin component interference while maintaining good analyte recovery (mean: 87%). However, in attempts to shorten the analytical run time, additional evaluations of increasing from a 1–2 ml sample wash with methanol/0.1% TFA in deionized water (50:50, v/v) did not reduce the amount of late eluting nonpolar components (e.g. 22–24 min) from the plasma or urine extracts. The Oasis® C_{18} sorbent (30 mg) capacity was evaluated using 250 and 500 μl of plasma fortified with 20 μg/ml bivalirudin and using methanol/0.1% TFA in deionized water (50:50, v/v). Using 500 μl of plasma, the absolute recovery of bivalirudin was reduced to approximately 60% versus 87% absolute recovery using 250 μl of plasma sample. If additional component sensitivity is required which may require larger sample volumes (e.g. 500 or 1000 μl), it is recommended using a larger sorbent mass with increased sample capacity (e.g. Oasis® 60 mg C_{18}).

The OPA reagent was chosen for the derivatization step for its unique selectivity in reacting with primary amine containing analytes. It is commonly used in HPLC methods for amino acid and peptide analysis [5,6]. The first amino acid in the bivalirudin sequence is phenylalanine, which has a free primary amine for the OPA to react with forming a derivatization product that will fluoresce. In addition, there are two other primary amine sites (e.g. arginine, asparagine) in the bivalirudin amino acid sequence for OPA to react with and form derivatization products. The fluorescent reaction product can be excited using light set at 330 nm wavelength and detected at its characteristic 445 nm emission wavelength. The amount of OPA reagent used for derivatization was optimized by evaluating various ratios of OPA reagent to sample extract volume (e.g. 5:1, 2:1, 1:1, and 0.5:1) and evaluating the bivalirudin response. It was determined that a 1:1 ratio of OPA reagent to sample extract (e.g. 500 μl OPA/500 μl sample extract) provided sufficient derivatization reagent for the reaction with bivalirudin without significant loss in sensitivity due to the dilution effect of adding OPA. However, it has been published that some OPA reaction products (e.g. amino acids) are not stable for long periods thus making either a post column derivatization step necessary or using a precolumn derivatization step with subsequent injection into the HPLC system [7]. Stability testing on the OPA derivatized bivalirudin was performed via repeated injections of the same derivatized sample and demonstrated stability for at least 2 h after OPA derivatization. To eliminate any potential problem of reaction product instability and to run unattended analysis overnight, the autosampler was programmed to perform the derivatization step and inject the derivatized product 5 min after OPA derivatization of the sample extract. This precolumn approach added approximately 5 min to the total analysis time but allowed for unattended analysis and eliminated the need for an additional pump required for post column derivatization.

The mobile phase aqueous component (0.1% TFA in deionized water) was chosen as it is commonly used in the HPLC analysis of peptides and proteins. The mobile phase organic modifiers (e.g. acetonitrile versus methanol) were evaluated to determine which organic solvent would provide the best chromatographic separation for bivalirudin. When evaluating acetonitrile at various concentration levels in the mobile phase gradient, the bivalirudin peak shape was broad (large peak width and peak tailing) which indicated poor analytical column selectivity. This observed peak shape effect may also have been caused from peptide degradation at the higher concentrations of acetonitrile (>50%) used in the mobile phase gradient. Methanol was chosen as the organic modifier as it provided excellent bivalirudin peak shape and selectivity from other endogenous peptides and proteins in the sample extract.

3.2. Linearity, limit of detection, and computations

The plasma method was linear throughout the concentration range of 3–20 μg/ml with a mean correlation coefficient of 0.9974 ($n = 9$ analytical runs). The urine method was calibrated using a single point calibration standard of 10 μg/ml. The limit of detection for the method (1 μg/ml) was determined using a spiked amount of bivalirudin in each matrix at 1 μg/ml ($n = 3$) and calculation from standard curves for plasma and urine. For plasma component calculations, weighted linear regression ($1/x$) utilizing external standardization and peak area was used. The lowest standard calibrator for plasma was used as the limit of quantitation for reporting calculated patient results.

3.3. Accuracy, precision, and recovery

The accuracy and precision for the method was determined by evaluation of replicate plasma control samples at concentrations of 5 and 15 μg/ml. The intra-day accuracy of the method was reported as the percent error of theoretical versus measured bivalirudin concentrations at 5 and 15 μg/ml and was 1.3 and −0.8% ($n = 6$), respectively. The inter-day accuracy of the method for bivalirudin concentrations at 5 and 15 μg/ml was −5.5 and −0.9% ($n = 16$), respectively. The precision of the method was reported as percent relative standard deviation. The intra-day precision of the method for bivalirudin concentrations of 5 and 15 μg/ml was 8.9 and 5.8% ($n = 6$), respectively. The inter-day precision of the method for bivalirudin concentrations of 5 and 15 μg/ml was 18.4 and 9.4% ($n = 16$), respectively. Absolute recovery for the plasma method was evaluated by comparing extracted standards prepared in blank plasma at 5 and 15 μg/ml versus unextracted standards prepared in deionized water. The absolute recovery for the plasma method at 5 and 15 μg/ml ($n = 3$) was determined to be 85 and 89%, respectively. In addition, standards and controls used for analysis were treated identical to the patient samples thus controlling for potential errors in sample handling and micropipetting.

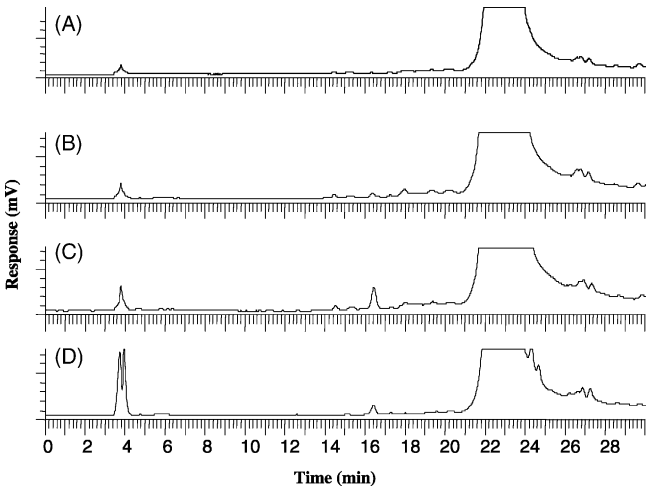

Fig. 1. Chromatograms of (A) prepared blank human plasma, (B) prepared blank human plasma fortified with 3 μg/ml bivalirudin, (C) prepared blank human plasma fortified with 20 μg/ml bivalirudin, and (D) subject plasma sample taken at 5 min post i.v. bolus treatment with bivalirudin (9 μg/ml plasma level). Retention time of bivalirudin: 16.3 min.

3.4. Chromatography

The method demonstrated excellent chromatographic selectivity with no endogenous interference at the retention time of bivalirudin (16.3 min) (Fig. 1A). Chromatograms of prepared blank human plasma containing low (3 μg/ml) and high (20 μg/ml) concentration of bivalirudin (Fig. 1B and C, respectively) indicated good detector response and baseline resolution from endogenous substances with an analytical run time of 30 min. A typical chromatogram for plasma from one subject dosed with bivalirudin (9 μg/ml plasma level) is shown in Fig. 1D. Chromatograms demonstrating the selectivity and sensitivity of the method for blank human urine and a 10 μg/ml urine standard are in Fig. 2A and B, respectively. To extend column lifetime, the analytical column was flushed after each analytical run (approximately 50 samples) for 2 h at 1 ml/min with acetonitrile/deionized water (85:15, v/v) to eliminate retained non-polar substances from the column.

4. Conclusions

A sensitive and selective method has been developed for evaluating bivalirudin in plasma and urine. We employed C_{18} solid-phase extraction sample preparation for plasma and urine samples and eliminated the need for an internal standard. In addition, this method utilized current bioanalytical HPLC column technology and an automated OPA precolumn derivatization step, which provided sufficient selectivity and sensitivity for measurement of this synthetic peptide. The method was employed without significant methodological problems in the evaluation of plasma and urine samples from a pharmacokinetic and pharmacodynamic study of bivalirudin used in the treatment of patients undergoing PCI. Since the method uses conventional HPLC

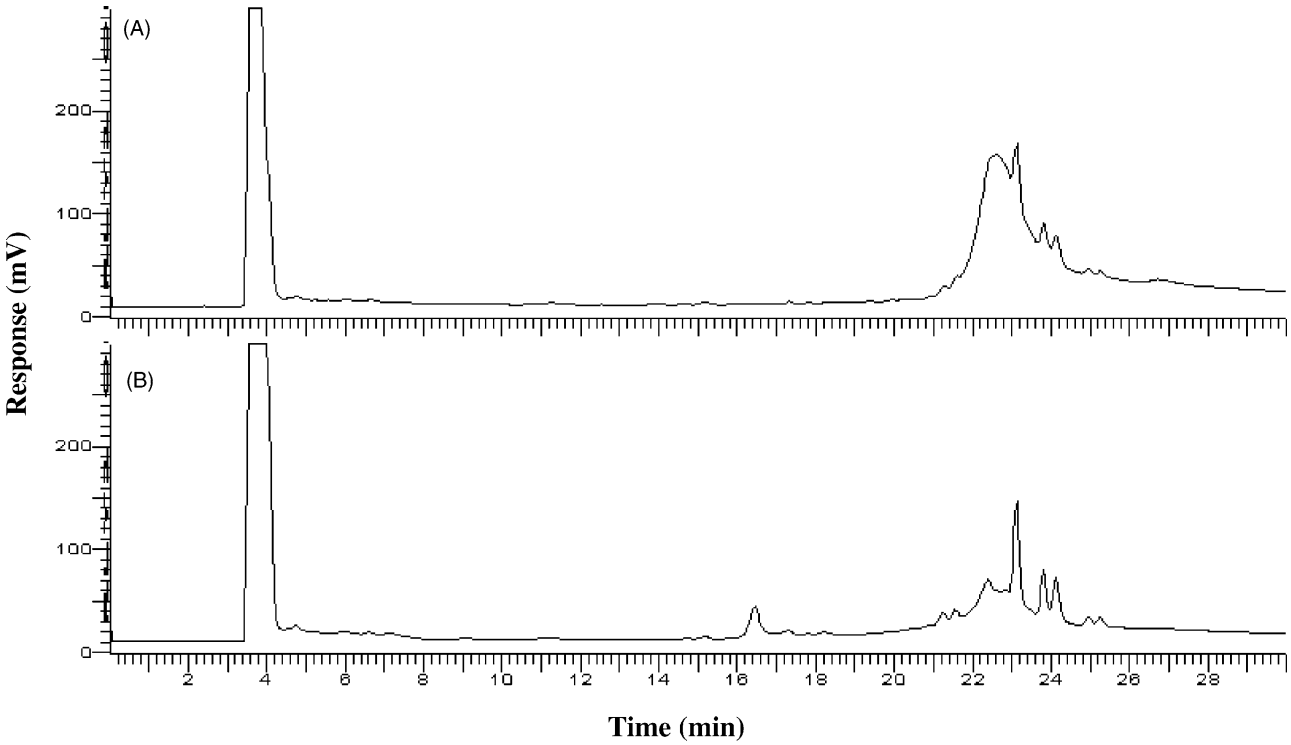

Fig. 2. Chromatograms of (A) prepared blank human urine and (B) prepared blank human urine fortified with 10 μg/ml bivalirudin. Retention time of bivalirudin: 16.3 min.

equipment and technology (e.g. conventional column parameters, OPA derivatization and fluorescence detector), it may offer an alternative analytical approach to the more expensive LC/MS/MS for pharmacokinetic and pharmacodynamic studies of peptides in biological fluids, and facilitate studies of bivalirudin as an anticoagulant for patients with acute coronary syndromes.

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