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Article

HPLC–Fluorescence Method for Measurement of the Uremic Toxin Indoxyl Sulfate in Plasma

Mohammed Al Za'abi*[1], Badreldin Ali[1] and Mohammed Al Toubi[2]

[1]Department of Pharmacology and Clinical Pharmacy, and [2]Department of Biochemistry, College of Medicine & Health Sciences, Sultan Qaboos University, Oman

*Author to whom correspondence should be addressed. Email: zaabi@squ.edu.om

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A sensitive and reproducible high-performance liquid chromatography–fluorescence method was developed and validated for quantitative determination of indoxyl sulfate in plasma using methyl paraben as an internal standard. The separation was carried out on an OSD-2 C18 Spherisorb column by isocratic elusion with sodium acetate buffer (pH 4.5) and acetonitrile (10:90, v/v) as the mobile phase. The method was validated and found to be linear in the range of 2.5 to 50 μM. The limit of quantification was 2.0 μM. The variations for intra-day and inter-day precision were less than 10.1%, and the accuracy values were between 93.4 and 102.5%. The extraction recoveries were more than 89%.

Introduction

Indoxyl sulfate (IS), a uremic toxin, plays an important role in many pathological conditions. It is synthesized in the liver from indole, which is produced from tryptophan by intestinal flora (1–2). More than 96% of IS is bound to albumin, thus excretion through the kidney occurs primarily via tubular secretion by an organic anion transport system (3–4).

Several studies have demonstrated an association between serum concentration of IS and progression of chronic kidney diseases, aortic calcification and vascular stiffness, diabetic nephropathy and cardiovascular events (5–6).

Despite the extensive literature on the role of IS in the pathology of renal diseases and other conditions, few published methods are available for the quantification of IS in biological fluids (7–11). Most of these require extensive preparation and long laboratory run time. In this communication, a rapid high-performance liquid chromatography (HPLC)–fluorescence method is described based on a simple protein precipitation approach.

Experimental

Materials and reagents

IS and the internal standard, methyl paraben, were obtained from Sigma (St. Louis, MO). Acetontirile and methanol (HPLC gradient grade) were from J.T. Baker (Deventer, Holland). Pure water was obtained by passage through a Mili-Q system (Millpore, Milford, MA) and was used to prepare all solutions. All other reagents were analytical grade and obtained through commercial sources.

Stock solutions and standards

Stock solution was made by dissolving a suitable amount of IS in methanol. Further standard solutions were obtained by serial dilutions of stock solution with the same solvent. The standard solutions were stored at −20°C and protected from light. Under these conditions, they were stable for at least six weeks. The calibration and quality control plasma samples were prepared by addition of standard solutions to drug-free plasma. The solution of the internal standard was obtained by dissolving methyl paraben (1 mg) in acetonitrile (1 mL), and the precipitation solution containing (10 ng/mL) of methyl paraben was obtained by further diluting this solution with acetonitrile.

Sample preparation

The plasma samples were stored in a −20°C freezer and thawed at room temperature before processing the sample. Two hundred and fifty microliters of plasma were pipetted to a polypropylene tube, 900 μL of the precipitation solution containing the internal standard was added and the tube was vortex-mixed for 30 s. The tube was then centrifuged for 5 min at 10,000 rpm and the supernatant was transferred to a 2-mL autosampler vial. A 2-μL aliquot was injected into the chromatographic system.

Liquid chromatographic conditions

HPLC was performed with a Waters HPLC system series 2695 (Waters, Milford, MA) equipped with an autosampler, Empower software data station, version 2.0.7, and multi fluorescence detector (2475 module). The chromatographic separation was performed on a Spherisorb OSD-2 C18 column (250 × 4.6 mm, 5 μm, Supelco, Bellefonte, PA) protected with a Pursuit XRs metagurad (C18, 4.6 mm, 5 μm, Varian, Palo Alto, CA) at room temperature. The mobile phase, which consisted of sodium acetate buffer (pH 4.5) and acetonitrile (10:90, v/v), was delivered at flow rate of 1.3 mL/min. The excitation and emission wavelengths were set at 280 and 375 nm, respectively.

Validation procedures

The selectivity of the method was assessed by comparing chromatograms of blank plasma control from different sources with that of plasma spiked with IS and internal standard. The linearity of the method was evaluated by constructing calibration curves in the range of 2.5–50 μM, (2.5, 5, 10, 15, 20, 30 and 50 μM of IS). Calibration curves were generated by linear

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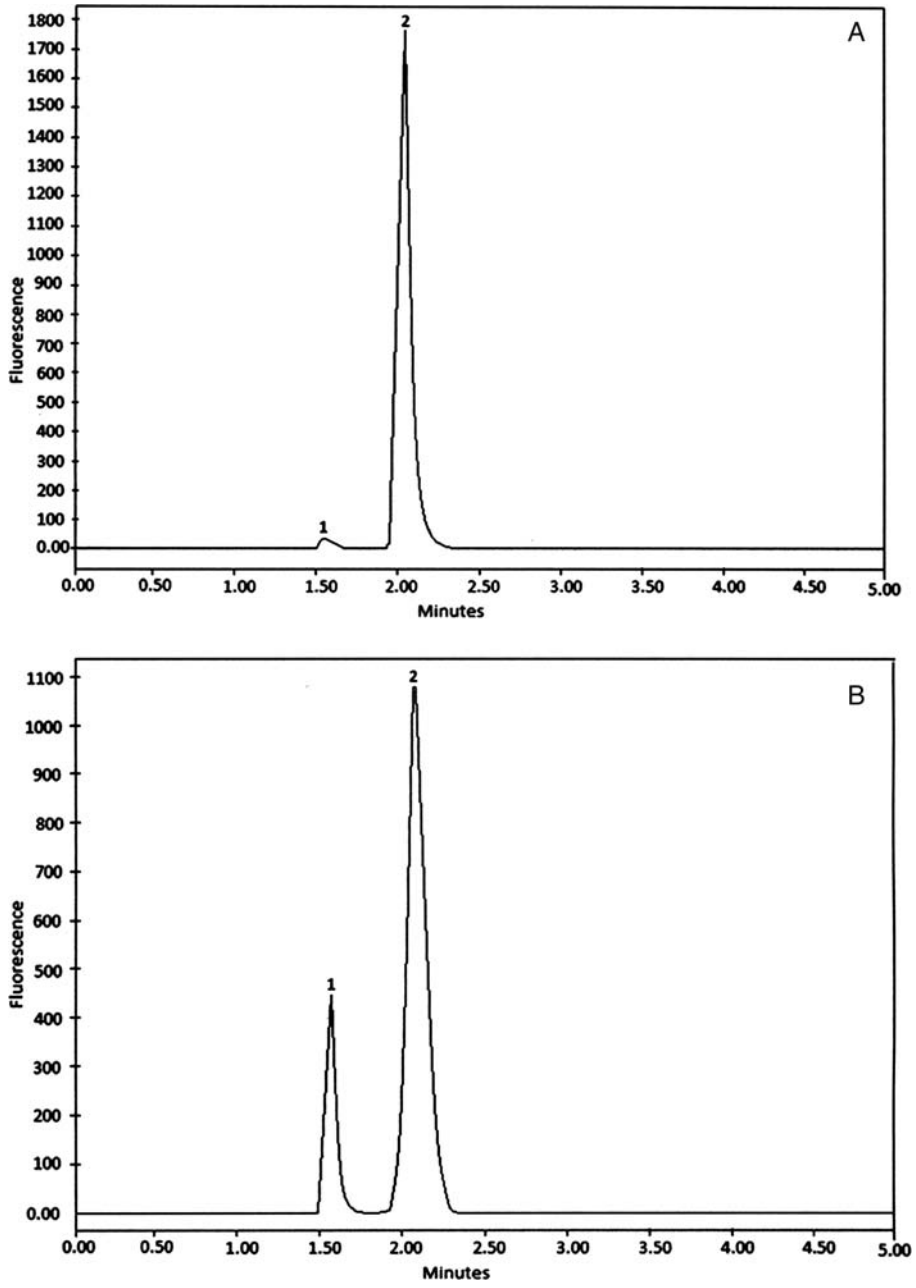

Figure 1. Chromatogram of: blank plasma with internal standard (A); blank plasma spiked with 25 μM indoxyl sulfate and internal standard (B) (1: indoxyl sulfate and 2: internal standard).

regression analysis of the peak height ratio of IS and internal standard against the nominal concentration. The regression line was not forced through the origin (0, 0). The calibration curve was then used to calculate the concentrations in quality controls (QCs) by using the generated calibration equations.

The intra-day and inter-day accuracy and precision were investigated by determining QC samples at three different concentrations (8, 25 and 40 μM) over four consecutive days. QC samples of three different concentrations were tested in six replicates and calculated with calibration curves obtained daily. Accuracy was expressed as percentage value [% accuracy = (detected concentration / nominal concentration) × 100%]. The precision was estimated as percentage relative standard deviation (RSD). For acceptable intra-day and inter-day values, accuracy should be within 85–115% and RSD values should be ≤15% over the

Table I
Accuracy, Intra-Day and Inter-Day Precision of IS in Plasma

Concentration (μM)	Intra-day ($n = 6$)		Inter-day ($n = 12$)	
	Accuracy (%)	RSD (%)*	Accuracy (%)	RSD (%)
8	102.5	10.1	97.7	7.8
25	99.9	3.4	93.4	5.4
40	97.6	1.6	93.6	5.2

*RSD: expressed as CV (%).

Table II
Stability Results, Expressed as Percentages of Freshly Prepared Quality Control Samples of IS ($n = 4$)

Theoretical concentration (μM)	Short-term study		Long-term study		Freeze-thaw study	
	Freshly prepared (mean ± SD)	After 24 h storage (mean ± SD) (%)*	Freshly prepared (mean ± SD)	After 3 weeks storage at −20°C (mean ± SD) (%)*	Freshly prepared (mean ± SD)	After 3 freeze thaw cycles (mean ± SD) (%)*
8	8.72 ± 0.16	8.66 ± 0.15 (99.3)	8.15 ± 0.33	8.233 ± 0.27 (100.3)	7.88 ± 0.21	8.44 ± 0.20 (104.5)
25	22.41 ± 0.31	21.67 ± 0.67 (96.7)	22.97 ± 0.82	23.05 ± 1.29 (100.3)	22.21 ± 0.39	23.89 ± 0.61 (104.1)
40	35.69 ± 1.34	34.78 ± 0.24 (94.4)	35.87 ± 6.1	35.94 ± 2.71 (95.0)	36.77 ± 0.45	36.70 ± 1.26 (99.8)

*Percentage calculated from freshly prepared QC.

calibration range, except at the limit of quantification (LOQ), where accuracy should be between 80 and 120% and RSD should not exceed 20%. The extraction recovery of IS was determined at three concentration levels by comparing the analyte peak height of the three extracted QC samples with those obtained from direct injection of standard solutions prepared at the same concentrations of the three replicates.

The stability of the QC samples at the three concentrations was also investigated. This included (i) stability of the extracted samples at room temperature for 24 h; (ii) stability after three freeze-thaw cycles with frozen temperature of −20°C and thawing temperature of 25°C; and (iii) stability of plasma samples at −20°C for three weeks. All samples were analyzed together with freshly processed QC samples.

The lower limit of detection (LOD) was the concentration for which the signal-to-noise ratio was at least three, across the retention window of IS. The lower limit of quantification (LLOQ) for the assay was the lowest concentration at which IS spiked in six different lots of plasma could be measured with accuracy greater than 80%.

Results and Discussion

The described HPLC method with fluorescence detection represents a relatively simple, sensitive and rapid technique for the measurement of IS in biological samples. It offers several advantages to sensitivity and sample preparation and requires a lower volume of sample requirements than the previously reported HPLC methods (7–11).

An appropriate internal standard is needed for accurate quantitative analysis of analytes in biological fluids. In this study, we chose methyl paraben, a methyl ester of p-hydroxybenzoic acid, as an internal standard. Methyl paraben is available commercially and displays similar solubility to the liquid extraction of IS. Moreover, the retention times and extraction recoveries for IS and internal standard were very close and resolution between their peaks was good.

During the method development, various mixtures of solvents were used to precipitate plasma proteins and/or to act as isocratic mobile phase. Acetonitrile–methanol with buffers such as phosphate and acetate were tested to improve peak shape and to eliminate interference from endogenous substances. Acetonitrile was selected as a precipitating agent because it resulted in a satisfactory peak shape and was able to dissolve and preserve the internal standard. Sodium acetate buffer in a mixture with acetonitrile (10:90, v/v) was used as mobile phase because it improved the resolutions, sensitivity and precision of the separation over the other tested buffers.

The chromatograms of the selected extraction procedure and HPLC running conditions resulted in a clear and satisfactory separation between IS and endogenous interferences from plasma. The retention times of IS and internal standard were approximately 1.6 and 2.2 min, respectively. No interfering peaks were detected at the retention times of either IS or internal standard. Figure 1 shows chromatographs of IS and internal standards. The calibration curve for IS was good in the concentration range 2.5–50 μM. The peak height ratio of IS to internal standard equations were linear with an $r^2 > 0.99$. The standard deviations from nominal concentration were all within ±7%. LOD and LLOQ for IS were 0.2 and 2.0 μM, respectively.

Table I summarizes the intra-day and inter-day precision and accuracy data. Overall precision gave RSD values of less than 10% and accuracy range of 93.4–102.5 of nominal values. These data suggest that the method is both accurate and precise.

The mean extraction recoveries for IS in plasma were 90.1 ± 5.4% at 8 μM, 89.4 ± 8.1% at 25 μM and 91.3 ± 6.3% at 40 μM.

IS was found to be stable after extraction from the plasma at room temperature for 24 h and at −20°C for three weeks. It was also stable after three freeze-thaw cycles. As shown from the results of the stability conditions (Table II), there were no significant deviations between QC samples in the tested conditions and the freshly prepared samples.

Conclusion

A sensitive and reproducible HPLC–fluorescence method was developed and validated for the quantitative determination of IS in plasma. It allows the determination of IS in the 2.5–50 μM range. The laboratory work time and preparation are simpler than previous methods. The precision and accuracy are within the limits required for biological analytical assays.

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