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Separation and sensitive determination of i-urobilin and l-stercobilin by high-performance liquid chromatography with fluorimetric detection

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ABSTRACT

i-Urobilin and l-stercobilin were separated by high-performance liquid chromatography on a reversed-phase octadecylsilane-bonded column and detected fluorimetrically through formation of phosphor with zinc ions in the eluent. The separation and the intensity of the fluorescence response were affected by concentrations of zinc acetate and sodium borate buffer, pH and methanol content in the eluent. The optimal eluent used consisted of 0.1% zinc acetate in 75 m*M* boric acid buffer (pH 6.0)–methanol (25:75). The detection limit was 0.2 μg/l for both i-urobilin and l-stercobilin (signal-to-noise ratio 2), which makes the method 250–2500 times more sensitive than conventional methods.

INTRODUCTION

Urobilinogens and urobilins, which are generally called urobilinoids, are known to exist in the faeces and urine [1]. Urobilinogens are formed from conjugated bilirubin in bile by hydrolysis and reduction by intestinal microflora [1]. Colourless urobilinogens are easily oxidized by air into brown urobilins. Urobilinoids are classified into three types, *i.e.* inactive (i), laevorotatory (l) and dextrorotatory (d) [1]. The composition of the urobilinoids in urine or faeces has been reported to be variable [1–3]. Urobilinoids in urine are known to be increased by hepatic dysfunction, haemolysis or constipation [1]. On the other hand, they are decreased when bilirubin excretion is hindered by obstructive jaundice. Accordingly, an assay method for urobilinoids should be very useful in clinical medicine.

At present, urobilinoids are usually quantified colorimetrically on the basis of Ehrlich's aldehyde reaction [4,5], the Jaffe–Schlesinger reaction [6] and Schmidt's mercuric chloride procedure [7]. These methods, however, are not specific for urobilinoids and cannot quantify the three types of urobilinoids. Recently, Bull Rosalind and coworkers [8–10] reported an assay for urobilinoids using high-performance liquid chromatography (HPLC) with a reversed-phase column and a UV detector. However, the sensitivity of their method was insufficient for clinical analysis.

The present paper presents a method for the separation and sensitive determination of urobilins, *i.e.* i-urobilin and l-stercobilin, using HPLC with fluorimetric detection which is based on the formation of phosphor from urobilins and zinc ions.

EXPERIMENTAL

Reagents

i-Urobilin and l-stercobilin used as standards were purchased from Porphyrin Products (Logan, UT, USA). Methanol, zinc acetate, sodium

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chloride and sodium hydroxide were from Kanto (Tokyo, Japan). Boric acid and hydrochloric acid were from Wako (Osaka, Japan). All other reagents were of analytical reagent grade, and Milli-Q-filtered water (Japan Millipore, Tokyo, Japan) was used.

Apparatus

The fluorescence spectra and intensity were measured using a Shimadzu (Tokyo, Japan) RF-502 spectrofluorimeter under the following conditions: light voltage, 900 V; slit width, 4 nm; cel, 10 × 10 mm. The HPLC system used was a Shimadzu LC-4A equipped with a Shimadzu RF-535 fluorescence detector.

HPLC conditions

The column was a Shiseido (Tokyo, Japan) Capcell Pak C_{18} (250 × 4.6 mm I.D., 5 μm particle diameter) packed with Hypersil ODS. Flow-rate, injection volume, excitation and emission wavelengths for fluorescence detection and column temperature were 1.0 ml/min, 10 μl, 475 nm and 513 nm and 20°C, respectively. The mobile phase consisted of methanol and sodium borate buffer containing zinc acetate. The resolution factor (R_s) of i-urobilin and l-stercobilin was estimated from retention times and baseline widths according to the following equation:

$$R_s = 2\,(t_{R2} - t_{R1})/(W_1 + W_2)$$

where t_{R1} and t_{R2} are retention times from the injection point of i-urobilin and l-stercobilin, respectively, and W_1 and W_2 are the baseline widths of the i-urobilin and l-stercobilin peaks on the chart, respectively [11]. When R_s was more than 1.0, the two peaks were completely separated. The detection sensitivity was estimated from peak area. A mixture of i-urobilin and l-stercobilin (0.5 mg/l each) in methanol was used for estimating the separation performance and detection sensitivity.

Pretreatment of clinical samples

Urine was treated with iodine to convert all urobilinogens into urobilins, and then filtered through a membrane filter (0.45 μm, Toyo Roshi, Tokyo, Japan) prior to the HPLC analysis.

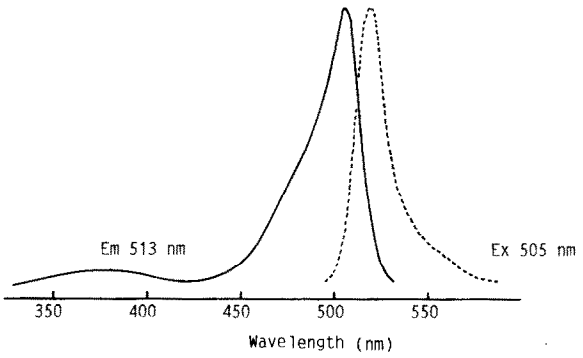

Fig. 1. Fluorescence excitation (full line) and emission (broken line) spectra of urobilins with zinc acetate. Solvent, methanol containing 0.2% zinc acetate.

RESULTS AND DISCUSSION

Factors affecting peak intensity and separation

i-Urobilin and l-stercobilin have been known to form phosphor by the Jaffe–Schlesinger reaction with zinc ions. Fig. 1 shows the fluorescence spectra for i-urobilin and l-stercobilin. The two spectra were identical, having emission and excitation maxima at 513 nm and 503 nm, respectively. Even if the zinc ion was replaced by another metal the spectra remained unaltered.

Table I shows the fluorescence intensities for i-urobilin and l-stercobilin in methanol solution containing some metal ions. The fluorescence intensities were measured with excitation at 475 nm and emission at 513 nm, in order to avoid back-

TABLE I

METAL EFFECTS ON FLUORESCENCE INTENSITIES OF i-UROBILIN AND l-STERCOBILIN

Concentration of urobilins, 2 mg/l; solvent, methanol; metal acetate, 0.2%; fluorescence spectrophotometer, Shimadzu RF 502; excitation wavelength, 475 nm; emission wavelength, 513 nm.

Metal	Fluorescence intensity (mm)	
	i-Urobilin	l-Stercobilin
Mercury	0	0
Copper	0	0
Calcium	21.8	6.7
Magnesium	96.9	70.8
Zinc	200.0	150.0

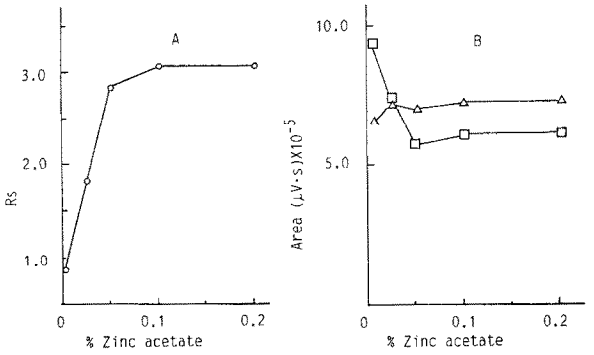

Fig. 2. Effects of zinc concentration (A) on R_s between i-urobilin and l-stercobilin and (B) on the fluorescence response of i-urobilin (△) and l-stercobilin (□). Eluent, methanol–75 m*M* (pH 6.0) sodium borate buffer (75:25) with zinc acetate; flow-rate, 1.0 ml/min; column, Shiseido Capcell Pak C_{18} (250 × 4.6 mm I.D., 5 μm particle diameter).

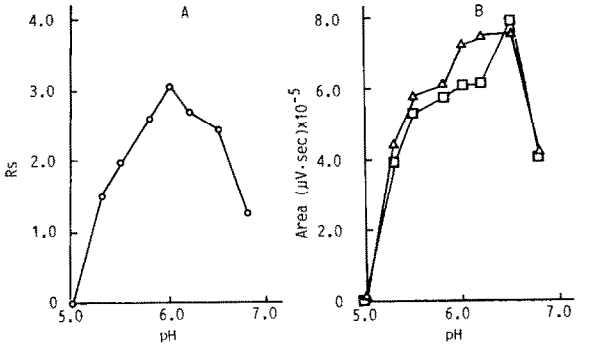

Fig. 3. Effects of pH (A) on R_s between i-urobilin and l-stercobilin and (B) on the fluorescence response of i-urobilin (△) and l-stercobilin (□). Eluent, methanol–75 m*M* sodium borate buffer (75:25) containing 0.1% zinc acetate; flow-rate, 1.0 ml/min; column, Shiseido Capcell Pak C_{18} (250 × 4.6 mm ID. 5 μm particle diameter).

ground noise arising from the scattered light. The fluorescence intensities of both urobilins were the strongest in the presence of zinc ions, indicating that the Jaffe–Schlesinger reaction was the most suited for formation of phosphor from urobilins.

Fig. 2 shows the effect of the concentration of zinc acetate in the eluent, which consisted of 75 m*M* sodium borate buffer (pH 6.0) and 75% methanol, on resolution and peak intensities of i-urobilin and l-stercobilin in HPLC. The R_s of both urobilins was increased with an increase in the concentration of zinc acetate in the eluent, though the increase was small, in the range 0.05–0.2%. The peak intensities were nearly constant in the range 0.05–0.2%. No fluorescence response was observed in the absence of zinc ions. These results indicate that the formation of phosphor from urobilins with zinc was effective for both the HPLC separation and sensitive detection by fluorescence.

The effects of the pH of the eluent on resolution and on the peak intensity are shown in Fig. 3. The R_s of both urobilins showed a maximum at pH 6.0. The peak intensities increased with a increase in pH of the eluent up to pH 6.5, though the increases were small, in the range pH 5.5–6.5.

The effects of the ionic strength of the sodium borate buffer in the eluent on R_s and the peak intensities are shown in Fig. 4. The best separation (R_s = 3.07) was achieved by using the eluent containing 75 m*M* sodium borate buffer. The ionic strength of sodium borate buffer in the eluent had little effect on detection sensitivities.

Fig. 5 shows the effect of methanol content in the eluent. The R_s of both urobilins decreased with an increase in methanol content. On the other hand, the peak intensities tended to increase with an increase in methanol content.

On the basis of the above-mentioned results, the most suitable composition of the eluent for HPLC with fluorescence detection of i-urobilin and l-sterobilin was as follows: 0.1% zinc acetate in 75 m*M* sodium borate buffer (pH 6.0)–metha-

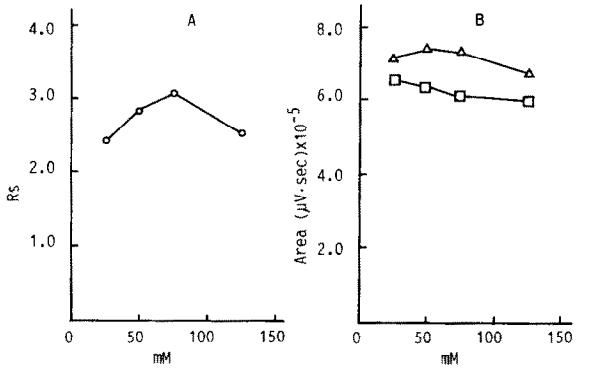

Fig. 4. Effects of concentration of sodium borate buffer in the eluent (A) on R_s between i-urobilin and l-stercobilin and (B) on the fluorescence response of i-urobilin (△) and l-stercobilin (□). Eluent, methanol–sodium borate buffer (pH 6.0) (75:25) containing 0.1% zinc acetate; flow-rate, 1.0 ml/min; column, Shiseido Capcell Pak C_{18} (250 × 4.6 mm I.D., 5 μm particle diameter).

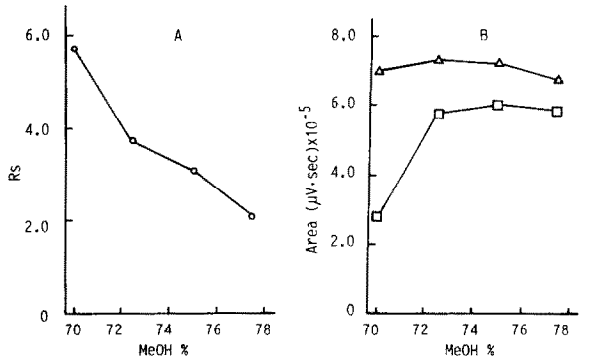

Fig. 5. Effects of the methanol (MeOH) content in the eluent (A) on R_s between i-urobilin and l-stercobilin and (B) on the fluorescence response of i-urobilin (△) and l-stercobilin (□). Eluent, methanol–75 m*M* sodium borate buffer containing 0.1% zinc acetate; flow-rate, 1.0 ml/min; column, Shiseido Capcell Pak C_{18} (250 × 4.6 mm I.D., 5 μm particle diameter).

nol (25:75). Column temperature in the range of 20–35°C affected neither the separation nor detection sensitivity.

Linearity and limit of detection

Linear relationships were obtained between peak area and the concentration of i-urobilin and l-stercobilin over the range 1.0–1000 μg/l, and correlation coefficients for both were 0.999. The detection limits for i-urobilin and l-stercobilin were 0.2 μg/l (signal-to-noise ratio 2), which is 250–2500 times higher than that of conventional analytical methods, *i.e.* 50 μg/l using the Jaffe–Schlesinger method [12], 250 μg/l using Ehrlich's aldehyde method [4,5] and 500 μg/l using Schmidt's mercuric chloride method [7].

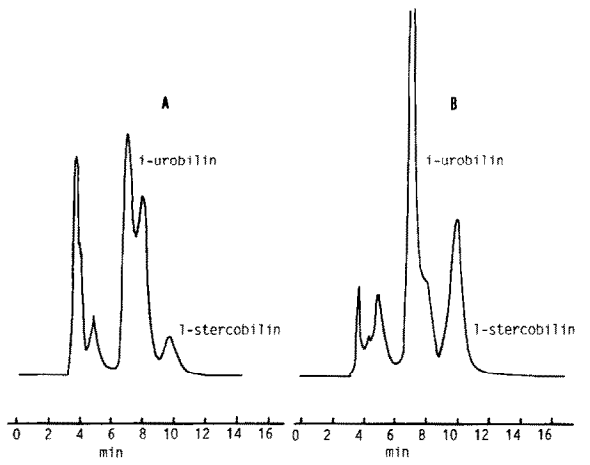

Fig. 6. HPLC profiles of a sample from human urine. (A) Normal, (B) hepatitis. Eluent, methanol–75 m*M* (pH 6.0) sodium borate buffer (75:25) containing 0.1% zinc acetate; flow-rate, 1.0 ml/min; column, Shiseido Capcell Pak C_{18} (250 × 4.6 mm I.D., 5 μm particle diameter); fluorescence detector, Shimadzu RF535; excitation wavelength 475 nm; emission wavelength, 513 nm.

TABLE II

i-UROBILIN AND l-STERCOBILIN IN HUMAN URINE SAMPLES

Urine sample	Concentration (μg/l)	
	i-Urobilin	l-Stercobilin
Normal	127	36
Normal	317	84
Hepatitis	1733	1248
Hepatitis	1050	1149

Application

The present HPLC method was applied to urine samples. Fig. 6 and Table II show the HPLC profiles and analytical results for some urine samples. i-Urobilin and l-stercobilin were detected in all urine samples. They were identified on the basis of their retention times and fluorescence spectra.

However, many other peaks were also observed in the urine samples. They may have been caused by glucuronides [2], sulphates [2], or stereoisomers of urobilins [13] or other tetrapyrrole compounds [14] capable of forming phosphor with zinc ions.

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