Urinary Excretion of α- and β-Isomers of Biliverdin-IX in Humans

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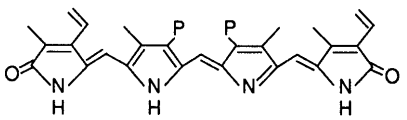
The distribution and relative proportions of the four isomers of α-, β-, γ- and δ-biliverdin-IX were investigated using urinary samples from seven healthy adults. The biliverdins from samples were adsorbed on talc, and the adsorbed biliverdins were then soaked in a solution of methanol and concentrated sulfuric acid for derivation to the dimethyl esters. The esters were passed to a reversed-phase cartridge for clean-up, then quantitated by HPLC on a reversed-phase column. The analyses revealed that the biliverdins in 24-h urine were comprised of 6.4±2.5 nmol α- and 9.3±5.4 nmol β-isomers. The HPLC analyses suggested the presence of a δ-isomer in a much smaller amount, but no γ-isomer was detected. The identifications of α- and β-isomers were performed with co-chromatography and absorption spectra.

Key words biliverdin; urine; HPLC; isomer; human

Due to the asymmetrical arrangement of the substituents of protoporphyrin-IX, the oxidative degradation of heme results in the formation of one or more of four structural isomers of biliverdin-IX. A non-enzymatic reaction *in vitro*, known as coupled oxidation using O_2 and a reductant such as ascorbate, cleavages heme at all four *meso* positions to produce α-, β-, γ-, and δ-isomers (**1**, **2**, **3**, and **4** respectively). In the physiological degradation of heme in mammals, the cleavage occurs predominantly at its α-*meso* position to produce α-biliverdin-IX which is reduced to α-bilirubin-IX by biliverdin reductase.[1,2] The cleavage at other positions, however, has been shown by the presence of non-α-isomers of bilirubin-IX and biliverdin-IX. β- and δ-Bilirubin-IX[3-6] in the body fluids of mammals and γ-bilirubin-IX[7,8] in some lower animals has been found, and all these isomers[9] have been detected in the bile of mutant rats. On the other hand, the quantity of biliverdin-IX has been reported to be varied under certain pathological conditions. An increased excretion of β-biliverdin-IX into rabbit urine has been observed following the administration of hemoglobin or phenylhydrazine as a hemolytic agent.[10] Abnormal accumulation, caused by the origin of a malfunction of biliverdin reductase, is known to lead to severe pathologies such as the bronze baby syndrome and green jaundice.[11,12]



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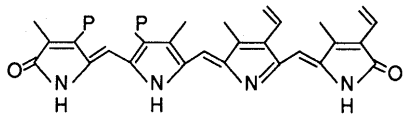

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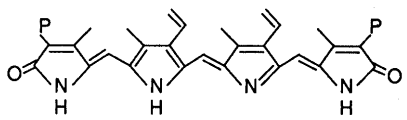

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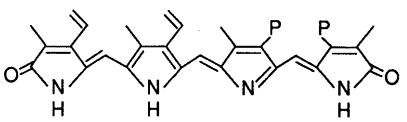

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P= -CH_2CH_2COOH

Analyses of the isomeric composition and quantity of biliverdin-IX are useful for the biochemical and metabolic study of bile pigments. The analysis for urine from healthy adults was carried out in this paper, and the isomers were determined by the use of high-performance liquid chromatography (HPLC) after the derivatization of the biliverdin to the dimethyl ester.

MATERIALS AND METHODS

TLC and Chemicals TLC was performed on a glass plate pre-coated with silica gel 60 (0.25 mm thickness, Merck). Chloroform used for TLC contained 0.8—1% ethanol as a stabilizer. Other reagents and solvents were of the best commercially available grades.

Preparation of the Dimethyl Esters and Free Acids of Biliverdin-IX The α-, β-, γ-, and δ-isomers of the ester prepared from the coupled oxidation of hemin[13] were separated into each isomer by preparative TLC. The development was carried out in the dark using 3% (v/v) acetone in chloroform. TLC plates were sufficiently exposed to the vapor of the solvent before the development. This exposure improved the separation for four isomers, especially for α- and γ-isomers, which have a tendency to overlap. Each band corresponding to the isomers was scraped off and eluted with 20% acetone in chloroform. The obtained eluate was concentrated and reapplied on new TLC plates. These procedures were repeated (usually three times) to obtain a single band. Separated isomers dissolved in methanol were stored at −10 °C and used for saponification (see below) within a week. Concentrations of the solutions of these isomers were calculated from the molar absorption coefficients of the isomers.[14]

The individual free acids of the isomers were obtained by saponification of the corresponding dimethyl esters.[15] Each ester (30—50 μg) in methanol (1.0 ml) was mixed

with 0.5 M sodium hydroxide (1.0 ml), and allowed to stand for 3 h at 4 °C in the dark. The alkaline solution was neutralized with hydrochloric acid and immediately used for the internal standard and preparation of calibration curves (see below).

HPLC HPLC was carried out on a model LC-6A Shimadzu liquid chromatograph with a UV detector (Shimadzu SPD-6AV). The detector was modified to detect biliverdin-IX dimethyl ester at 656 nm, the wavelength of the strong line emitted by the deuterium lamp. The second-order emission was removed by a colored glass filter (Toshiba) placed between the lamp and the detector cell. The area of the peak corresponding to the ester was measured with a data processor (Shimadzu C-R4A) connected to the detector. Separation was obtained on a Zorbax ODS column (15 cm × 0.64 cm, particle size 5—6 μm) from du Pont (Wilmington, DE, U.S.A.). The mobile phase was a solution of sodium acetate buffer (0.01 M, pH 3.65) and acetonitrile (1 : 1.2, v/v). The flow rate was 1.0 ml/min at a column temperature of 46 °C.

Quantitative Analysis of Biliverdin-IX The biliverdin was derivatized to the dimethyl ester and quantitated by HPLC, according to the method previously reported with some modifications.[10] All operations were carried out in subdued light. 24 h-Urine was collected from healthy adults, sterilized with toluene, and used immediately. A 12-ml sample of the urine was placed in a centrifuge tube and spiked with 0.5 ml of a solution (see above) containing γ-biliverdin-IX (150 ng, internal standard). The solution was acidified to pH 3.5 with acetic acid. Talc (0.5 g) was added and the resultant suspension was gently shaken at 4 °C for 1 h to adsorb the biliverdin. The tube was centrifuged at 2500 × *g* for 5 min and the resultant supernatant was totally removed by inverting (15 min) the tube to lead to the following quantitative esterification of biliverdin. The talc was mixed with 7.5 ml of 5% (v/v) sulfuric acid in methanol. The resultant suspension was left overnight at 4 °C, and centrifuged to separate the supernatant and the talc. The supernatant was set aside for further use. The talc was homogenized with methanol (1.0 ml) to extract the remaining ester. The supernatant obtained by centrifugation was combined with the above supernatant. The combined supernatant was diluted with an equal volume of water. The diluted solution was passed through a reversed-phase cartridge of Sep-Pak-C-18 (Waters Associates), which had been regenerated by the successive passing of 10 ml of methanol and 6 ml of 50% methanol in water. The cartridge was washed with 10 ml of 50% methanol in water to eliminate brown materials. The ester, which had been retained on the cartridge, was eluted with methanol (4 ml), and the eluate was evaporated to dryness under a nitrogen stream. The residue was dissolved in the mobile phase (400 μl) for HPLC, and an aliquot of 300 μl was injected onto the HPLC column.

Respective concentrations of α- and β-isomers of biliverdin were directly obtained from the calibration curves for each isomer, which were determined for each analysis. The curves were prepared by analyses of urine (12 ml) from a person, to which 0.5 ml of a solution (see above) containing the standard the α- or β-isomer (20—100 ng) and γ-isomer (150 ng) was added. The peak area ratio of the α- or β-isomer/γ-isomer was used for the preparation. There was almost no difference between the curves for both isomers.

TLC and Spectral Measurements of Urinary Biliverdin-IX Sufficient amounts of the isomers to measure *Rf*-values on TLC and absorption spectra of the biliverdin were collected from urine by repetition of the following procedures. The adsorption and esterification of the biliverdin were carried out at ten-times the scale of the above quantitative procedure. The obtained esters were treated with a cartridge of Sep-Pak and heavily overloaded on TLC plates. A blue region including α-, β- and δ-isomers of the biliverdin was scraped off, eluted, and concentrated. The concentrated solution was reapplied as bands on new TLC plates. Respective blue bands corresponding to α- and β-isomers were scraped off and eluted. A very small fraction of the δ-isomer was included in the band of α-isomer finally obtained. The eluates thus obtained from the bands were used for the determination of *Rf*-value. However, those were not directly used for spectral measurements because a contaminant was still present. Then, the spectra were recorded on the flow separating it by the HPLC column using a UV-VIS diode array spectrophotometer (Shimadzu SPD-M1A). The measuring point was at the peak of biliverdin on the HPLC chromatogram.

RESULTS AND DISCUSSION

Identification of Urinary Biliverdin-IX The dimethyl esters isolated from urine as described above were identified by chromatographic and spectrophotometric analyses. HPLC was carried out using two systems of the mobile phase for the column (Table 1, legend), which provided elution patterns differing from one another in retention times and elution sequences of the four isomers (*cf.* Fig. 2). The retention times of α-, β-, and δ-isomers were in agreement, respectively, with those of the standard α-, β-, and δ-isomers prepared by the coupled oxidation of hemin (Table 1). TLC was also carried out using two systems of the developing solvent (Table 1, legend), which showed different chromatographic behaviors in *Rf*-values.

Table 1. Chromatographic Parameters of Biliverdin Dimethyl Ester

Source of ester	Isomer	HPLC, retention time (min)		TLC, *Rf*-value	
		Mobile phase A[a]	Mobile phase B[b]	Solvent A[c]	Solvent B[d]
Coupled oxidation of hemin	α	31.8	20.1	0.19	0.05
	β	37.5	24.8	0.23	0.23
	γ	57.0	39.0	0.17	0.23
	δ	34.5	25.8	0.15	0.05
Urine[e]	α	31.8	20.1	0.19	0.05
	β	37.5	24.8	0.23	0.23
	δ	34.5	25.8	N.D.[f]	N.D.[f]

a) Sodium acetate buffer (0.01 M, pH 3.65)/acetonitrile (1/1.2, v/v). *b*) Acetonitrile/methanol/sodium acetate buffer (0.01 M, pH 3.65) (1/1/1, v/v/v). *c*) Chloroform/acetone (97/3, v/v). *d*) *n*-Heptane/methylethylketone/acetic acid (5/2/0.2, v/v/v). *e*) α- and β-Isomers isolated from urine were used. *f*) Not determined because sufficient amounts to measure were not isolated.

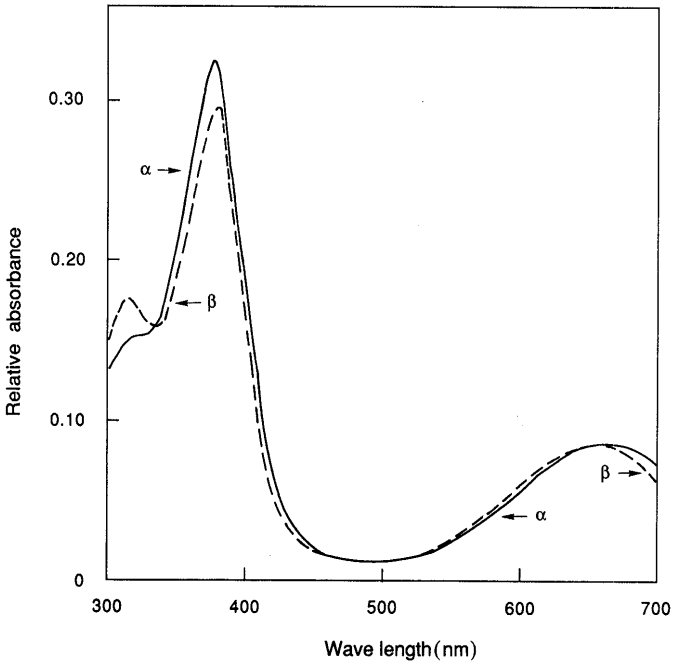

Fig. 1. Absorption Spectra of α- and β-Biliverdin Dimethyl Esters Derived from Human Urine

Spectra were recorded on-line after the flow of the HPLC separation.

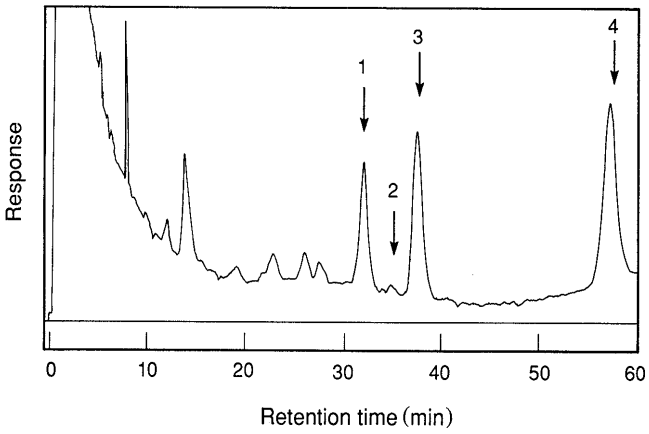

Fig. 2. A Typical HPLC Chromatogram of Biliverdin Dimethyl Esters Derived from Human Urine

Biliverdins in urine were esterified and analyzed by HPLC (see Materials and Methods for HPLC conditions). Peaks labelled 1, 2, and 3 are those of the α-, δ-, and β-isomers respectively. The peak labelled 4 indicates the peak of γ-isomer as an internal standard.

Spots of biliverdins on TLC were detected by the blue color characteristic of these compounds. The obtained values in each system were identical to those of standard α- and β-isomers (Table 1). Similar identification of a δ-isomer could not be performed. This isomer was not available in sufficient amounts and purity to permit the measurement because of the trace constituent present in urine (*cf.* Fig. 2).

The spectra of α- and β-isomers from the urine, as shown in Fig. 1, are virtually similar in shape to the relatively sharp absorption band at near 378 nm and a broad band at 650—675 nm. The respective spectra of the α- and β-isomers were compared with those of their standard isomers which were measured in the same way, and the comparison resulted in identity.

Analysis of the Method and Quantitation of Urinary Biliverdin-IX The effect of various pre-treatments on the results of isomeric analysis of the biliverdin was examined. The adsorption of biliverdin by talc was examined over the range of pH, showing a maximal capacity at pH 3.5. The substantial yellow pigment in urine was eliminated during the adsorption of biliverdin to talc. Further elimination by passing through a reversed-phase cartridge was necessary for the prevention of an abnormal increase in back pressure in the course of the HPLC analysis. Such elimination made it possible to continue the analysis of a large number of samples. Figure 2 shows a typical chromatogram from a urinary sample spiked with γ-biliverdin as an internal standard. No inclusion of the compound was confirmed in all the samples tested, which permitted it to be used as the standard. Calibration curves for α- and β-biliverdins (not listed) were linear in the range of 20—100 ng.

The recovery of urinary biliverdins was examined. The dimethyl ester of α-biliverdin dissolved in the HPLC mobile phase, without any pre-treatment, was analyzed by HPLC. The results were compared to analytical results of the urine spiked with α-biliverdin. The recovery of the isomer was $34.5 \pm 4.4\%$ ($n=4$), and that obtained by a separated run was $31.0 \pm 6.5\%$ ($n=4$). These relatively low recoveries are due to a poor adsorption capacity of biliverdin on talc, since a high recovery, of 90%, on the passage of the esters through the cartridge and almost quantitative esterification of biliverdins were attained. The isomer composition during treatments with both talc and cartridge did not alter significantly.

Oxidation of endogenous bilirubin to biliverdin in urine during analysis was tested using the urine samples spiked with α-bilirubin. Analytical results showed that no additional increase in α-biliverdin in the samples was observed, indicating no oxidation.

The distribution and relative proportion of biliverdins excreted into human urine are summarized in Table 2. The biliverdins were found to consist entirely of α- and β-isomers, and the amounts excreted in 24 h-urine were 2.6—9.6 nmol (6.4 ± 2.5 nmol) α-isomer and 2.7—18.6 nmol (9.3 ± 5.4 nmol) β-isomer. These isomers differed largely between individuals, but the proportion of the β-isomer, except for in one sample, was larger than the α-isomer (43%, α-isomer and 57%, β-isomer). The δ-isomer was present in amounts too small to permit quantification (*cf.* Fig. 2), and the γ-isomer was not detected, as described above. Biliverdin is equivalent to only a very small fraction of the heme from hemoproteins. Fractions of urinary biliverdins, of the estimated total physiological degradation of the heme, were calculated and resulted in $2.0 \times 10^{-3}\%$ and $2.8 \times 10^{-3}\%$ as the recovery of α- and β-isomers, respectively (Table 2). This extremely low recovery can be explained on the basis that the bulk of biliverdin is rapidly reduced to bilirubin by biliverdin reductase for both isomers,[1,2,16] and virtually eliminated by excretion into bile. Human duodenal bile includes both α- and β-isomers of bilirubin. The latter is the minor component, with a fraction of 0.6% of the total bilirubin.[5]

The physiological cleavage of the heme of hemoprotein

Table 2. Biliverdin Isomers in Human Urine

	Sex	Age	1 d urine (ml)	Body weight (kg) (A)	Heme physiologically degraded (nmol/d) ($B^{a)}$)	Urinary biliverdin excreted (nmol/d) (C)	Proportion of physiological bilirubin excreted $\times 10^{-3}$ (%) ($D^{b)}$)
1	M	47	760	63	409500	α, 2.6 (49[c)])	0.6
						β, 2.7 (51)	0.7
2	M	23	1680	58	377000	α, 9.6 (34)	2.5
						β, 18.6 (66)	4.9
3	M	23	1210	56	364000	α, 7.4 (39)	2.0
						β, 11.5 (61)	3.2
4	F	22	1060	45	292500	α, 8.7 (43)	3.0
						β, 11.7 (57)	4.0
5	F	22	535	48	312000	α, 3.8 (44)	1.2
						β, 4.8 (56)	1.5
6	F	21	1100	43	279500	α, 6.2 (52)	2.2
						β, 5.7 (48)	2.0
7	F	21	730	45	292500	α, 6.3 (39)	2.2
						β, 9.8 (61)	3.4
	Mean ± S.D.		1011 ± 380	51 ± 8	332429 ± 50548	α, 6.4 ± 2.5 (42.9 ± 6.2)	2.0 ± 0.8
						β, 9.3 ± 5.4 (57.1 ± 6.2)	2.8 ± 1.5

a) $B = A \times 6500$. *b*) $D = (C/B) \times 100$. *c*) Isomer proportion (%). The isomer contents (C) of urinary samples (24 h) were estimated as described under Materials and Methods. Heme physiological degradation of 6500 nmol of heme/d per kg of body weight was estimated on the value obtained from red-cell survival studies or from kinetic measurements using radiolabelled bilirubin.[19]

controlled by heme oxygenase is specific for the α-*meso* position and produces only the α-isomer. However, the presence of the β-isomer indicates that protoporphyrin-IX in the physiological degradation is not cleaved exclusively at its α-*meso* position to convert the α-isomer, but at least the cleavage at the β-position is included in the degradation. Degradation of hemoglobin by a non-enzymatic reaction *in vitro*, known as a coupled oxidation, produces α- and β-isomers by cleavage at the α- and β-*meso* positions.[10,17] This oxidation is thought to involve the same chemical reactions as the physiological conversion of heme into biliverdin, although it differs from the oxidation by heme oxygenase in the specificity of the cleavage site on the porphyrin ring of heme. The major portion of bilirubin (70%) is derived from red cell hemoglobin.[18] The excretion of the β-isomer, therefore, would account for the non-enzymatic destruction of part of the heme from hemoglobin.

The heme cleavage pattern is dependent on species and altered under certain pathological conditions. This analysis of urinary biliverdin may serve as a biochemical and metabolic study of bile pigments because urinary samples are obtainable non-surgically.

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