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Short communication

Determination of isatin in urine and plasma by high-performance liquid chromatography

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Abstract

A method for the detection and determination of isatin (indole-2,3-dione) in urine and plasma by high-performance liquid chromatography has been developed. It consists of a two-step purification using two different columns with UV detection. With this method, we have reconfirmed that isatin is present in human urine. We have also demonstrated that isatin is present in human plasma and that the isatin levels in spot urine samples reflect the plasma isatin levels. In the present report we describe a rapid and sensitive means of determining urine and plasma isatin for laboratories equipped with a high-performance liquid chromatography system.

Keywords: Isatin; Indole-2,3-dione

1. Introduction

Isatin (indole-2,3-dione) is a heterocyclic compound with an indole nucleus. The pharmacological effects of this compound include induction of arousal, reduction in the duration of slow-wave sleep and an increase of the seizure threshold in rats [1–4]. The most potent in vitro action of isatin is that of the monoamine oxidase (MAO) inhibitor. Endogenous MAO inhibitory activity was first discovered in normal human urine by Glover et al. [5] and the compound responsible for the activity was subsequently given the name "tribulin" [6]. In 1988, isatin was identified as a major constituent of tribulin, a naturally occurring, low-molecular-mass inhibitor of MAO type B [7]. It should be noted that isatin is anxiogenic at low doses in rodents [8] and its urinary excretion is increased after cold restraint stress [9]. Tribulin output in urine is increased in a range of states associated with stress or anxiety in humans [10–13]. Sandler et al. have proposed that tribulin may serve as an endocoid marker for stress and anxiety in humans [14]. Isatin may be a stress- or anxiety-related compound. However, little information is available about its physiological roles and mechanism of formation.

Quantitative assay methods for isatin involving the use of gas chromatography–mass spectrometry (GC–MS) have been described [7,15]. All information about isatin levels in biological samples has been collected using GC–MS methods. In the present paper a high-performance liquid chromatographic

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(HPLC) method for quantitative assaying of isatin is described for the first time. This sensitive and specific method may be useful for investigating the mechanism of formation and physiological roles of isatin.

2. Experimental

2.1. Materials

HPLC-grade acetonitrile, methanol and ethyl acetate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Isatin was obtained from Kanto Chemical (Tokyo, Japan). All other chemicals were of analytical grade.

2.2. Equipment

All chromatographic experiments were carried out using a Hitachi 655A chromatograph (Hitachi, Tokyo, Japan) equipped with a Hitachi L-4200 variable-wavelength detector.

2.3. Extraction of isatin

Plasma and spot urine samples were collected from 20 normal subjects [10 males, 10 females; age 43.75±3.19 years (mean±S.D.); age range 38–48 years].

Urine (5 ml) was acidified with 6 *M* HCl to pH 1 and heated for 10 min in a boiling water bath to solubilize urine sediment. After cooling at room temperature, isatin was extracted with 10 ml of ethyl acetate. The organic layer was then evaporated using a stream of nitrogen. The residue was dissolved in 0.3 ml of methanol and then diluted with 5 ml of 50 m*M* potassium phosphate buffer, pH 7.4. Isatin was extracted using a disposable solid-phase column, the Mega Bond Elut C_{18} column (Varian, Harbor City, CA, USA). Each column was conditioned by washing with 6 ml of acetonitrile followed by washing with 6 ml of distilled water and 6 ml of 50 m*M* potassium phosphate buffer, pH 7.4. Then, each sample was applied on a Bond Elut C_{18} column, and rinsed with 6 ml of 50 m*M* potassium phosphate buffer, pH 7.4 and 6 ml of distilled water, and then eluted with 6 ml of 50 m*M* potassium phosphate buffer (pH 7.4)–acetonitrile (85:15, v/v). The eluate was used for HPLC analyses.

Plasma (5 ml) was diluted with 10 ml of distilled water and acidified with 6 *M* HCl to pH 1. After heating for 10 min in boiling water, the plasma sample was cooled at room temperature. Then, the sample was washed twice with 20 ml of heptane and extracted with 20 ml of ethyl acetate. The subsequent solid-phase extraction procedure was carried out as described above.

2.4. Liquid chromatography

In order to obtain the necessary selectivity in the assay a two-step procedure was performed. Partial purification was carried out with a Shodex ES-502C column (100×7.6 mm I.D., 9.0 μm particle size; Showa Denko, Tokyo, Japan) under the following conditions: mobile phase, 50 m*M* potassium phosphate buffer (pH 7.4)–acetonitrile (85:15, v/v); flow-rate, 1.0 ml/min; 50°C. The eluate was monitored by UV detection at 240 nm. After injection of the extract of a sample, the fraction corresponding to isatin (3 ml) was collected. The final HPLC analysis was carried out on a Kaseisorb LC ODS Super column (250×4.6 mm I.D., 5 μm particle size and 120 Å pore size; Tokyo Chemical Industries, Tokyo, Japan). The column was equilibrated using 50 m*M* potassium phosphate buffer (pH 7.4)–acetonitrile (85:15, v/v) at a flow-rate of 1 ml/min. The fraction corresponding to isatin in the first-step analysis was injected directly. Note that only one sixth (500 μl) to one twentieth (150 μl) of the isatin fraction was analysed owing to the high concentration of isatin. Separations were made at 50°C and the eluate was monitored by UV detection at 240 nm.

2.5. Spectrophotometric analyses

Spectrometric measurements were performed on a Shimadzu UV 260 spectrophotometer (Shimadzu, Kyoto, Japan) after the samples had been dissolved in methanol. The mass spectral analyses were conducted in the electron-impact (EI) mode using the direct insertion probe on the TSQ-70 Finnigan MAT (San-Jose, CA, USA) mass spectrometer. The mass spectra were measured under the conditions of an ionizing voltage of 70 eV, an ionizing current of 200 mA and an ion-source temperature of 200°C.

3. Results and discussion

Typical chromatograms for human urine and plasma are shown in Fig. 1. For example, a chromatogram obtained after the first-step purification of isatin from a human urine sample is shown in Fig. 1A, where one sixth of the extract (1 ml) of a human urine sample was analyzed. In the final-step analysis, a sharp peak corresponding to isatin was clearly identifiable (Fig. 1B). On the final chromatograms, the retention time of isatin is stable and there is no interfering peak. Therefore, we did not use an internal standard. The calibration graph for isatin was found to be linear within the range employed (0, 1.0, 3.1, 9.2, 18.4, 36.8 ng). There was a significant correlation between the amount of isatin (ng) (y-axis) and the peak height (mm) (x-axis) ($y=0.235x$, $r=0.991$). The lower limit of detection was 3.2 pmol (0.47 ng) at a signal-to-noise ratio of 2. The fraction corresponding to isatin was collected and subjected to spectrometric analyses. The compound purified

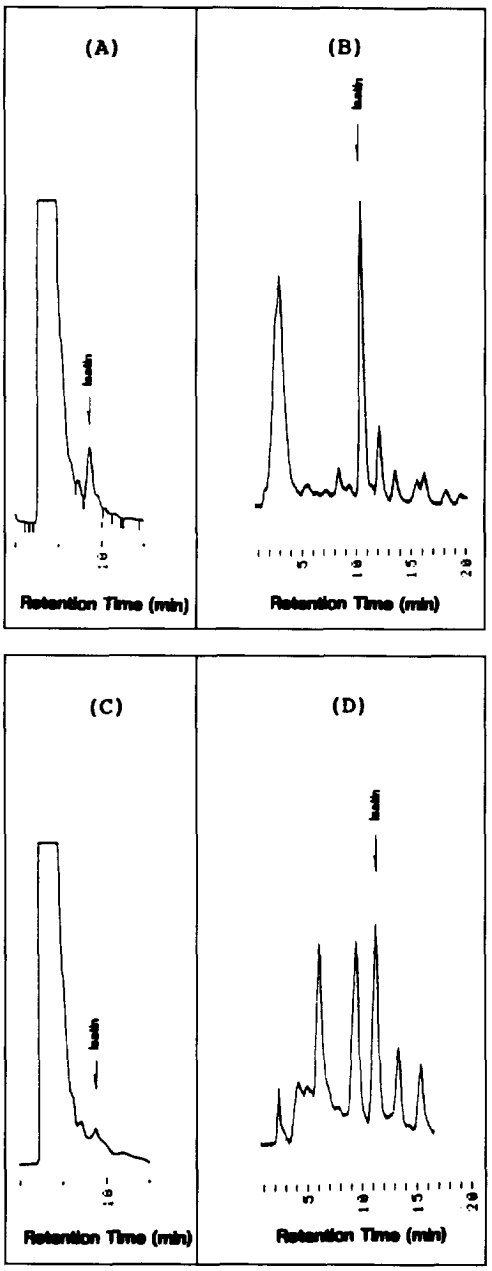

Fig. 1. Chromatograms of isatin in human urine and plasma. Partial purification was carried out with a Shodex ES-502C column (100×7.6 mm I.D.) under the following conditions: mobile phase, 50 mM potassium phosphate buffer (pH 7.4)–acetonitrile (85:15, v/v); flow-rate, 1.0 ml/min; 50°C. The fraction corresponding to isatin was collected. The final analysis was carried out on a Kaseisorb LC ODS Super column (250×4.6 mm I.D.) under the same conditions used in the partial purification step. The fraction corresponding to isatin in the first purification step was analysed. (A) Preparative chromatogram of a urine sample on an ES-502C column. (B) Final chromatographic profile of isatin in a urine sample on an ODS- Super column. (C) Preparative chromatogram of a plasma sample on an ES-502C column. (D) Final chromatographic profile of isatin in a plasma sample on an ODS-Super column.

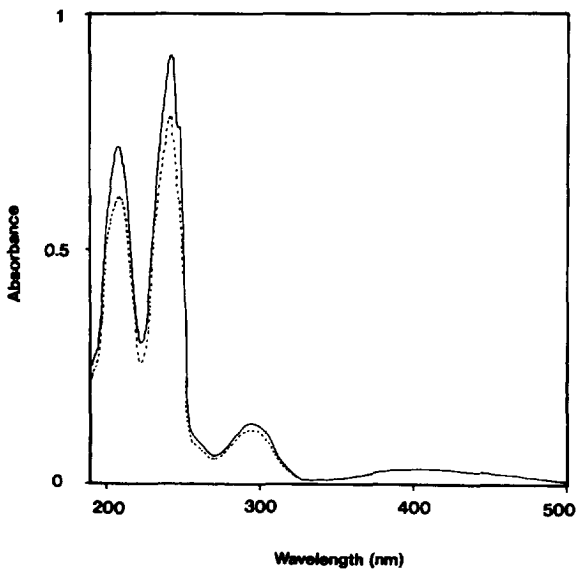

Fig. 2. Absorbance spectra of authentic isatin and the compound purified with HPLC. The compound purified with HPLC(——) and authentic isatin (41 μM) (····) were dissolved in methanol.

with HPLC was confirmed to be isatin from its absorbance spectrum (Fig. 2) and mass spectrum (Fig. 3).

The precision of the extraction procedure and the determination was estimated from the difference between duplicate urine and plasma samples (samples No.1–3 in Table 1). The coefficient of variation for six paired urine samples was 6.0% and that for six paired plasma samples was 6.5%.

Recovery experiments were conducted as follows. The amount of isatin in pooled human urine or plasma was determined. A sufficient amount of isatin in a small volume was then added to the sample to double the amount present and the amount of isatin

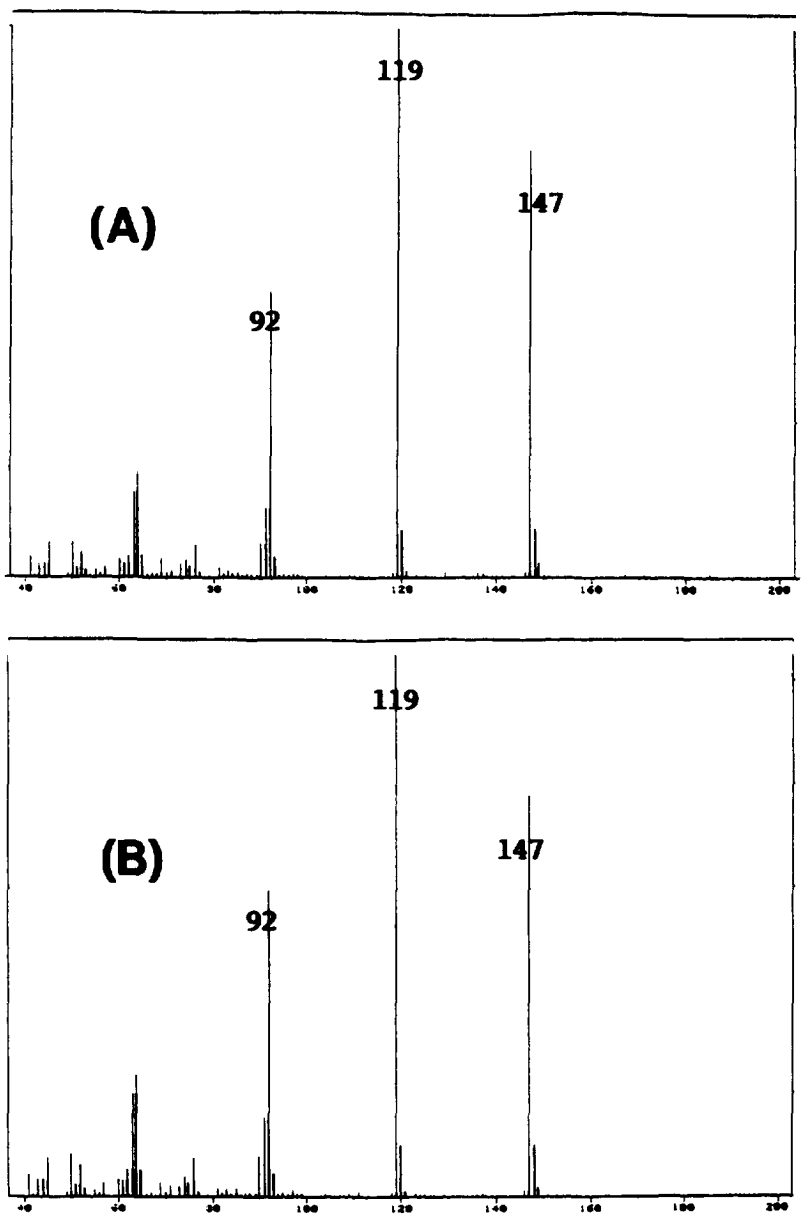

Fig. 3. Mass spectra of authentic isatin (A) and the compound purified with HPLC (B).

Table 1
Isatin levels in human urine and plasma

Subject No.	Sex	Age	Isatin in urine		Isatin in plasma
			mg/l	mg/g creatinine	μg/l
1	M	38	163.0	0.266	31.2
2	M	40	77.5	1.233	44.4
3	M	42	948.5	2.395	245.6
4	M	44	319.1	0.293	62.3
5	M	45	1240.4	2.064	246.0
6	M	45	401.3	0.130	65.1
7	M	46	260.3	0.433	53.4
8	M	47	2754.3	5.564	256.5
9	M	47	1395.5	4.796	311.7
10	M	48	18.2	0.067	30.4
11	F	38	2298.3	1.103	428.5
12	F	40	273.5	1.040	48.1
13	F	40	912.0	3.815	304.0
14	F	42	456.0	0.427	55.6
15	F	44	483.4	0.948	57.2
16	F	44	1423.0	1.194	178.1
17	F	45	355.6	0.316	104.0
18	F	45	301.0	0.163	88.4
19	F	47	45.6	0.563	41.2
20	F	48	41.1	0.038	31.9
Mean±S.D.		43.75±3.19	708.4±766.9	1.342±1.616	134.2±120.6

in the spiked sample was determined. The difference between the first and second determinations was divided by the added amount to give the recovery rate. With additions in the range 100–1500 ng (100 ng, 200 ng, 500 ng, 1000 ng and 1500 ng), the recoveries for human urine and plasma were 68.2±3.0% (mean±S.D., $n=10$) and 65.2±3.5% ($n=10$), respectively.

Isatin was detected in all urine samples. Table 1 shows the data corrected for the recovery. The mean amount of isatin in human urine was 708.4±766.9 μg/l (mean±S.D., $n=20$). When expressed as mg/g creatinine, isatin levels were 1.342±1.616 (mean±S.D., $n=20$). There are marked individual differences in isatin levels in urine although there is no sex difference in urine isatin levels. Halket et al. [15] reported that urinary excretion levels for isatin in adult humans ($n=5$) range from 5 to 30 mg per 24 h (mean±S.D., 14.6±9.9 mg per 24 h). Assuming a urinary excretion volume of 2 l, the mean level of isatin in adult urine is calculated to be 7.3 mg/l. This value is approximately ten times higher than the mean level of isatin in urine (708.4 μg/l) shown in Table 1. However, our data indicate the presence of marked individual differences in isatin levels in urine. For example, the highest level of isatin in urine was 2754.3 μg/l while the lowest was 18.2 μg/l. Considering the marked individual differences, it seems that our data on urine isatin levels are not inconsistent with the data reported by Halket et al. The presence of marked individual differences might suggest differences in states of stress or anxiety, since the urinary excretion of tribulin is increased in various states of stress or anxiety and isatin is a major constituent of tribulin [7,10–14].

In the present study, we demonstrated that isatin is present in human plasma.The mean level of isatin in plasma was 134.2±120.6 μg/l (mean±S.D., $n=20$). The plasma isatin levels were much lower than the urine isatin levels (Table 1). There are marked individual differences in plasma isatin levels as well as in urine isatin levels. However, there is a correlation between plasma isatin level (μg/l) and urine isatin level (μg/l) ($r=0.851$, $p<0.001$). This suggests that isatin levels in spot urine samples reflect the plasma isatin levels.

In conclusion, the method provides a rapid and sensitive means of determining urine and plasma isatin for laboratories equipped with an HPLC system. This method will be useful for investigating the physiological roles and mechanism of formation of isatin.

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