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High-performance liquid chromatographic determination of the new quinolone antibacterial agent DU-6859a in human serum and urine using solid-phase extraction with photolysis–fluorescence detection

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Abstract

A sensitive and specific HPLC method for the determination of DU-6859a (I), a fluoroquinolone antibacterial agent, in human serum and urine was developed. Compound I and the internal standard extracted from serum and urine by means of a Bond Elut C_8 LRC cartridge showed recoveries of 96%. The extracts were chromatographed on a reversed-phase column with photolysis–fluorescence detection. This unique detection method was 42.5 times more sensitive than intrinsic fluorescence detection, the limits of detection being in 3.43 ng/ml for serum and 4.35 ng/ml for urine. In addition, I was stable in serum and urine for at least 1 month at −20°C. The proposed method was sensitive and selective enough to apply to pharmacokinetic studies of I in humans after a single oral dose of 100 mg.

1. Introduction

DU-6859a, 7-[(7*S*)-7-amino-5-azaspiro[2.4]heptan-5-yl]-8-chloro-6-fluoro-1-[(1*R*,2*S*)-2-fluoro-1-cyclopropyl]-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid sesquihydrate (I), is a fluoroquinolone antibacterial agent synthesized by Daiichi Pharmaceutical (Tokyo, Japan). Compound I exhibits a broad spectrum of antibacterial activity and is 8–64 times more potent than ofloxacin (OFLX) against Gram-positive and Gram-negative bacteria, especially against *Streptococcus pneumoniae*, quinolone-resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) [1,2]. The pharmacokinetics of ^{14}C-labelled I have been clarified in experimental animals. Compound I administered orally to monkeys is well absorbed and is excreted mainly in the unchanged form into urine [3,4].

Most quinolone compounds are potently

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fluorescent, which is often used for their sensitive detection in HPLC [5–7]. Compound I has three halogen atoms in the 1-fluorocyclopropyl, 6-fluoro and 8-chloro moieties of the molecule. While introduction of a halogen atom into the 8-position usually enhances the antibacterial activity of quinolones [8], it often decreases the intrinsic fluorescence of the compound, as shown in other chemicals [9]. As a result, conventional HPLC with fluorescence detection was not sensitive enough to determine concentrations of I in serum and urine. There is no method to determine the biological concentration of I except by measuring radiolabelled I.

We found that photoproducts of I have more intense fluorescence than the intrinsic fluorescence of I. This suggested the possibility that we might be able to detect I very sensitively by determining its photoproducts instead.

Detection involving a photochemical reaction is a variation of on-line postcolumn derivatization, which has been found to be particularly useful for the analysis of drugs [10–13]. Combined with a photo-derivatization technique, the selectivity and sensitivity of fluorescence detection is advantageously enhanced.

To determine I in biological samples sensitively, it is necessary to develop a preparation method that yields good recoveries of I and the internal standard (I.S.). We previously established a sensitive and selective HPLC method for the determination of (*S*)-(−)-OFLX (levofloxacin) in human serum and urine using solid-phase extraction with fluorescence detection [7]. This extraction method proved very effective for preparing biological samples of quinolone compounds prior to HPLC. We therefore utilized this solid-phase extraction for the development of an HPLC method for I.

This paper describes a method for the determination of I in human serum and urine using solid-phase extraction followed by HPLC with photolysis–fluorescence detection. With high sensitivity and specificity and good precision, accuracy and recovery, the method is applicable to non-clinical and clinical pharmacokinetic studies of I.

2. Experimental

2.1. Reagents and materials

Compound I (Lot. 104) and the internal standard (I.S.), DX-9484 (Lot. K1158), were synthesized by Daiichi Pharmaceutical. The structures of these compounds are shown in Fig. 1. Compound I represents the sesquihydrate of DU-6859. All concentrations were expressed as the equivalent of anhydrate of I (DU-6859) unless specified otherwise. Tetrahydrofuran (THF) was an HPLC-grade solvent (Kanto Chemical, Tokyo, Japan). Sodium phosphate buffer (50 m*M*, pH 2) was prepared by dissolving 50 m*M* sodium hydrogenphosphate (KH_2PO_4) in water and adjusting the pH to 2 with orthophosphoric acid (H_3PO_4). Other chemicals were of analytical-reagent grade and used as received. Water obtained with a Milli-Q water-purification system (Millipore, Milford, MA, USA) was used to prepare all aqueous solutions. Human control serum and urine were obtained from healthy male volunteers. Consera (Nissui Seiyaku, Tokyo, Japan) was also used as a control human serum.

2.2. Apparatus

The chromatographic system consisted of an LC-10AD HPLC pump (Shimadzu, Kyoto, Japan), a DGU-4A degasser (Shimadzu), an F-1150 fluorescence detector (Hitachi, Tokyo, Japan), an SPD-10AV UV detector (Shimadzu) and an AS-8000 sample autoinjector with a 50-μl fixed loop (Tosoh, Tokyo, Japan). A CTO-2A column oven (Shimadzu) was used to regulate

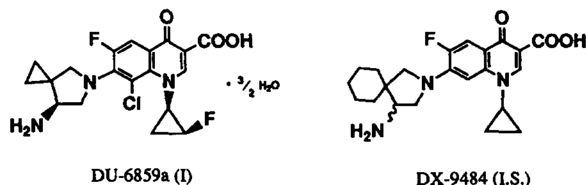

Fig. 1. Structures of I (DU-6859a) and the internal standard (DX-9484).

the temperature of the column. Data integration was performed on a Maxima 820 DO chromatography workstation (Millipore).

2.3. Post-column photolysis

The intrinsic fluorescence (spectrum and intensity) and UV spectra of I and the I.S. in the HPLC mobile phase were measured on a Model 650-60 spectrofluorimeter (Hitachi) and a Model 557 spectrophotometer (Hitachi), respectively.

To determine the optimum fluorescence wavelength for postcolumn photolysis, the fluorescence spectrum of photolytic substance(s) of I was also measured. Compound I was dissolved in the HPLC mobile phase at 1 μg/ml and transferred into a 50-ml beaker. The solution was exposed to UV radiation in an LX-210 photochamber (Taitec, Saitama, Japan) for 15 min (ca. 4000 Lx/h) to produce photolytic substance(s) and the fluorescence spectrum of this solution was measured.

In the "on-line" analysis, a Photo Deriver Σ08 photochemical reactor (Irica, Kyoto, Japan), consisting of a reaction coil (20 m × 0.3 mm I.D.) made of a PTFE capillary wound around a UV lamp (254 nm), was employed for postcolumn photolysis. The column eluate passed through a PTFE capillary which was irradiated by UV radiation entered the fluorescence detector. The effect of postcolumn photolysis on the fluorescence intensity of I and the I.S. was examined by assaying a sample mixture (0.50 μg/ml of I and 0.55 μg/ml of I.S.) for five determinations each with and without UV irradiation.

2.4. Chromatographic conditions

A reversed-phase Inertsil ODS-2 column (150 × 4.6 mm I.D.; 5-μm particle size) (GL Sciences, Tokyo, Japan) was used and the fluorescence detector was operated at an excitation wavelength of 280 nm and an emission wavelength of 430 nm. A mixture of THF, 50 m*M* sodium phosphate buffer (pH 2) and 1 *M* ammonium acetate (19:81:1, v/v/v) was used isocratically as the mobile phase at a flow-rate of 1 ml/min. The column temperature was kept at 25°C using the column oven. UV detection was performed at 295 nm.

2.5. Preparation of standard solutions

Stock standard solutions of I and the I.S. were prepared by dissolving ca. 100 mg of each compound in 100 ml of water and adding 2*M* acetic acid (2–3 drops) in a volumetric flask. The solutions were stored at 4°C for a maximum of 1 month. Working standard solutions were prepared by diluting the stock standard solution with water. Serum and urine standards were prepared by diluting appropriate aliquots of the working standard solution with control human serum or urine.

2.6. Assay procedure

The sample preparation procedure was a modification of the previously developed solid-phase extraction of (*S*)-(−)-OFLX from human serum and urine [7]. Briefly, Bond Elut C_8 LRC columns (200 mg) (Varian, Harbor City, CA, USA) were placed in a Vac Elut manifold (Varian) and activated prior to use by passing 4 ml of methanol, 6 ml of water and 6 ml of 50 m*M* KH_2PO_4. Aliquots of serum sample (0.2 ml) were diluted with 0.4 ml of 50 m*M* KH_2PO_4 and 0.2 ml of I.S. solution (0.1 μg/ml) was added. The resulting mixture was applied to a Bond Elut C_8 LRC column and drawn through by a Vac Elut manifold (ca. 0.67 kPa of suction). The column was washed successively with 6 ml of 50 m*M* KH_2PO_4 and 2 ml of THF–water (20:80, v/v).

Compound I and the I.S. were eluted from the column with 3 ml of THF–0.15% H_3PO_4 (50:50, v/v). The eluate was evaporated to dryness in vacuo in a CVE-200D centrifugal evaporator (Tokyo Rikakikai, Tokyo, Japan). The residue was dissolved in 0.3 ml of THF–water (20:80, v/v) and a 0.05-ml aliquot of the solution was injected to the HPLC system. Urine samples were treated using the same procedure as for the

serum samples, except that a 0.2-ml aliquot of human urine and 1.0 μg/ml of the I.S. solution were used.

2.7. Recovery

The absolute recoveries of I and the I.S. were calculated by comparing the peak area obtained from the injection of the theoretical amount of the compound with that obtained from the injection of extracts of serum and urine samples. The overall recoveries were determined by analysing the human serum and urine samples, which were spiked with 0.938 μg/ml of I and 2.0 μg/ml of the I.S. for six determinations.

2.8. Calibration

Determinations were performed employing the internal standard method. The calibration graph for I was constructed by linear regression using the least-squares method with reference to the obtained peak-area ratios of the standards. The drug concentrations in human serum and urine were expressed as the equivalent of the anhydrate of I.

2.9. Validation study

The intra-day precision and accuracy of the method were evaluated by replicate analyses ($n = 6$) on serum and urine samples that contained 0.938 and 2.350 μg/ml of I, respectively. The assay precision was based on the calculation of the relative standard deviation (R.S.D.). An indication of accuracy was based on the relative error (R.E.) of samples, i.e., $[(F - A)/A] \cdot 100$, representing the deviation of the found concentration (F) from theoretical concentration (A). The samples used to construct calibration graphs for serum and urine were independent of the samples utilized to evaluate the precision and accuracy of the method. The inter-day precision and accuracy for serum and urine were assessed by the results of intra-day assays on four separate days. The precision and accuracy were based on the geometrical mean of the R.S.D. and R.E. values of each day of determination.

2.10. Detection limits

The detection limit, based on the standard deviations of the serum and urinary concentrations of I and the noise level, was obtained by the following equation, described elsewhere [14]:

$$\text{detection limit} = \text{noise level} + 3(\text{S.D.}_1 + \text{S.D.}_2 + \cdots + \text{S.D.}_n)/n$$

where the noise level represents the mean of the blank signal, S.D. is the standard deviation of an individual concentration range assayed and n is the number of concentration ranges assayed.

2.11. Stability

The stability of I in human serum and urine was investigated with pooled samples spiked with known amounts of the compounds. Aliquots of the pooled serum and urine samples were pipetted into capped plastic tubes and stored at room temperature or at −20°C. These samples containing 0.938 and 2.350 μg/ml of I were assayed after 1 day, 1 week and 1 month of storage at −20°C. In addition, serum samples containing 0.019 and 0.938 μg/ml of I and urine samples containing 0.188 and 9.380 μg/ml were assayed after 1 and 2 days of storage at room temperature. The effect of freezing and thawing on the stability of I was also investigated by measuring serum and urine samples containing 0.019 and 0.938 μg/ml of I that had been frozen and thawed twice.

3. Results and discussion

3.1. Post-column photolysis

We first measured the UV and fluorescence spectra of I and the I.S. at a concentration of 2.34 μg/ml. The maximum wavelength (λ_{max}) of excitation (λ_{ex}) and emission (λ_{em}) for I was 281 and 440 nm, respectively, whereas that for the I.S. was 290 and 455 nm. The intrinsic fluorescence of the I.S. was, however, 40 times greater

than that of I. Also, both compounds had the same UV λ_{max} of 295 nm.

Compound I and the I.S. have three and one halogen atoms in the molecule, respectively. It has been suggested that the introduction of halogen into the molecule results in decreased fluorescence intensity [9]. Halogen therefore may play an important role in the decrease in the fluorescence intensity of these compounds. In other words, drastic enhancement of the fluorescence intensity may occur if halogen(s) or the halogen-containing moiety of I is removed by any chemical reactions such as photochemical or photothermal reaction.

We next examined the chemical properties of the photoproducts of I in order to employ the photochemical reaction for the determination of I by HPLC. Compound I dissolved in the HPLC mobile phase was converted into the photo derivative(s) by UV irradiation, and these derivatives exhibited maximum λ_{ex} and λ_{em} at 280 and 430 nm, respectively. Table 1 shows the effect of postcolumn photolysis on fluorescence intensity (based on the peak area) of I and the I.S. Postcolumn photolysis improved the sensitivity for the detection of I by a factor of more than 42.5, while minimizing interfering peaks or eliminating them altogether. On the other hand, no increase in the fluorescence intensity of the I.S. was observed on postcolumn UV irradiation.

It has been shown that the new quinolone compounds possessing a fluorine or a chlorine substituent in the 8-position are photosensitive and that UV irradiation induces their structural degradation, thereby leading to changes in the absorption spectrum [15]. In addition, it is known that alkyl or aryl bromides and aromatic chlorinated compounds liberate anionic bromide or chloride by postcolumn photolysis [16,17]. It therefore seems likely that the enhanced fluorescence intensity of I induced by photolysis is due to elimination of a chlorine atom at the 8-position or a 1-fluorocyclopropyl moiety at the 1-position of I. The I.S. was stable against UV irradiation because there is no halogen substituent at the 1- or 8-position. Although its fluorescence intensity was not enhanced by postcolumn photolysis, the I.S. could be used as an internal standard compound on account of its strong intrinsic fluorescence.

The lifetime of the PTFE capillary was more than 1 month of operation. To maintain the optimum conditions of the photoreactor, we changed the capillary periodically at 1-month intervals in these experiments.

3.3. Chromatography

Sharp and well defined chromatographic peaks for I and the I.S. were obtained on the isocratic reversed-phase ODS column. Fig. 2 shows the comparative chromatograms of I and the I.S. with intrinsic fluorescence detection and with photolysis–fluorescence detection. The concentrations of I and the I.S. were 0.055 and 0.050 μg/ml, respectively, and the injection volume was 0.05 ml. No peak was observed at the retention time of I with fluorescence detection

Table 1
Effect of postcolumn photolysis on fluorescence intensity of I and the internal standard in HPLC determination

UV lamp of photoreactor	I			I.S.		
	Peak area (mV s)	Relative standard deviation (%)	On/off ratio	Peak area (mV s)	Relative standard deviation (%)	On/off ratio
Off	118.0 ± 6.1 (1.0)	5.1	1.0	3060 ± 199.0 (23.4)	6.5	1.0
On	5010.0 ± 203.0 (1.0)	1.7	42.5	3400.0 ± 150.0 (0.7)	4.4	1.1

Values in parentheses represent the peak area ratio of I.S. to I. Peak area represents the mean ± standard deviation for five determinations. The analytical conditions are described in the text.

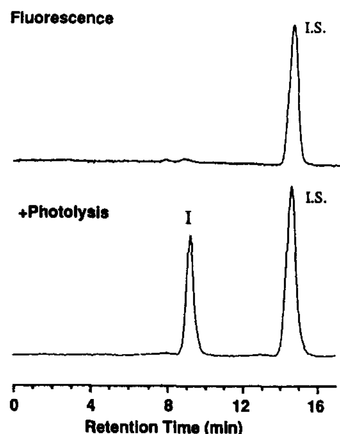

Fig. 2. Comparative chromatograms of 0.05 ml of sample mixture containing I (0.055 μg/ml) and the I.S. (0.050 μg/ml) with intrinsic fluorescence detection (top) and photolysis–fluorescence detection (bottom). The analaytical conditions are described in the text.

(top), and sensitive and specific detection of I was achieved with photolysis–fluorescence detection (bottom). Typical chromatograms of extracts from drug-free serum and urine are shown in Figs. 3A and 4A, respectively. These chromatograms indicated that no endogenous compounds interfered at the retention times of I and I.S. Typical chromatograms for serum and urine samples spiked with I and the I.S. are shown in Figs. 3B and 4B, respectively. Compound I and the I.S. were well resolved from each other with a resolution factor (R_s) of 1.5, and the retention times of I and the I.S. were approximately 9.6 and 14.4 min, respectively. The overall chromatographic run time was 17.0 min.

3.4. Recovery

Compound I and the I.S. were extracted from serum and urine by means of a Bond Elut C_8 LRC cartridge with recoveries of >96%. The absolute recoveries of I, which contained 0.938 μg/ml of I and 2.0 μg/ml of the I.S., using solid-phase extraction were 96.1 ± 2.3% (mean ±S.D., $n = 6$) in serum and 105.5 ± 2.1% in urine, and the recoveries of the I.S. were 97.1 ± 2.6% and 97.3 ± 2.2%, respectively. This method gave good recoveries of I and the I.S. from biological fluids, resulting in a noticeable contribution to high sensitivity of these compounds.

3.5. Assay validation

The intra-day and inter-day precision and accuracy for serum and urine samples spiked with I are shown in Table 2. The R.S.D.s for 0.938 μg/ml in serum samples analysed on different days were within 4.1% and the R.E.s ranged from −3.4 to 2.1%. The R.S.D.s for 2.350 μg/ml in urine samples analyased likewise were within 7.6% and the R.E.s ranged from 0.2 to 3.5%. The mean R.S.D. of the inter-day precision for serum and urine indicated good

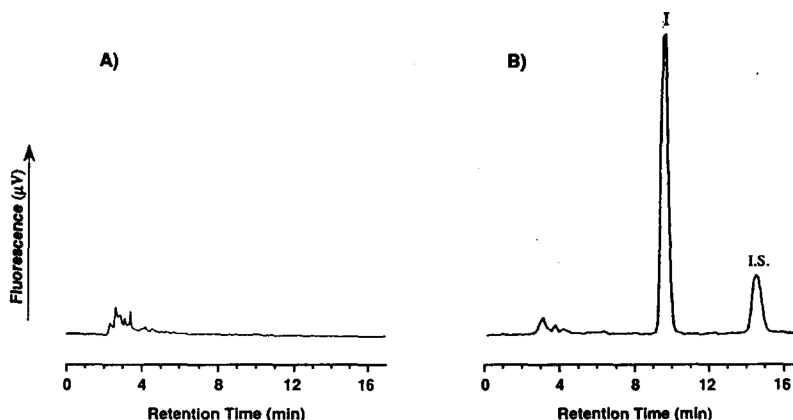

Fig. 3. Typical chromatograms of 0.2-ml serum extracts obtained from (A) drug-free human serum and (B) control serum spiked with I (0.235 μg/ml) and the I.S. (0.050 μg/ml). The analytical conditions are described in the text.

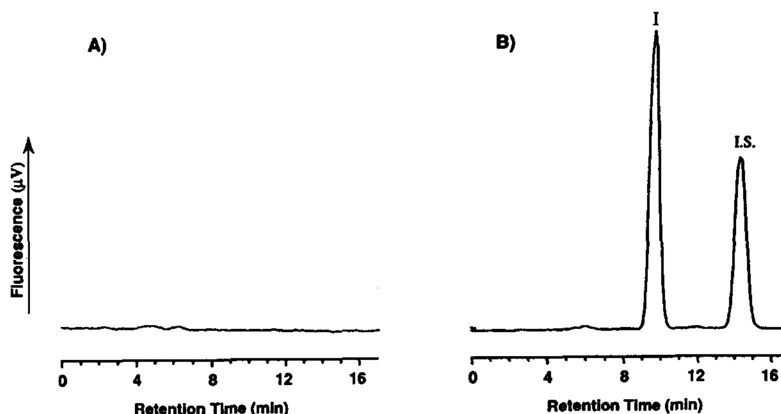

Fig. 4. Typical chromatograms of 0.2-ml urine extracts obtained from (A) drug-free human urine and (B) control serum spiked with I (5.863 µg/ml) and the I.S. (5.000 µg/ml). The analytical conditions are described in the text.

reproducibility with mean values of 3.3 and 3.9%, respectively. The inter-day R.E.s for serum and urine were −0.1 and 1.8%, respectively. Consequently, this method using photolysis–fluorescence detection showed acceptable precision and accuracy for four consecutive occasions.

3.6. Calibration

Calibration graphs for serum based on analyses performed over four occasions were linear and reproducible with mean ±S.D. values for the constants in the regression equation $y = (0.037 \pm 0.002)x + (-0.00337 \pm 0.00408)$, where y and x represent the concentration (µg/ml) and peak-area ratio, respectively. The correlation coefficients were >0.9996. The inter-day R.S.D. of the slope of the calibration graph for serum was 4.9%. The least-squares linear regression equation obtained for human urine over four occasions was $y = (0.419 \pm 0.045)x + (-0.0231 \pm 0.0224)$, with correlation coefficients >0.9998. The inter-day R.S.D. of the slope of the calibration graph for urine was 10.8%. The linearity of the graphs was demonstrated in the concentration ranges 0.004–1.880 µg/ml for serum 0.073–9.380 µg/ml for urine.

3.7. Detection limits

The concentrations of I used to determine the detection limits were 7.33, 14.66 and 29.32 ng/ml for serum and 11.73, 23.45 and 49.91 ng/ml for urine. The detection limits of this method, calculated on the basis of the equation in Ref. 14, were found to be 3.43 and 4.39 ng/ml in human serum and urine, respectively.

3.8. Stability

The stability data on I in serum and urine are given in Table 3 for −20°C and Table 4 for room temperature. Compound I was found to be stable for at least 2 days at room temperature and for up to 1 month at −20°C in human serum (0.938 µg/ml of I) and urine (2.350 µg/ml of I). The effect of freezing and thawing on the stability of I, in human serum and urine is shown in Table 5. After two cycles of freezing and thawing, I remained at 114.7 ± 7.4% and 105.8 ± 5.7% in the serum samples at concentrations of 0.019 and 0.938 µg/ml, respectively, and also remained at 102.8 ± 3.7% and 109.1 ± 3.3% in urine at concentrations of 0.188 and 9.380 µg/ml, respectively. These results indicated that the biological samples containing I could be handled under normal laboratory conditions without a significant loss of the compound.

3.9. Application to biological samples

This method was applied to a pharmacokinetic study of I in humans. Fig. 5 shows the serum

Table 2
Intra-day and inter-day reproducibility of the determination of I in human serum and urine

Replication day	Concentration found (μg/ml)	Relative error (%)	Relative standard deviation (%)
Serum			
1	0.938 ± 0.026	0.0 ± 2.7	2.7
2	0.958 ± 0.031	2.1 ± 3.3	3.2
3	0.948 ± 0.031	1.0 ± 3.3	3.3
4	0.906 ± 0.037	−3.4 ± 4.0	4.1
Mean	0.938	−0.1	3.3
S.D.	0.023	2.4	0.6
Urine			
1	2.381 ± 0.066	1.3 ± 2.8	2.8
2	2.355 ± 0.178	0.2 ± 7.6	7.6
3	2.400 ± 0.076	2.1 ± 3.3	3.2
4	2.432 ± 0.052	3.5 ± 2.2	2.1
Mean	2.392	1.8	3.9
S.D.	0.033	1.4	2.5

Each value represents the mean ± standard deviation for six determinations. Theoretical concentrations of I in serum and urine were 0.938 and 2.350 μg/ml, respectively.

Table 3
Stability of I in human serum and urine at −20°C

Storage time	Serum		Urine	
	Remaining (%)	Relative standard deviation (%)	Remaining (%)	Relative standard deviation (%)
Initial	100.0	3.2	100.0	3.5
1 day	99.3 ± 5.8	5.8	96.2 ± 5.9	6.1
1 week	99.2 ± 2.0	2.0	117.5 ± 4.9	4.2
1 month	101.1 ± 3.5	3.4	104.7 ± 3.0	2.9

Each value represents the mean ± standard deviation for seven determinations. Theoretical concentrations of I in serum and urine were 0.938 and 2.350 μg/ml, respectively.

concentration versus time profile of I after a single oral dose of I to a healthy male Japanese volunteer. No peak that interfered with the determination of I was observed in a typical chromatogram of a serum sample from the volunteer. The peak concentration of I in serum was 1.012 μg/ml. It was attained 1 h after oral administration, and thereafter declined gradually. The proposed method proved to be applicable to pharmacokinetic studies of I in humans.

4. Conclusions

An HPLC method for the determination of I in human serum and urine has been developed. Solid-phase extraction was successfully applied as the initial step for extraction of I from human serum and urine with recoveries of more than 96%. Photolysis–fluorescence detection was 42.5 times more sensitive than intrinsic fluorescence detection, so that the limit of detection was

Table 4
Stability of I in human serum and urine at room temperature

Storage Time	Serum			Urine		
	Concentration added (μg/ml)	Remaining (%)	Relative standard deviation (%)	Concentration added (μg/ml)	Remaining (%)	Relative standard deviation (%)
Initial	0.019	100.0	8.5	0.188	100.0	15.5
1 day		125.6 ± 16.7	13.3		105.6 ± 13.9	13.2
2 days		118.9 ± 8.1	6.8		118.8 ± 21.7	18.3
Initial	0.938	100.0	3.5	9.380	100.0	3.0
1 day		112.2 ± 6.0	5.3		92.6 ± 4.2	4.5
2 days		112.4 ± 11.0	9.8		90.1 ± 3.8	4.2

Each value represents the mean ± standard deviation for seven determinations.

Table 5
Influence of freeze–thaw cycles on stability of I in human serum and urine

Freezing and Thawing cycle	Serum			Urine		
	Concentration added (μg/ml)	Remaining (%)	Relative standard deviation (%)	Concentration added (μg/ml)	Remaining (%)	Relative standard deviation (%)
None	0.019	100.0	8.5	0.188	100.0	4.6
1st		119.4 ± 18.5	7.1		100.3 ± 9.8	9.8
2nd		114.7 ± 7.4	6.4		102.8 ± 3.7	3.6
None	0.938	100.0	3.5	9.380	100.0	3.4
1st		106.0 ± 5.5	5.2		99.3 ± 2.8	2.8
2nd		105.8 ± 5.7	5.4		109.1 ± 3.3	3.0

Each value represents the mean ± standard deviation for seven determinations.

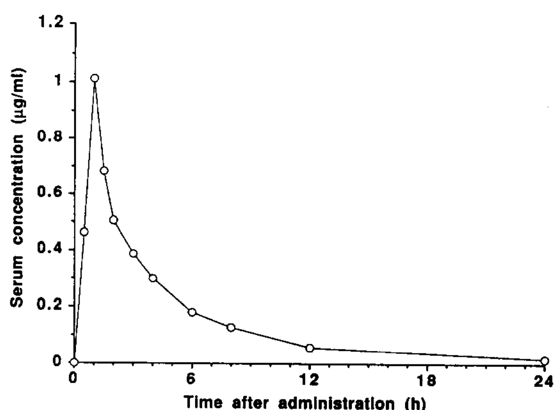

Fig. 5. Serum concentration of I versus time profile after single oral administration of I to a human subject at a dose of 100 mg. The analytical conditions are described in the text.

distinctly improved. This method is satisfactory with respect to precision and accuracy, and will be useful for pharmacokinetic studies of I in humans. The determination of I in biological samples has been achieved for the first time by means of HPLC with photolysis–fluorescence detection.

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