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Determination of ZD9583, a thromboxane receptor antagonist, in human plasma and urine by liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry

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Abstract

A liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry (LC–APCI-MS–MS) method is described for the determination of a thromboxane receptor antagonist (4*Z*)-6-((2*S*,4*S*,5*R*)-2-(1-(2-cyano-4-methylphenoxy)-1-methylethyl)-4-(3-pyridyl)-3-dioxan-5-yl)hex-4-enoic acid (ZD9583, I) in human plasma and urine. Proteins in plasma and urine samples are precipitated using acidified acetonitrile. The resulting supernatant is chromatographed on a C_8 reversed-phase chromatography column. Following the diversion of the solvent front from the mass spectrometer by a switching valve, the column eluate is passed on to the mass spectrometer via a heated nebulizer interface where the analyte is detected by multiple reaction monitoring (MRM). The method has a chromatographic run time of less than 2 min, a linear calibration curve with a range of 1–500 ng ml^{-1} and intra- and inter-day precision estimates of less than 10% over the calibration range. © 1997 Elsevier Science B.V.

Keywords: Thromboxane receptor antagonist; ZD9583

1. Introduction

Recently, LC–APCI-MS–MS has been gaining acceptance as a rapid, robust, sensitive and selective analytical method for the determination and characterization of drug substances and their metabolites in complex biological matrices [1–7].

(4*Z*)-6-((2*S*,4*S*,5*R*)-2-(1-(2-Cyano-4-methylphenoxy)-1-methylethyl)-4-(3-pyridyl)-3-dioxan-5-yl)-hex-4-enoic acid (ZD9583, I) is a thromboxane receptor antagonist and synthetase inhibitor targeted for disease states associated with thromboxane vascular mediators. To support toxicology studies, an HPLC method with fluorescence detection had been developed for the bioanalysis of I in animal plasma. This method, however, required a labor intensive liquid–liquid extraction and had a chromatographic run time of 12 min. The lower limit of quantitation (LLOQ) was 50 ng ml^{-1}. The analysis of clinical samples requires higher sensitivity, selectivity and sample throughput. The following method describes a rapid, highly sensitive and selective LC–APCI-MS–MS method for the determination of I in human plasma and urine.

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2. Experimental

2.1. Materials

HPLC grade solvents and analytical grade reagents were purchased from Aldrich (Milwaukee, WI, USA) and Fisher (Pittsburgh, PA, USA). Compound I was synthesized by Zeneca Pharmaceuticals (Alderley Park, UK).

2.1.1. Human plasma and urine samples

Blank human blood and urine samples were collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with the anticoagulant sodium heparin. Pooled plasma and urine were prepared and stored at approximately −70°C until needed.

2.2. Sample preparation

Aliquots (0.500 ml) of human plasma or urine samples were diluted two-fold with 0.1% trifluoroacetic acid (TFA) in acetonitrile (ACN). The sample was then vortexed to mix and centrifuged at approximately 13 000 *g* for 2 min. The supernatant was transferred to a glass autosampler vial for analysis.

2.3. Calibration standards

Compound I calibration standards, over the range 1–500 ng ml^{-1} were prepared, adding stock solutions (5–25 μl) to 0.500 ml of blank human plasma or urine. The spiked samples were then extracted as described above.

2.4. LC conditions

An aliquot (10 μl) of the plasma or urine extract was injected into a C_8 column (4×33 mm, 3.5 μm) (Perkin–Elmer, Newford, CT, USA) using an HP 1050 LC system (Hewlett–Packard, Wilmington, DE, USA). Separation and elution were achieved using 60% of ACN–methanol (1:1, v/v) and 40% of 0.1% TFA in water as the mobile phase, at a flow-rate of 1.5 ml min^{-1}. The time between injections was 2 min.

2.5. Mass spectrometric conditions

Mass spectrometric detection was performed using a Perkin–Elmer Sciex (Toronto, Ontario, Canada) API III^{plus} triple quadrupole mass spectrometer, equipped with a Sciex heated nebulizer as the APCI source. The temperature of the nebulizer was set at 550°C. Nitrogen was used as the nebulizing (80 p.s.i.), auxiliary (1 l min^{-1}) and curtain (1.2 l min^{-1}) gas. The APCI source was operated in the positive ionization mode, and MRM (*m/z* 451–218) was used for quantitation. The dwell time for the transition was 500 ms. Argon was used as the collision gas (250×10^{12} molecules cm^{-2}). Data were acquired by Sciex RAD software and the sample concentrations were calculated using the Sciex MACQUAN software. The mass spectrometer was connected with an automatic shutdown and recycle mode inducing device from MGT Systems (MasterGate and Master-Link, Milton Keynes, UK).

2.6. Column switching operation

The column switching device (Valco, Houston, TX, USA) was controlled by the HP1050 LC system. Following injection under position 1 (Fig. 1), the solvent front from the sample is directed through the UV detector to the waste while another pump (pump 2) delivers the same mobile phase to the mass spectrometer. At 0.6 min, when the solvent front containing most of the endogenous components of the sample has eluted from the column, the valve is switched to position 2 and the analyte is introduced to the mass spectrometer. At 1.6 min, the valve is switched back to position 1 in preparation for another sample.

2.7. Validation procedures

2.7.1. Interferences

Over the counter (OTC) drugs, aspirin, acetaminophen and ibuprofen, and caffeine in the form of coffee, were given to healthy volunteers. Blood samples were taken predose and at 1 h post-dose. Urine samples were obtained predose and at 4 h post-dose. Potential interferences by the proposed I metabolites were tested using aliquots of in vitro I

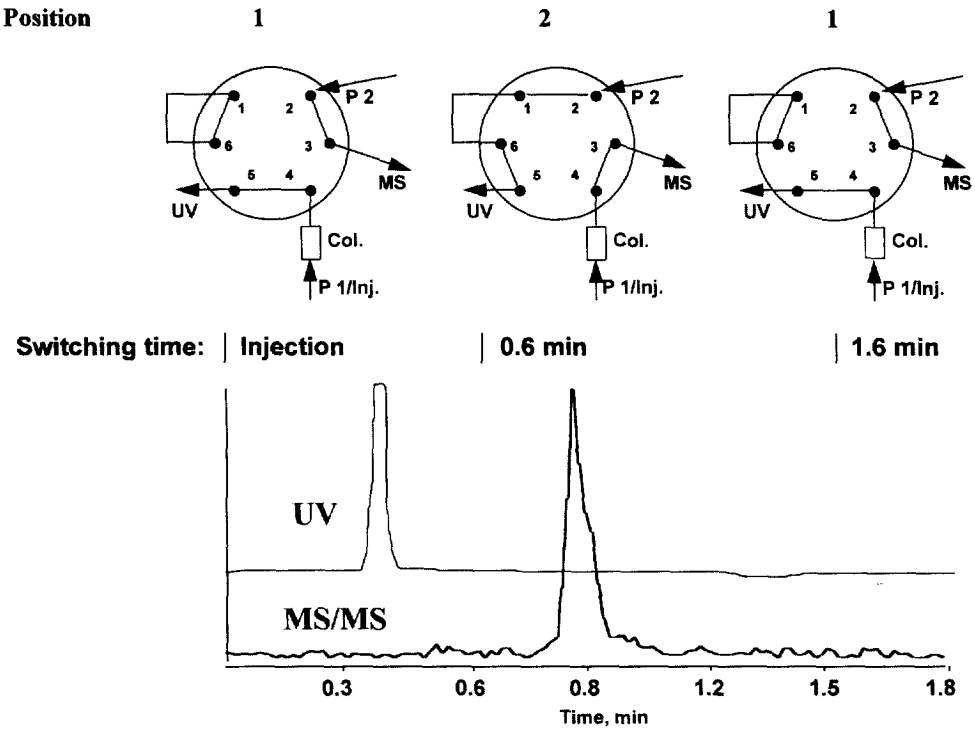

Fig. 1. Valve switching diagram and the corresponding UV and MRM profiles. P1/inj. = pump 1/injector; P2 = pump 2; col. = analytical column; MS = mass spectrometer.

incubations of dog and rat hepatocytes. The samples were extracted as described in Section 2.2.

2.7.2. Precision, accuracy, and recovery

Precision and accuracy were examined using quality control (QC) samples at 1.00, 5.00, 75.0, 150 and 400 ng ml^{-1} of I, analyzed in quadruplicate on each of 4 days. Precision was calculated by analysis of variance (ANOVA) using the following equations:

$$\%\text{R.S.D.}_w = (\text{MS}_w)^{1/2}/X_b \times 100$$

$$\%\text{R.S.D.}_b = [(\text{MS}_b - \text{MS}_w)/n]^{1/2}/X_b \times 100$$

where R.S.D.$_w$ = within-day R.S.D., R.S.D.$_b$ = between-day R.S.D., MS$_w$ = within-day variance, MS$_b$ = between-day variance, X_b = between-day mean, n = number of groups (days).

Random error was assessed from the relative standard deviation (R.S.D.). Process recovery was determined by comparison of I peak height from quality control samples and analytical standard samples prepared at the same concentrations, in 4 validation runs.

2.7.3. Blood distribution and hemolysis

Compound I was added to blank human blood samples to final concentrations of 2.00, 150 and 400 ng ml^{-1}. The blood was then centrifuged to isolate the plasma for analysis. Hemolyzed human plasma samples were prepared by freezing whole blood, and isolating the resulting hemolysate by centrifugation. Compound I was added to the hemolyzed blood at concentrations of 2.00 and 400 ng ml^{-1}. The samples were stored frozen at approximately −70°C until analyzed.

2.7.4. Stability test

Freeze–thaw stability was tested using plasma and urine samples to which I was added to final concentrations of 5.00 and 400 ng ml^{-1}. Four freeze–

thaw cycles were analyzed (−70°C to 25°C). The samples were assayed in duplicate.

Stability of I in both matrices was tested using QC samples at 5.00, 150 and 400 ng ml^{-1}. To test the stability of I in extraction/injection solvent and in thawed plasma and urine at room temperature, samples were allowed to stand on a bench top for approximately 72 h at room temperature with incidence of natural light cycles. Storage stability at −10°C was tested by placing two sets of samples in a −10°C freezer, and then analyzing after 7 days and 4 weeks storage. Stability in the spiking solutions was tested by storing the spiking solutions at approximately 4°C for four weeks. These solutions were then used to prepare plasma and urine samples, which were tested against a standard curve prepared with freshly made spiked solutions.

3. Results and discussion

3.1. LC–MS–MS

The product mass spectrum of I and its molecular structure are shown in Fig. 2. The spectrum was obtained by a flow injection of I standard into the MS system. The predominant fragment ion at m/z 218 was most likely formed from breaking the cyclic ether bond at the position indicated in Fig. 2. The proposed fragment ions at m/z 218 are shown in Fig. 3. The transition of m/z 481 to 218 was chosen for MRM. Other fragment ions were most likely formed from the breakage of C–O bonds at different positions [2]. The MRM unsmoothed ion chromatograms of plasma and urine extracts containing the matrix blank and I at 1.00 and 3.00 ng ml^{-1} are shown in Figs. 4 and 5. The signal-to-noise ratio (S/N) was at least 5 to 1 at 1 ng ml^{-1}. Compound I was eluted within 2 min with symmetric peak shape and narrow band. The mobile phase composition and the flow-rate were optimized for satisfactory peak shape and capacity factor (k'). The k' for I was calculated to be at least 1.03 so that the solvent front can be completely diverted to waste prior to the elution of I.

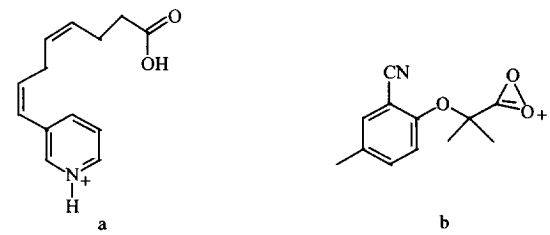

Fig. 3. Proposed fragment ions of I at m/z 218.

3.2. Calibration curve fitting

The overall mean accuracy over the calibration range was greater than 98% for both plasma and urine, using a linear fit and weightings of either $1/X$

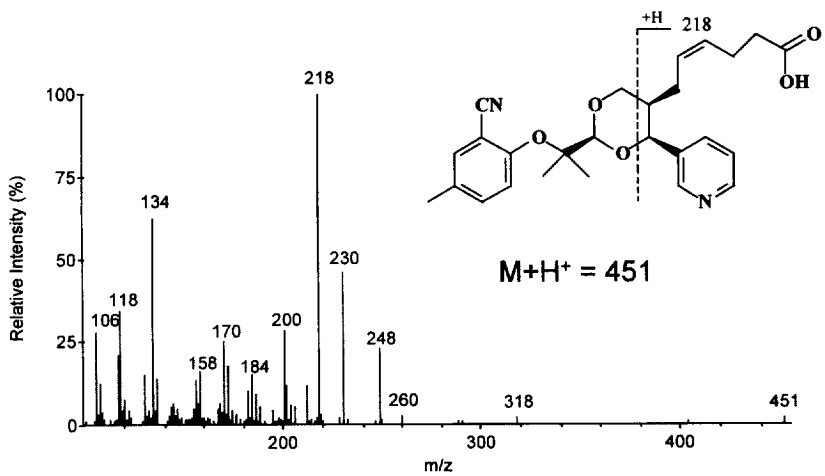

Fig. 2. Positive product ion spectrum of I.

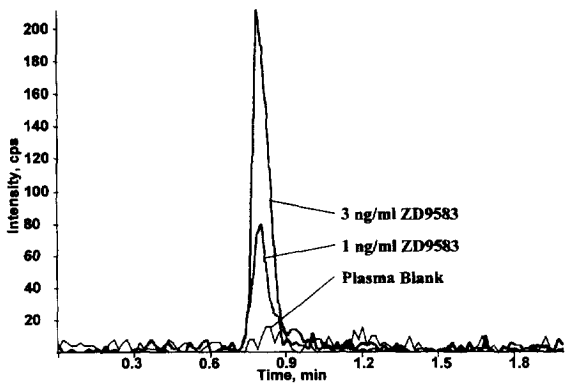

Fig. 4. Overlaid ion chromatograms of I spiked in plasma. For chromatographic and mass spectrometric conditions see Section 2.4 Section 2.5.

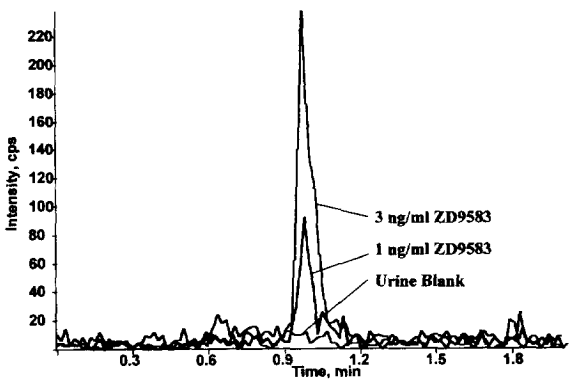

Fig. 5. Overlaid ion chromatograms of I spiked in urine. For chromatographic and mass spectrometric conditions see Section 2.4 Section 2.5.

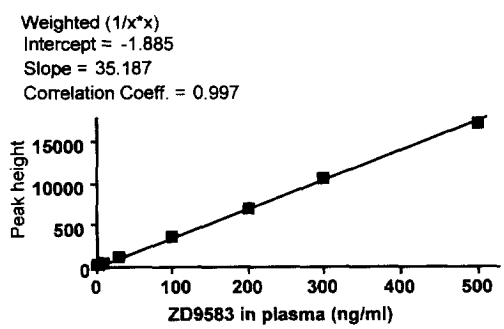

Fig. 6. Calibration curve for the determination of I in plasma. The linear range was from 1–500 ng ml^{-1}.

or $1/X^2$. However, the use of $1/X^2$ weighting gave better precision over the standard curve range, especially in urine samples. The linear fit with $1/X^2$ weighting was determined to best describe the data (Figs. 6 and 7).

3.3. Specificity

No interferences with the assay was observed in the blank plasma from twenty human volunteers, or in the plasma of volunteers after taking three OTC drugs.

Due to the lack of authentic metabolite standards, samples from in vitro I incubations of dog and rat hepatocytes were used to test potential metabolite interferences. These samples contained the following proposed metabolites: I glucuronide, I methyl ester, hydroxy I and methoxy I [8]. Samples were pretreated as described in Section 2.2 before they were analyzed by LC–MS using selected ion monitoring (SIM). The four proposed metabolites were well separated from I in both dog and rat samples (Figs. 8 and 9). There was no evidence of interferences due to the four proposed metabolites.

3.4. Assay sensitivity, precision, accuracy and recovery

The LLOQ was 1.00 ng ml^{-1} I in plasma or urine. The upper quantifiable limit was 500 ng ml^{-1}. Samples with concentrations in excess of this limit were diluted with blank plasma or urine. The method accuracy and precision were determined from QC samples at 5.00, 75.0, 150 and 400 ng ml^{-1} I. The overall mean absolute percent difference from nomi-

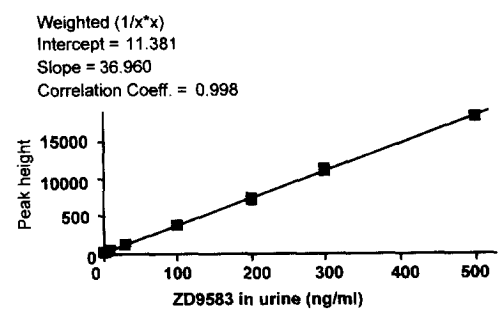

Fig. 7. Calibration curve for the determination of I in urine. The linear range was from 1–500 ng ml^{-1}.

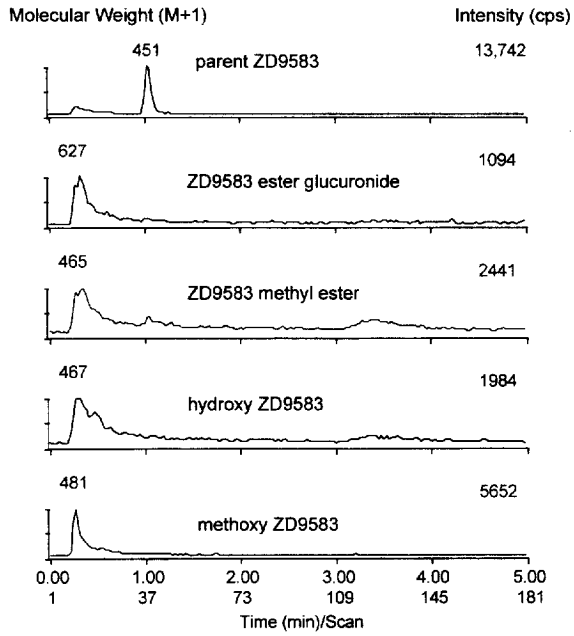

Fig. 8. Extracted ion chromatograms of proposed I metabolites found in vitro I incubations of dog hepatocytes. The sample extract was analyzed by LC–MS. For chromatographic and mass spectrometric conditions see Section 2.4 Section 2.5.

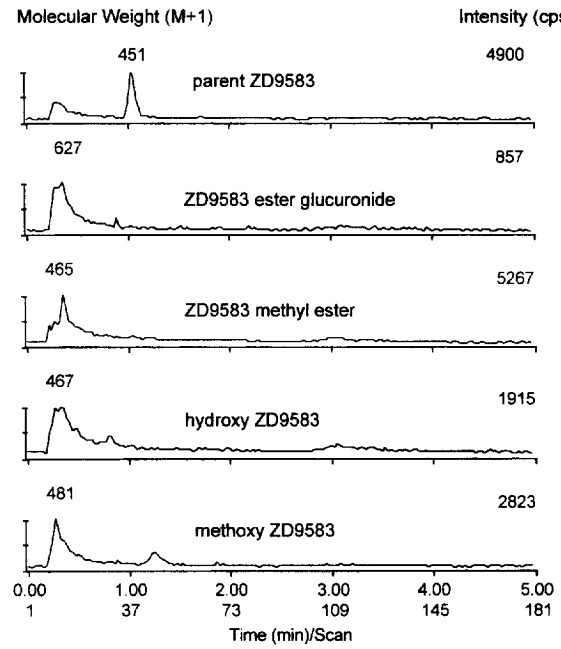

Fig. 9. Extracted ion chromatograms of proposed I metabolites found in vitro I incubations of rat hepatocytes. The sample extract was analyzed by LC–MS. For chromatographic and mass spectrometric conditions see Section 2.4 Section 2.5.

Table 1
Accuracy, and precision of the method in human plasma

Nominal concentration (ng ml^{-1})	Day	Within-day		Between-day		Difference from nominal[b] (%)
		Mean ($n=4$) (ng ml^{-1})	R.S.D.[a] (%)	Mean ($n=16$) (ng ml^{-1})	R.S.D.[a] (%)	
5.00	1	5.17	2.8	4.82	5.1	5.5
	2	4.63	6.0			
	3	4.87	2.7			
	4	4.59	6.2			
75.0	1	76.3	2.7	76.9	NC[c]	3.9
	2	76.3	2.3			
	3	78.8	0.9			
	4	76.3	6.7			
150	1	154	1.8	155	1.0	4.3
	2	155	2.7			
	3	159	2.6			
	4	152	5.5			
400	1	396	1.8	397	1.4	3.1
	2	394	2.5			
	3	410	0.5			
	4	388	7.5			
Overall mean			3.5		2.5	4.2

[a] ANOVA was used to calculate percentage R.S.D.
[b] Mean absolute percent difference.
[c] Not calculable: between-day variance is less than within-day variance.

nal concentration was ±4.2% for plasma and ±5.0% for urine. The mean within-day R.S.D. over all levels was 3.5% for plasma and 3.2% for urine. The mean between-day R.S.D. was 2.5% and 3.7% for plasma and urine, respectively (Tables 1 and 2). The overall mean I extraction recovery was 99.6% for plasma and 101% for urine samples (Table 3).

3.5. Blood distribution and hemolysis

The amount of I recovered in the plasma in the blood distribution determination ranged from 1.8–2.0 times the nominal concentration. The concentration of I in plasma was approximately twice the nominal concentration of spiked whole blood. In normal human subjects, the hematocrit is approximately 40–50%. Assuming complete separation from the red blood cells, a I concentration in plasma of 2.0–2.5 times that of spiked whole blood should be expected. The data generated were close to the theoretical prediction, supporting the choice of plasma as a matrix. The I concentrations determined in grossly hemolyzed samples were within 7% of the nominal values with R.S.D. less than 2%. Thus, gross hemolysis had no adverse effect on sample analysis.

Table 3
Extraction recovery of I from human plasma and urine

Nominal concentration (ng ml^{-1})	Mean recovery (%) (n=12)	R.S.D. (%)
I in plasma		
1.00	84.7	22.4
5.00	97.2	3.7
75.0	104	1.4
150	110	15.2
400	102	3.7
Overall mean	99.6	9.5
I in urine		
1.00	72.4	3.3
5.00	99.5	7.8
75.0	107	4.0
150	118	25.7
400	107	6.0
Overall mean	101	17.0

Table 2
Accuracy and precision of the method in human urine

Nominal concentration (ng ml^{-1})	Day	Within-day		Between-day		Difference from nominal[b] (%)
		Mean (n=4) (ng ml^{-1})	R.S.D.[a] (%)	Mean (n=16) (ng ml^{-1})	R.S.D.[a] (%)	
5.00	1	4.91	3.1	4.63	3.2	7.8
	2	4.58	2.1			
	3	4.53	6.3			
	4	4.50	7.3			
75.0	1	73.3	6.0	75.3	3.6	4.0
	2	72.3	1.8			
	3	77.1	1.6			
	4	78.5	2.7			
150	1	144	3.6	146	3.9	4.1
	2	139	3.1			
	3	148	1.9			
	4	153	2.7			
400	1	383	3.0	391	4.1	3.9
	2	372	2.0			
	3	398	0.4			
	4	410	3.9			
Overall mean			3.2		3.7	5.0

[a] ANOVA was used to calculate percentage R.S.D.
[b] Mean absolute percent difference

Table 4
Four-week stability of I in human plasma and urine stored at −70°C

Before storage		After storage		Difference (%)
Mean ($n=16$) (ng ml^{-1})	R.S.D. (%)	Mean ($n=2$) (ng ml^{-1})	R.S.D. (%)	
I in plasma				
4.82	5.1	4.86	13.5	0.8
155	1.0	155	0.9	0
397	1.4	403	2.5	1.5
I in urine				
4.63	3.2	4.38	3.0	−5.4
146	3.9	134	2.0	−8.2
391	4.1	373	0.4	−4.6

3.6. Stability studies

3.6.1. Stability under freeze–thaw cycles

Two QC samples in plasma spiked with I at 5.00 and 400 ng ml^{-1} were subjected to four freeze–thaw cycles. The concentrations (R.S.D.) at completion of the experiment were 5.04 (0.9%) and 402 (2.2%) ng ml^{-1} I. The results suggest that I is stable in human plasma after 4 freeze–thaw cycles.

3.6.2. Stability of the stock standard solution

Stock solutions of I in methanol were stored at 4°C for four weeks. Plasma and urine QC samples were prepared using these stock solutions at concentrations of 5.00, 150 and 400 ng ml^{-1} I. The sample extracts were then assayed in duplicate against freshly prepared standard solutions. The overall mean absolute percent difference from nominal concentration was ±9.9% for plasma and ±4.3% for urine. The mean R.S.D. over all levels was 1.9% for plasma and 2.5% for urine.

3.6.3. Stability under storage at −70°C

Aliquots of plasma and urine QC samples were assayed immediately when they were prepared. The rest of samples were stored at −70°C for four weeks and re-assayed. Statistical data, given in Table 4, showed no significant differences between the data sets before and after the storage. It was concluded that I showed satisfactory stability in human plasma and urine at 70°C for four weeks.

3.6.4. Light and room temperature stability

The stability of I in extraction/injection solvent and in thawed plasma and urine at room temperature was tested. Samples were exposed to incidence of natural light cycles at room temperature for 72 h. The mean absolute percent difference was less than 2.5% for both plasma and urine samples, suggesting that I was stable under these conditions.

4. Conclusions

A fast and reliable LC–MS–MS method for the determination of I in human plasma and urine was developed and validated. The validation results indicated that the method is rugged, precise and accurate and is suitable for the routine analysis of I in human plasma and urine.

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