ADVANCED
BIOANALYTICAL
SERVICES, INC.

ANALYTICAL REPORT

TITLE: **Additional Characterization of Metabolites of T-6292, T-6293 and T-6294 from Rat and Human Hepatocytes by TurboIonSpray LC/MS and LC/MS/MS. Semi-Quantitative Analysis of T-6295 in Rat and Human Hepatocytes Incubated with T-6292, T-6293 and T-6294 by LC/MS/MS.**

DATE: 27 January, 1998

REPORT: 98AGKP01.3M

AUTHOR: Grace K. Poon, PhD
Steve Lowes, PhD

PREPARED FOR: 3M Medical Department,
Toxicology Services,
St. Paul, MN 55133

NO. OF PAGES: 69

15 Catherwood Road ■ Ithaca. New York 14850 ■ (607) 266-0665 ■ Fax (607) 266-0749

ADVANCED BIOANALYTICAL SERVICES

SIGNATURE PAGE

Title: **Additional Characterization of Metabolites of T-6292, T-6293 and T-6294 from Rat and Human Hepatocytes by TurboIonSpray LC/MS and LC/MS/MS. Semi-Quantitative Analysis of T-6295 in Rat and Human Hepatocytes Incubated with T-6292, T-6293 and T-6294, by LC/MS/MS.**

Reported by: Grace Poon — 27th Jan. 1998

Grace K. Poon, PhD.
Senior Research Scientist

Date

Reviewed and Approved by: M. D Lentham — Jan. 27. 1998

Mark Lentham, B.A.
Auditor

Date

Authorized for Release by: S Lowes — 27-JAN-98

Steve Lowes, PhD.
Scientific Director
Advanced BioAnalytical Services

Date

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PART A: CHARACTERIZATION OF METABOLITES OF T-6292, T-6293 AND T-6294 FROM RAT AND HUMAN HEPATOCYTES BY TURBOIONSPRAY LC/MS AND LC/MS/MS.

1. INTRODUCTION

1.1. BACKGROUND

The metabolism of T-6292, T-6293 and T-6294 in rat hepatocytes was investigated at Advanced BioAnalytical Services, Inc. using liquid chromatography ionspray tandem mass spectrometry (Reference 1). Several metabolites had been identified, namely the glucuronide acid conjugate, the carboxylic acid, the sulfonamido alcohol, the *N*-dealkyl sulfonamido alcohol and the sulphonamide of T-6293.

1.2. STUDY OBJECTIVE

The principle aim of this work was

(1) To complete the characterization of the metabolic products of T-6292, T-6293 and T-6294 which were formed by incubating each compound with rat and human hepatocytes;

(2) structural characterization of the carboxylic acid and alcohol analogues using tandem mass spectrometry;

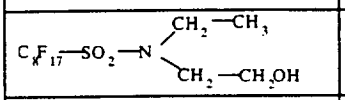
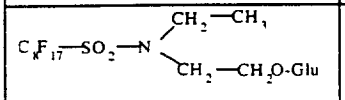
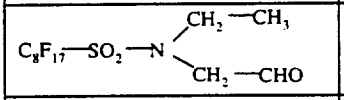
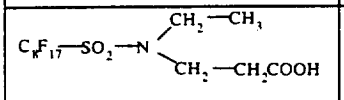
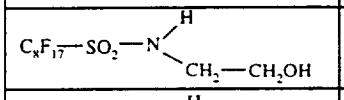
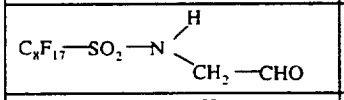
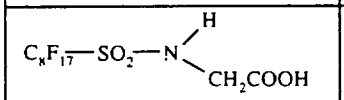
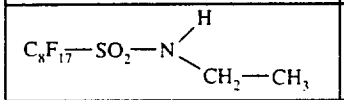
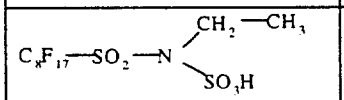
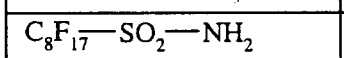
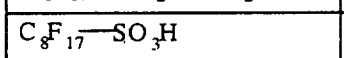
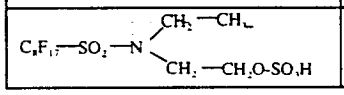
(3) identification of additional metabolites by LC/MS, using positive ionization ionspray;

(4) identification of the aldehyde and *N*-dealkyl aldehyde analogues of T-6293;

(5) semi-quantitative analysis to determine the concentration of T-6295 in the rat and human hepatocytes after incubating with T-6292, T-6293 and T-6294.

The quenched incubates were directly injected and separated on an HPLC column. Chromatographic separation of the various constituents was accomplished using gradient elution. Metabolites were characterized by LC/MS and LC/MS/MS, and the results are summarized in Tables I – III. The mass spectrometer was operated in the full scan single MS mode (*m/z* 150-800). Tandem mass spectrometry was used for further structural elucidation and confirmation.

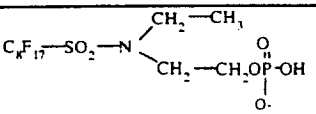
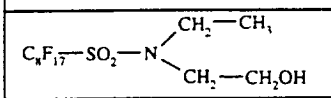
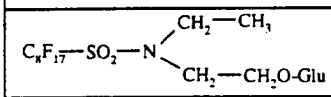
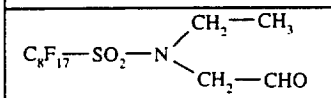
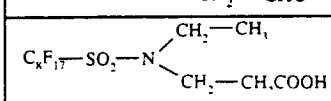
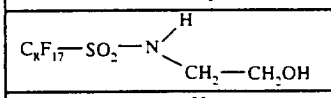
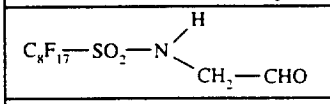
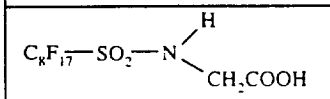
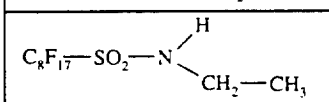
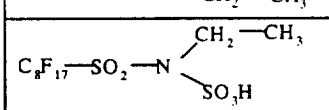
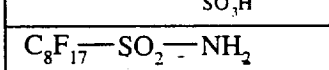
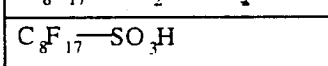
Table I: Summary of Proposed Structures of Metabolites of T-6292 Incubated with Rat and Human Hepatocytes

Proposed Structure	Abbreviation	$[M-H]^-$	Rat		Human	
			0 hour	6 hour	0 hour	6 hour
	II (T-6292)	570	No MS response	No MS response	No MS response	No MS response
	III	746	x	✓	x	✓
	IV	568	x	x	x	x
	V	584	x	✓	x	✓
	VI	542	x	✓	x	✓
	VII	540	x	trace	x	trace
	VIII	556	x	✓	x	✓
	IX (T-6294)	526	✓	✓	✓	✓
	X	606	x	trace	x	trace
	XI	498	✓	✓	✓	✓
	XII (T-6295)	499	✓	✓	x	✓
	XIII	650	x	✓	x	✓

✓ Observed

X Not observed

Table II: Summary of Proposed Structures of Metabolites of T-6293 Incubated with Rat and Human Hepatocytes

Proposed Structure	Abbreviation	$[M-H]^-$	Rat		Human	
			0 hour	6 hour	0 hour	6 hour
	I (T-6293)	650	✓	✓	✓	✓
	II (T-6292)	570	No MS response	No MS response	No MS response	No MS response
	III	746	x	✓	x	✓
	IV	568	x	x	x	x
	V	584	x	✓	x	✓
	VI	542	x	✓	x	✓
	VII	540	x	trace	x	trace
	VIII	556	x	✓	x	✓
	IX (T-6294)	526	x	✓	✓	✓
	X	606	x	x	x	x
	XI	498	x	✓	x	✓
	XII (T-6295)	499	✓	✓	✓	✓

✓ Observed

X Not observed

Table III: Summary of Proposed Structures of Metabolites of T-6294 Incubated in Rat and Human Hepatocytes

Proposed Structure	Abbreviation	$[M-H]^-$	Rat		Human	
			0 hour	6 hour	0 hour	6 hour
$C_8F_{17}—SO_2—N(H)—CH_2—CH_2OH$	VI	542	x	✓	x	✓
$C_8F_{17}—SO_2—N(H)—CH_2—CHO$	VII	540	x	x	x	x
$C_8F_{17}—SO_2—N(H)—CH_2COOH$	VIII	556	x	✓	x	✓
$C_8F_{17}—SO_2—N(H)—CH_2—CH_3$	IX (T-6294)	526	✓	✓	✓	✓
$C_8F_{17}—SO_2—N(CH_2—CH_3)—SO_3H$	X	606	x	x	x	x
$C_8F_{17}—SO_2—NH_2$	XI	498	✓	✓	✓	✓
$C_8F_{17}—SO_3H$	XII (T-6295)	499	✓	✓	✓	✓

✓ Observed
X Not observed

2. EXPERIMENTAL

2.1. CHEMICALS AND MATERIALS

T-6292, T-6293, T-6294 and T-6295 were provided by 3M Medical Department, Toxicology Services, St. Paul, MN 55133.

Polypropylene autosampler vial:	Cat # 66008-014, VWR Scientific Inc., Bridgeport, NJ 08014
Polypropylene screw-cap tubes:	16 x 100 mm, Cat # 500 765, Sun Brokers Inc., Wilmington, NC 28402
Ethanol	Cat # 112000200CSPP, Pharmaco Products, Brookefield, CT 06804

HPLC Grade Methanol	Cat # 230-4, Baxter/Scientific Products, McGaw Park, IL 60085
Dimethyl Sulfoxide	Cat # 27043-1, Sigma-Aldrich Inc., Milwaukee, WI 53233
Ammonium Acetate	Cat # 24019-2, Sigma-Aldrich Inc., Milwaukee, WI 53233

2.2. STANDARD SOLUTIONS

Stock solutions of T-6292, T-6293, T-6294 and T-6295 were prepared at a concentration of 100 µg/mL in dimethyl sulfoxide (DMSO). Working solution (10 µg/mL) of each standard were prepared by diluting 1mL of the stock solution into 9 mL ethanol. All stock solutions and working solutions were stored at 4° C and were allowed to equilibrate to room temperature before use.

Preparation of Analytical Standard Stock Solutions

Stock Solutions of T-6292, T-6293, T-6294 and T-6295 (100 µg/mL):
Each test article was accurately weighed on a micro balance and transferred to a 16 x 100 mm polypropylene tube. The solid samples were dissolved in appropriate amount of DMSO to yield a 100 µg/mL solution.

Working Solutions of T-6292, T-6293, T-6294 and T-6295 (10 µg/mL):

Each standard working solution was prepared by diluting the stock solution (1mL) with ethanol (9 mL) in a 16 x 100 mm polypropylene tube .

2.3. HPLC ELUENT PREPARATION

1 M Ammonium Acetate Solution: Ammonium acetate (77g) was added to NANOpure water (1L) in a volumetric flask. The solution was filtered through a 0.45 µm filter and stored at 4° C.

2 mM Ammonium Acetate Solution: 1 M ammonium acetate solution (2 mL) was diluted to 1L with NANOpure water. The solution was filtered through a 0.45 mm filter and stored at ambient temperature. A fresh solution was prepared every month.

Methanol : 2 mM Ammonium Acetate (90:10 v/v), Eluent: Methanol (450 mL) was mixed with 2 mM ammonium acetate solution (50 mL). The solution was stored at ambient temperature. A fresh solution was prepared every month.

Methanol : 2 mM Ammonium Acetate (10:90 v/v), Eluent: Methanol (50 mL) was mixed with 2 mM ammonium acetate solution (450 mL). The solution was stored at ambient temperature. A fresh solution was prepared every month.

2.4. SAMPLE PREPARATION

The metabolism study was carried out at SRI International, Menlo Park, CA. The incubates were quenched with an equal volume of ice-cold methanol, centrifuged and shipped in dry ice to Advanced BioAnalytical Services (ABS). The samples were stored at ABS (-20° C). During sample analysis, the supernatant was transferred to an autosampler vial. The autosampler vials were maintained in an ice-water bath at all times except during injection.

2.5. LC/MS AND LC/MS/MS INSTRUMENTATION

Liquid chromatography was performed on Shimadzu LC-10AD pumps and Shimadzu SCL10A gradient controller (Shimadzu Scientific Instruments, Inc., Columbia MD 21046). Samples were introduced with a Perkin-Elmer 200 series autosampler. The HPLC column (Betasil C18, 2 x 100 mm) was obtained from Keystone Scientific, Inc., Bellefonte, PA 16823. A Sciex API III$^+$ triple quadrupole mass spectrometer was used for sample analysis.

LC Conditions:
LC Gradient:

Time (min)	%A (10:90 CH_3OH:2mM NH_4OAc)	% B (90:10 CH_3OH:2mM NH_4OAc)
0 – 5	80 to 0 %	20 to 100 %
5 – 10	0 %	100 %
10 – 10.5	0 to 80 %	100 to 20 %
10.5 – 15	80 %	20 %

Flow rate: 200 µL/min

Typical Mass Spectrometer Conditions:

	MS Mode	**MS/MS Mode**
Source:	TurboIonSpray	TurboIonSpray
Ion Spray Voltage:	-3800 V	-3900 V
Orifice:	-78 V	-60 V
Declustering Potential:	-48 V	-30 V
Ionization Mode:	Negative	Negative
Curtain Gas (U.H.P. N_2)	1.2 LPM	1.2 LPM
Nebulizer Gas (N_2)	60 PSI	60 PSI
Collision Gas Thickness:	N/A	280 x 10^{13} atoms/square cm
Collision Energy:	N/A	35 eV
Dwell Time:	5 msec	200 msec
Step Size:	1 amu	
Scan Range:	150-800 amu	variable

Metabolite IV Transitions: m/z 568 -> 269; 526 -> 169; 526 -> 119; 526 -> 83; 526 -> 65

Metabolite VII Transitions: m/z 540 -> 269; 540 -> 169; 540 -> 119; 540 -> 83; 540 -> 65

Metabolite IX Transitions: m/z 526 -> 419; 526 -> 269; 526 -> 169;

	526 -> 123; 526 -> 97
Metabolite X Transitions:	m/z 606 -> 269; 606 -> 169; 606 -> 119; 606 -> 83; 606 -> 65
Data System:	RAD Version 2.6; LC Tune Version 2.4; Multiview Version 1.2; MacSpec Version 3.3; MacQuan Version 1.4

PPG Calibration

Instrument mass axis calibration was performed by infusion of PPG-425 calibration solution (polypropylene glycols, average molecular weight 425, dissolved in 1:1 methanol : 2 mM ammonium acetate containing 0.1 % formic acid and 0.1 % acetonitrile) at a flow rate of 10 µL/min in the positive ion mode of detection. Peak widths were approximately 0.6 amu at half-height in the single MS mode. A calibration check was performed for the analytes on Q1 by infusion of a 1 µg/mL solution in ethanol at a flow rate of 10 µL/min. The mass spectrometric parameters and sensitivity were optimized using 60% B of the HPLC mobile phase at 200 µL/min.

2.6. DATA HANDLING

All raw chromatographic and mass spectrometric data were stored on the ABS file server ABS1_FS.DATA in the file hierarchy "RAWDATA:Validation:3MSRI:3MSRI Qualitative". All experimental information was stored in ABS notebook No: 2070.

3. RESULTS AND DISCUSSION

T-6293, T-6294 and T-6295 displayed $[M-H]^-$ ions at m/z 650, 526 and 499 respectively.

The reconstructed ion chromatograms and negative ionization product ion mass spectra of compounds T-6293, T-6294 and T-6295 are shown in Figures 1 –3. Their fragmentation patterns were described in details in previous study [reference 1]. As observed before, T-6292 did not show any signal in positive or negative ionization modes

[reference 1]. Therefore, no attempt was made to characterize T-6292 as a parent drug or a metabolite in this study.

Rat hepatocytes (reference # 4) and human hepatocytes (Female 1, reference #HH357) were used for the study.

3.1. CONTROL EXPERIMENTS

The full scan reconstructed ion chromatograms of the incubation media which contained rat and human hepatocytes but no test articles are displayed in Figures 4 and 5 respectively. These samples were analysed as control samples. The blank samples did not show any signal at the retention times expected for the T-6292, T-6293 and T-6294 metabolites.

The full scan reconstructed ion chromatograms of the incubation media which contained T-6292, T-6293 and T-6294 but no hepatocytes are displayed in Figures 6, 7 and 8 respectively. These samples were analysed as control samples. However, compounds IX, and XI were present in the T-6292 sample (Figure 6). In the T-6293 sample, apart from the parent drug, compound XII was observed (Figure 7). In the T-6294 sample, apart from the parent drug, compound XI was detected (Figure 8).

3.2. METABOLISM OF T-6292

3.2.1. Metabolism of T-6292 by Rat Hepatocytes at 0 hour

The full scan LC/MS reconstructed ion chromatograms of the rat hepatocytes sample incubated with T-6292 for 0 hour are shown in Figures 9. Figure 9 (i – x) displays the ion chromatograms for the following: the parent compound (*m/z* 570), metabolite III (*m/z* 746), metabolite V (*m/z* 584), metabolite VI (*m/z* 542), metabolite VIII (*m/z* 556), metabolite IX (*m/z* 526), metabolite X (*m/z* 606), metabolite XI (*m/z* 498), metabolite XII (*m/z* 499) and metabolite XIII (*m/z* 650). Even though compound IX, XI and XII were detected in this sample, compound IX and XI were observed in the control sample containing T-6292 and no hepatocytes (Figure 6).

3.2.2. Metabolism of T-6292 by Rat Hepatocytes at 6 hour

The full scan LC/MS reconstructed ion chromatograms of the rat hepatocytes sample incubated with T-6292 for 6 hour are shown in Figures 10. Figure 10 (i – x) displays the ion chromatograms for the following: the parent compound (*m/z* 570), metabolite III (*m/z* 746), metabolite V (*m/z* 584), metabolite VI (*m/z* 542), metabolite VIII (*m/z* 556), metabolite IX (*m/z* 526), metabolite X (*m/z* 606), metabolite XI (*m/z* 498), metabolite XII (*m/z* 499) and metabolite XIII (*m/z* 650). This sample contained metabolites III, V, VI, VIII, IX, XI, XII and XIII.

Full scan mass spectra were not obtained for metabolites IV (*m/z* 568), VII (*m/z* 540), metabolite IX (*m/z* 526) and X (*m/z* 606). As an alternative, a selected reaction monitoring (SRM) analysis was performed. For metabolite IV, the formation of *m/z* 269, 169, 119, 83 and 65 ions from the precursor ion at *m/z* 568 were monitored. For metabolite VII, the formation of *m/z* 269, 169, 119, 83 and 65 ions from the precursor ion at *m/z* 540 were monitored. For metabolite IX, the formation of *m/z* 269, 169, 119, 123 and 97 ions from the precursor ion at *m/z* 526 were monitored. For metabolite X, the formation of *m/z* 269, 169, 119, 97, 83 and 65 ions from the precursor ion at *m/z* 606 were monitored. Since these product ions were characteristic of this class of compounds. Tentative identification of metabolites IV and VII was achieved in the study samples when signals were observed at their appropriate transitions.

No metabolite IV was detected in the 0 or 6 hour rat study samples (Figure 14 i and ii). Metabolite VII was present in the rat hepatocytes treated with T-6292 for 6 hours (Figure 15 iii), but absent in the 0 hour sample (Figure 15 ii), and the no test article or no hepatocytes control samples (Figures 13 i and 15 i).

Figure 16 (ii) and (iii) represent the SRM total ion chromatograms (TIC) of rat hepatocytes incubated with T-6292 for 0 and 6 hour respectively, showing the presence of metabolite IX in the samples. However, this component could also be observed in the control sample containing T-6292 and no hepatocytes (Figure 16 i).

Metabolite X was present in the rat hepatocytes treated with T-6292 for 6 hours (Figure 17 iii) but absent in the 0 hour sample (Figure 17 ii) and the no hepatocytes control sample (Figure 17 i).

A potential metabolite (XIII) was observed at 6.1 min and displayed an $[M-H]^-$ ion at *m/z* 650 (Figure 10 x). An addition of 80 mass units relative to T6292 could correspond to the addition of a sulphate moiety. The product-ion mass spectrum of metabolite XIII in Figure 33(i) showed fragment ions at *m/z* 526, 419 ($C_8F_{17}^-$), 319 ($C_6F_{13}^-$), 269 ($C_5F_{11}^-$), 219 ($C_4F_9^-$), 169 ($C_3F_7^-$), 97 (HSO_4^- or $FNSO_2^-$) and 80 (SO_3^-). The fragment ions at *m/z* 80 is consistent with the presence of the sulphate moiety.

3.2.3. Metabolism of T-6292 in Human Hepatocytes at 0 hour

The full scan LC/MS reconstructed ion chromatograms of the human hepatocytes sample incubated with T-6292 for 0 hour are shown in Figures 11. Figure 11 (i – x) displays the ion chromatograms for the following: the parent compound (*m/z* 570), metabolite III (*m/z* 746), metabolite V (*m/z* 584), metabolite VI (*m/z* 542), metabolite VIII (*m/z* 556), metabolite IX (*m/z* 526), metabolite X (*m/z* 606), metabolite XI (*m/z* 498), metabolite XII (*m/z* 499) and metabolite XIII (*m/z* 650). Compounds IX and XI were detected in this sample, as they were observed in the control sample containing T-6292 and no hepatocytes (Figure 6 vii and ix).

3.2.4. Metabolism of T-6292 by Human Hepatocytes at 6 hour

The full scan LC/MS reconstructed ion chromatograms of the human hepatocytes sample incubated with T-6292 for 6 hour are shown in Figures 12. Figure 12 (i – x) displays the ion chromatograms for the following: the parent compound (*m/z* 570), metabolite III (*m/z* 746), metabolite V (*m/z* 584), metabolite VI (*m/z* 542), metabolite VIII (*m/z* 556), metabolite XI (*m/z* 498), metabolite IX (*m/z* 526), metabolite X (*m/z* 606), metabolite XII (*m/z* 499) and metabolite XIII (*m/z* 650). This sample contained the metabolites III, V, VI, VIII, IX, XI, XII and XIII.

The SRM total ion chromatograms for metabolites IV (*m/z* 568), VII (*m/z* 540) and X were displayed in Figures 14, 15, 16 and 17. No metabolite IV was detected in the study samples (Figure 14 iii and iv). Metabolite VII was present in the human hepatocytes sample treated with T-6292 for 6 hours (Figure 15 v), but absent in the 0 hour sample (Figure 15 iv), and the no test article or no hepatocytes control samples (Figures 13 iii and 15 i).

The SRM total ion chromatograms of human hepatocytes incubated with T-6292 for 0 and 6 hour are displayed in Figure 16 (iv and v), respectively, showing the presence of metabolite IX in both samples. However, this component could also be observed in the control sample containing T-6292 and no hepatocytes (Figure 16 i).
Metabolite X was observed in the human hepatocytes sample incubated for 6 hours with T-6292 (Figure 17 v), but absent in the 0 hour sample (Figure 17 iv), and the no hepatocytes control sample (Figure 17 i).

3.3. METABOLISM OF T-6293

3.3.1. Metabolism of T-6293 by Rat Hepatocytes at 0 hour

The full scan LC/MS reconstructed ion chromatograms of the rat hepatocytes sample incubated with T-6293 for 0 hour are shown in Figure 18. Figure 18 (i – x) displays the ion chromatograms for the following: the parent compound (*m/z* 650), metabolite II (*m/z* 570), metabolite III (*m/z* 746), metabolite V (*m/z* 584), metabolite VI (*m/z* 542), metabolite VIII (*m/z* 556), metabolite IX (*m/z* 526), metabolite X (*m/z* 606), metabolite XI (*m/z* 498) and metabolite XII (*m/z* 499). Apart from the parent drug, compound XII was detected in this sample. However compound XII was also observed in the control sample containing T-6293 and no hepatocytes (Figure 7x).

3.3.2. Metabolism of T-6293 by Rat Hepatocytes at 6 hour

The full scan LC/MS reconstructed ion chromatograms of the rat hepatocytes sample incubated with T-6293 for 6 hour are shown in Figure 19. Figure 19 (i – x) displays the ion chromatograms for the following: the parent compound (*m/z* 650), metabolite II

(*m/z* 570), metabolite III (*m/z* 746), metabolite V (*m/z* 584), metabolite VI (*m/z* 542), metabolite VIII (*m/z* 556), metabolite IX (*m/z* 526), metabolite X (*m/z* 606), metabolite XI (*m/z* 498) and metabolite XII (*m/z* 499). This sample contained metabolites III, V, VI, VIII, IX, XI and XII.

SRM analyses were performed for metabolites IV (*m/z* 568), VII (*m/z* 540) and IX (*m/z* 526) and X (*m/z* 606). No metabolite IV was detected in the study samples (Figure 22 i and ii). Metabolite VII was present in the rat hepatocytes treated with T-6293 for 6 hours (Figure 23 iii), but absent in the 0 hour sample (Figure 23 ii) and the no test article or no hepatocytes control samples (Figures 13 ii and 23 i). Compound IX was found in the T-6293 standard solution and the 6 hour rat hepatocytes sample incubated with T-6293 (Figure 24 i, iv and v). No metabolite X was observed in the rat hepatocytes study samples (Figure 25 ii and iii).

3.3.3. Metabolism of T-6293 by Human Hepatocytes at 0 hour

The full scan LC/MS reconstructed ion chromatograms of the human hepatocytes sample incubated with T-6293 for 0 hour are shown in Figures 20. Figure 20 (i – x) displays the ion chromatograms for the following: the parent compound (*m/z* 650), metabolite II (*m/z* 570), metabolite III (*m/z* 746), metabolite V (*m/z* 584), metabolite VI (*m/z* 542), metabolite VIII (*m/z* 556), metabolite IX (*m/z* 526), metabolite X (*m/z* 606), metabolite XI (*m/z* 498) and metabolite XII (*m/z* 499). Apart from the parent drug, compound XII was detected in this sample, as was observed in the control sample containing T-6293 and no hepatocytes (Figure 7 x).

3.3.4. Metabolism of T-6293 by Human Hepatocytes at 6 hour

The full scan LC/MS reconstructed ion chromatograms of the human hepatocytes sample incubated with T-6293 for 6 hour are shown in Figures 21. Figure 21 (i – x) displays the ion chromatograms for the following: the parent compound (*m/z* 650), metabolite II (*m/z* 570), metabolite III (*m/z* 746), metabolite V (*m/z* 584), metabolite VI (*m/z* 542), metabolite VIII (*m/z* 556), metabolite IX (*m/z* 526), metabolite X (*m/z* 606), metabolite

XI (*m/z* 498) and metabolite XII (*m/z* 499). This sample contained metabolites III, V, VI, VIII, IX, XI and XII.

SRM analyses were performed for metabolites IV (*m/z* 568), VII (*m/z* 540), IX (*m/z* 526) and X (*m/z* 606). No metabolite IV was detected in the study samples (Figure 22 iii and iv). Metabolite VII was present in the human hepatocytes treated with T-6293 for 6 hours (Figure 23 v) but absent in the 0 hour sample (Figure 23 iv) and the no hepatocytes control sample (Figure 23 i). Metabolite IX was found in the 0 and 6 hour study samples (Figure 24 iv and v). No metabolite X was observed in the human hepatocytes study samples (Figure 25 iv and v).

3.4. METABOLISM OF T-6294

3.4.1. Metabolism of T-6294 by Rat Hepatocytes at 0 hour

The full scan LC/MS reconstructed ion chromatograms of the rat hepatocytes sample incubated with T-6294 for 0 hour are shown in Figure 26. Figure 26 (i – vii) displays the ion chromatograms for the following: metabolite VI (*m/z* 542), metabolite VII (*m/z* 540), metabolite VIII (*m/z* 556), the parent compound (*m/z* 526), metabolite X (*m/z* 606), metabolite XI (*m/z* 498) and metabolite XII (*m/z* 499). Apart from the parent drug, compounds XI and XII were detected in this sample. However compound XI was also observed in the control sample containing T-6294 and no hepatocytes (Figure 8v).

3.4.2. Metabolism of T-6294 by Rat Hepatocytes at 6 hour

The full scan LC/MS reconstructed ion chromatograms of the rat hepatocytes sample incubated with T-6294 for 6 hour are shown in Figure 27. Figure 27 (i – vii) displays the ion chromatograms for the following: metabolite VI (*m/z* 542), metabolite VII (*m/z* 540), metabolite VIII (*m/z* 556), the parent compound (*m/z* 526), metabolite X (*m/z* 606), metabolite XI (*m/z* 498) and metabolite XII (*m/z* 499). This sample contained metabolites VI, VIII, IX, XI and XII.

SRM analyses were performed for metabolites VII (*m/z* 540) and X (*m/z* 606). No metabolite VII was present in the 0 or 6 hours rat hepatocytes samples (Figure 30 ii and iii). No metabolite X was observed in the 0 or 6 hours rat hepatocytes study samples (Figure 31 ii and iii).

3.4.3. Metabolism of T-6294 by Human Hepatocytes at 0 hour

The full scan LC/MS reconstructed ion chromatograms of the human hepatocytes sample incubated with T-6294 for 0 hour are shown in Figure 28. Figure 28 (i – vii) displays the ion chromatograms for the following: metabolite VI (*m/z* 542), metabolite VII (*m/z* 540), metabolite VIII (*m/z* 556), the parent compound (*m/z* 526), metabolite X (*m/z* 606), metabolite XI (*m/z* 498) and metabolite XII (*m/z* 499). Apart from the parent drug, compounds XI and XII were detected in this sample. However compound XI was also observed in the control sample containing T-6294 and no hepatocytes (Figure 8v).

3.4.4. Metabolism of T-6294 by Human Hepatocytes at 6 hour

The full scan LC/MS reconstructed ion chromatograms of the human hepatocytes sample incubated with T-6294 for 6 hour are shown in Figure 29. Figure 29 (i – vii) displays the ion chromatograms for the following: metabolite VI (*m/z* 542), metabolite VII (*m/z* 540), metabolite VIII (*m/z* 556), the parent compound (*m/z* 526), metabolite X (*m/z* 606), metabolite XI (*m/z* 498) and metabolite XII (*m/z* 499). This sample contained metabolites VI, VIII, IX, XI and XII.

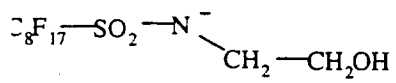
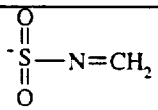
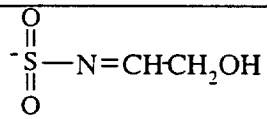
SRM analyses were performed for metabolites VII (*m/z* 540) and X (*m/z* 606). No metabolite VII was present in the 0 or 6 hours human hepatocytes samples (Figure 30 iv and v). No metabolite X was observed in the 0 or 6 hours human hepatocytes study samples (Figure 31 iv and v).

3.5. PRODUCT-ION MASS SPECTRA

The product-ion mass spectrum of metabolite III ($[M-H]^-$ at *m/z* 746) is displayed in Figure 32 i. The base peak is at *m/z* 526 corresponds to the loss of $-CH_2CH_2OGlu$ fragment. This negative ion mass spectrum contained a fragment ion at *m/z* 113, due to

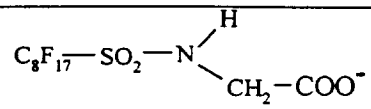
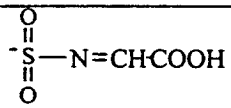
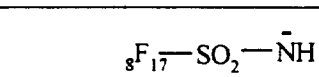
internal fragmentation of the glucuronide acid and it indicates the presence of a glucuronide conjugate.

Table IV: Proposed product-ion fragmentation for Metabolite VI

Proposed Structure	*m/z*
	542
HSO_2^-	65
	92
	122
$C_3F_7^-$	169
$C_4F_9^-$	219
$C_5F_{11}^-$	269
$C_6F_{13}^-$	319
$C_8F_{17}^-$	419

The product-ion mass spectrum of metabolite VI is displayed in Figure 32 ii and the corresponding fragment ions are illustrated in Table IV.

Table V: Proposed product-ion fragmentation for Metabolite VIII

Proposed Structure	*m/z*
	556
HSO_2^-	65
FSO_2^-	83
	136
$C_4F_9^-$	219
$C_5F_{11}^-$	269
	498

The product-ion mass spectrum of metabolite VIII is displayed in Figure 32 iii. The fragmentation pattern at m/z 498, 219, 169, 83 and 65 is comparable to that observed for Metabolite VI (Figure 32 ii), and the corresponding fragment ions are shown in Table V.

The product-ion mass spectrum of metabolite XI is shown in Figure 33 ii. The fragment ions at *m/z* 478 and 78 represent a loss of HF and $C_8F_{17}H$ moieties from the deprotonated molecule, respectively.

3.6. Positive Ionization LC/MS Analysis of Human Hepatocytes Incubated with T-6292 and T-6293

The human hepatocytes incubated with T-6292 and T-6293 for 6 hours were also examined by full scan LC/MS using positive ionization detection. However, the compounds of interest could not be detected in the positive ionization mode, and there were endogenous materials present in the samples at high concentration; therefore neither the parent drug nor the metabolite was observed in this analysis.

PART B: Semi-Quantitative Analysis of T-6295 in Rat and Human Hepatocytes Incubated with T-6292, T-6293 and T-6294 by LC/MS/MS

4. INTRODUCTION

T-6292, T-6293 and T-6294 undergo metabolic biotransformation in rat and human hepatocytes to form T-6295. The purpose of this study was to determine the concentration of T-6295 in human and rat hepatocytes after incubating with T-6292, T-6293 and T-6295, using LC/MS/MS analysis.

5. EXPERIMENTAL

5.1. STANDARD SOLUTIONS

Preparation of Analytical Standard Solutions for T-6292, T-6293 and T-6294:
See Experimental Section 2.2.

Preparation of Internal Standard Stock Solution (1 mg/mL): 1H,1H,2H,2H perfluoro octane sulfonic acid -
Accurately weigh 2 mg of the 1H,1H,2H,2H perfluoro octane sulfonic acid on a micro balance and transfer the chemical to a polypropylene tube. Dilute with an appropriate volume of methanol to yield a 1 mg/mL solution. Store the solution at 4° C and bring to ambient temperature before use.

Preparation of Internal Standard Working Solution (8 µg/mL): 1H,1H,2H,2H perfluoro octane sulfonic acid -
Dilute 0.8 mL of analytical standard stock solution with water in a 100 mL volumetric flask to yield a 8 µg/mL solution. Store the solution at 4° C and bring to ambient temperature before use.

Preparation of Internal Standard Spiking Solution (800 ng/mL):
1H,1H,2H,2H perfluoro octane sulfonic acid (10 mL at 8 µg/mL concentration) was diluted with NANOpure water (90 mL) in a 100 mL volumetric flask. 25 µL of this solution was added to each study sample.

5.2. PREPARATION OF QUENCHING SOLUTION:

Methanol was mixed with hepatocytes control media solution for B011-95 (supplied by 3M) in the ratio of 2:1 (v/v).

5.3. HPLC ELUENT PREPARATION:

See Experimental Section 2.3.

5.4. SAMPLE PREPARATION

The metabolism study was carried out at SRI International, Menlo Park, CA. The incubates were quenched with an equal volume of ice-cold methanol, centrifuged and shipped in dry ice to ABS. The samples were stored at -20° C.

Hepatocytes isolated from Male Rat #2, Female Rat #5, Male Human #341 and Female Human #2, each incubated with 0.1 mM of T-6292, T-6293 and T-6294, respectively, were analysed for T-6295.

Rat and Human Hepatocytes incubated with no test articles for 0 and 6 hours were used as control samples.

Five stock solutions prepared in DMSO with no hepatocytes (supplied by SRI International): T-6292 from rat 2 and rat 5 studies; T-6293 from rat 2 study; T-6294 from rat 2 and rat 5 studies (10 µL each) were also used as control samples. Each sample (10 µL) containing the analytical standard was diluted with the quenching solution (90 µL), then spiked with the internal standard solution (25 µL).

The internal standard solution (25 µL of 1H,1H,2H,2H perfluoro octane sulfonic acid, at 800 ng/mL concentration) was added per study sample (100 µL).

5.5. PREPARATION OF RAT HEPATOCYTES STANDARD CURVE SAMPLES

Appropriate volumes of T-6295 working solution was spiked to control rat hepatocytes according to the scheme outlined below:

Plasma Standard Prep.	T-6295 Conc.	Soln #	mL added	Control rat Hepatocytes (mL)	Volume (mL)
7	500	Working Soln	0.025	0.475	0.5
6	250	Std#7	0.150	0.150	0.3
5	100	Std#7	0.048	0.192	0.24
4	50	Std#7	0.048	0.432	0.48
3	10	Std#4	0.096	0.384	0.48
2	2	Std#3	0.048	0.192	0.24
1	1	Std#3	0.024	0.216	0.24
0	0	0	0	0.250	0.25

One standard curve was injected at the beginning of the analytical run and a second standard curve was injected at the end of the analytical run.

5.6. LC/MS/MS INSTRUMENTATION

5.6.1. HPLC conditions

HPLC Column Betasil C18 (2 x 100 mm)

Mobile Phase 10:90 MeOH:2mM NH_4OAc (A)
90:10 MeOH:2mM NH_4OAc (B)

LC Gradient

Time	%B
0 to 2 min	30%
2 to 4 min	30 to 100%
4 to 7 min	100%
7 to 8 min	100 to 30%

Flow rate 200 µL/min

5.6.2. MS conditions

Sciex API III$^+$ TurboIonSpray™ Interface

Ion Spray Voltage: -3900 V

Orifice: -60 V

Declustering Potential -30 V

Ionization mode	Negative
Collision Gas Thickness	280 x 10^{13} atoms/square cm
Collision Energy	35 eV
Dwell Time	200 msec
Transitions Monitored:	*m/z* 499 -> 99 for T-6295
	m/z 499 -> 80 for T-6295
	m/z 427 -> 81 for internal standard

The product-ion mass spectrum of T-6295 ($[M-H]^-$ at *m/z* 499) produced two fragment ions at *m/z* 80 and 99 (see Figure 3 iii), which are likely due to (SO_3^-) and (FSO_3^-) respectively. Both transitions were monitored.

Data System	RAD Version 2.6; LC Tune Version 2.4;
	Multiview Version 1.2; MacQuan Version 1.4

5.6.3. Data Handling

All raw chromatographic and mass spectrometric data were stored on the ABS file server ABS1_FS.DATA in the file hierarchy "RAWDATA:Validation:3MSRI:3MSRI Semi-Quan". All experimental information was stored in ABS notebook No: 2070.

6. RESULTS AND DISCUSSION:

One set of male rat hepatocytes, one set of female rat hepatocytes, one set of male human hepatocytes and one set of female human hepatocyte samples were analysed by LC/MS/MS. The results are shown in Table VI.

The same set of standard calibration curve samples was used to analyse the five stock solutions. The results are shown in Table VII.

Table VI **Quantitative Analysis of T-6295 in Rat and Human Hepatocytes after Incubation with T-6292, T-6293 and T-6294**

Weighted (1/x)				
Intercept =	0.00193			
Slope =	0.00331			
Correlation Coeff.	0.9961			
Filename	**Sample Name**	**Conc. (ng/mL)**	**Calc. Conc. (ng/mL)**	**Accuracy**
DB_1	Double Blank	n/a	n/a	n/a
DB_2	Double Blank	n/a	n/a	n/a
H341920	H341/T6292 t=0	n/a	0.36	n/a
H341926	H341/T6292 t=6	n/a	n/a	n/a
H341930	H341/T6293 t=0	n/a	7.319	n/a
H341936	H341/T6293 t=6	n/a	n/a	n/a
H341940	H341/T6294 t=0	n/a	1.051	n/a
H341946	H341/T6294 t=6	n/a	n/a	n/a
H341C920	H341 No Hep T6292 t=0	n/a	n/a	n/a
H341C926	H341 No Hep T6292 t=6	n/a	n/a	n/a
H341C930	H341 No Hep T6293 t=0	n/a	6.574	n/a
H341C936	H341 No Hep T6293 t=6	n/a	6.042	n/a
H341C940	H341 No Hep T6294 t=0	n/a	0.491	n/a
H341C946	H341 No Hep T6294 t=6	n/a	0.603	n/a
H341NTA0	H341 No Test Article t=0	n/a	n/a	n/a
H341NTA6	H341 No Test Article t=6	n/a	n/a	n/a
HF2920	Human Female 2/T6292 t=0	n/a	n/a	n/a
HF2926	Human Female 2/T6292 t=6	n/a	15.049	n/a
HF2930	Human Female 2/T6293 t=0	n/a	6.946	n/a
HF2936	Human Female 2/T6293 t=6	n/a	9.294	n/a
HF2940	Human Female 2/T6294 t=0	n/a	6.418	n/a
HF2946	Human Female 2/T6294 t=6	n/a	5.373	n/a
HF2C920	Human Female 2 No Hep T6292 t=0	n/a	n/a	n/a
HF2C926	Human Female 2 No Hep T6292 t=6	n/a	n/a	n/a
HF2C930	Human Female 2 No Hep T6293 t=0	n/a	7.706	n/a
HF2C936	Human Female 2 No Hep T6293 t=6	n/a	8.478	n/a
HF2C940	Human Female 2 No Hep T6294 t=0	n/a	6.739	n/a
HF2C946	Human Female 2 No Hep T6294 t=6	n/a	5.888	n/a
HF2NTA0	Human Female 2 No Test Article t=0	n/a	n/a	n/a
HF2NTA6	Human Female 2 No Test Article t=6	n/a	n/a	n/a
R2920	Rat Male 2/T6292 t=0	n/a	0.993	n/a
R2926	Rat Male 2/T6292 t=6	n/a	28.785	n/a
R2930	Rat Male 2/T6293 t=0	n/a	10.621	n/a
R2936	Rat Male 2/T6293 t=6	n/a	16.526	n/a
R2940	Rat Male 2/T6294 t=0	n/a	6.168	n/a
R2946	Rat Male 2/T6294 t=6	n/a	5.439	n/a
R2C920	Rat Male 2 No Hep T6292 t=0	n/a	1.041	n/a
R2C926	Rat Male 2 No Hep T6292 t=6	n/a	n/a	n/a
R2C930	Rat Male 2 No Hep T6293 t=0	n/a	6.714	n/a

Table VI Quantitative Analysis of T-6295 in Rat and Human Hepatocytes after Incubation with T-6292, T-6293 and T-6294 (continued)

Filename	Sample Name	Conc. (ng/mL)	Calc. Conc. (ng/mL)	Accuracy
R2C936	Rat Male 2 No Hep T6293 t=6	n/a	8.454	n/a
R2C940	Rat Male 2 No Hep T6294 t=0	n/a	4.446	n/a
R2C946	Rat Male 2 No Hep T6294 t=6	n/a	3.615	n/a
R2NTA0	Rat Male 2 No Test Article t=0	n/a	n/a	n/a
R2NTA6	Rat Male 2 No Test Article t=6	n/a	n/a	n/a
R5920	Rat female 5/T6292 t=0	n/a	n/a	n/a
R5926	Rat female 5/T6292 t=6	n/a	33.934	n/a
R5930	Rat female 5/T6293 t=0	n/a	7.862	n/a
R5936	Rat female 5/T6293 t=6	n/a	14.147	n/a
R5940	Rat female 5/T6294 t=0	n/a	0.856	n/a
R5946	Rat female 5/T6294 t=6	n/a	8.957	n/a
R5C920	Rat female 5 No Hep T6292 t=0	n/a	n/a	n/a
R5C926	Rat female 5 No Hep T6292 t=6	n/a	7.871	n/a
R5C930	Rat female 5 No Hep T6293 t=0	n/a	7.777	n/a
R5C936	Rat female 5 No Hep T6293 t=6	n/a	6.96	n/a
R5C940	Rat female 5 No Hep T6294 t=0	n/a	n/a	n/a
R5C946	Rat female 5 No Hep T6294 t=6	n/a	4.095	n/a
R5NTA0	Rat female 5 No Test Article t=0	n/a	n/a	n/a
R5NTA6	Rat female 5 No Test Article t=6	n/a	n/a	n/a
Std_1 1		n/a	11.643	n/a
Std_1 2		1	1.054	105.367
Std_2 1		2	1.907	95.35
Std_2 2		2	1.732	86.576
Std_3 1		10	9.98	99.799
Std_3 2		10	10.588	105.883
Std_4 1		50	57.979	115.958
Std_4 2		50	54.431	108.862
Std_5 1		100	98.732	98.732
Std_5 2		100	80.054	80.054
Std_6 1		250	259.142	103.657
Std_6 2		250	249.402	99.761
Zero_1		n/a	n/a	n/a
Zero_2		n/a	n/a	n/a

T-6295 was not detected in the control samples which contained only the hepatocytes and no test articles (Table VI).

For the rat and human hepatocytes, T-6295 was detected in the control samples which were incubated with T-6293 and T-6294 but with no hepatocytes (Table VI).

For the male human hepatocytes study samples, T-6295 was present in the 0 hour samples incubated with T-6292, T-6293 and T-6294. T-6295 was not observed in the 6 hour samples.

For the female human hepatocytes study samples, T-6295 was present in the 0 hour sample incubated with T-6292, 0 and 6 hour samples incubated with T-6293 and T-6294, and was not observed in the 6 hour samples incubated with T-6292 (Table VI).

For the male rat hepatocytes study samples, T-6295 was detected in the 0 and 6 hour samples incubated with T-6292, T-6293 and T-6294 (Table VI).

For the female rat hepatocytes study samples, T-6295 was present in the 6 hour sample incubated with T-6292, 0 and 6 hour samples incubated with T-6293 and T-6294, and was not observed in the 0 hour sample incubated with T-6292 (Table VI).

LC/MS/MS analyses of the stock solutions showed T-6295 was present at high concentration in all the samples (Table VII). Since carryover for T-6295 was observed during sample analysis, this affects the values of the second set of standard curve, particularly the calibration standards at low concentrations.

Table VII Quantitative Analysis of T-6295 in T-6292, T-6293 and T-6294

Stock Solutions

Weighted (1/x) Intercept = Slope = Correlation Coeff. =	 0.00150 0.00359 0.9910		
Filename	Conc.	Calc. Conc. (ng/mL)	Accuracy (ng/mL)
DB_1	0	n / a	n / a
DB_2	0	n / a	n / a
R2T6292Stock	n / a	125.713	n / a
R2T6293Stock	n / a	961.102	n / a
R2T6294Stock	n / a	671.542	n / a
R5T6292Stock	n / a	160.661	n / a
R5T6294Stock	n / a	210.365	n / a
Std_1 1	1	1.256	125.56
Std_1 2	n / a	5.563	n / a
Std_2 1	2	1.569	78.46
Std_2 2	n / a	4.772	n / a
Std_3 1	10	9.307	93.07
Std_3 2	n / a	12.66	n / a
Std_4 1	50	53.853	107.71
Std_4 2	50	59.95	119.9
Std_5 1	100	97.415	97.41
Std_5 2	100	70.035	70.04
Std_6 1	250	245.415	98.17
Std_6 2	250	274.2	109.68
Zero_1	0	n / a	n / a
Zero_2	0	3.096	n / a

7. REFERENCES :

1 D.E. Mulvana and J. Henion
Qualitative investigation of the in vitro metabolism of T-6292, T-6293, T-6294 and T-6295 by rat and human hepatocytes using ion spray LC/MS and LC/MS/MS.
Report No. 96ADEM01.3M
November 12, 1996.

2 ABS Laboratory Notebook No. 2070

Figure 1. Reconstructed ion chromatogram (i), negative ionization mass spectrum (ii) and LC/MS/MS product-ion mass spectrum (iii) of authentic T-6293 .

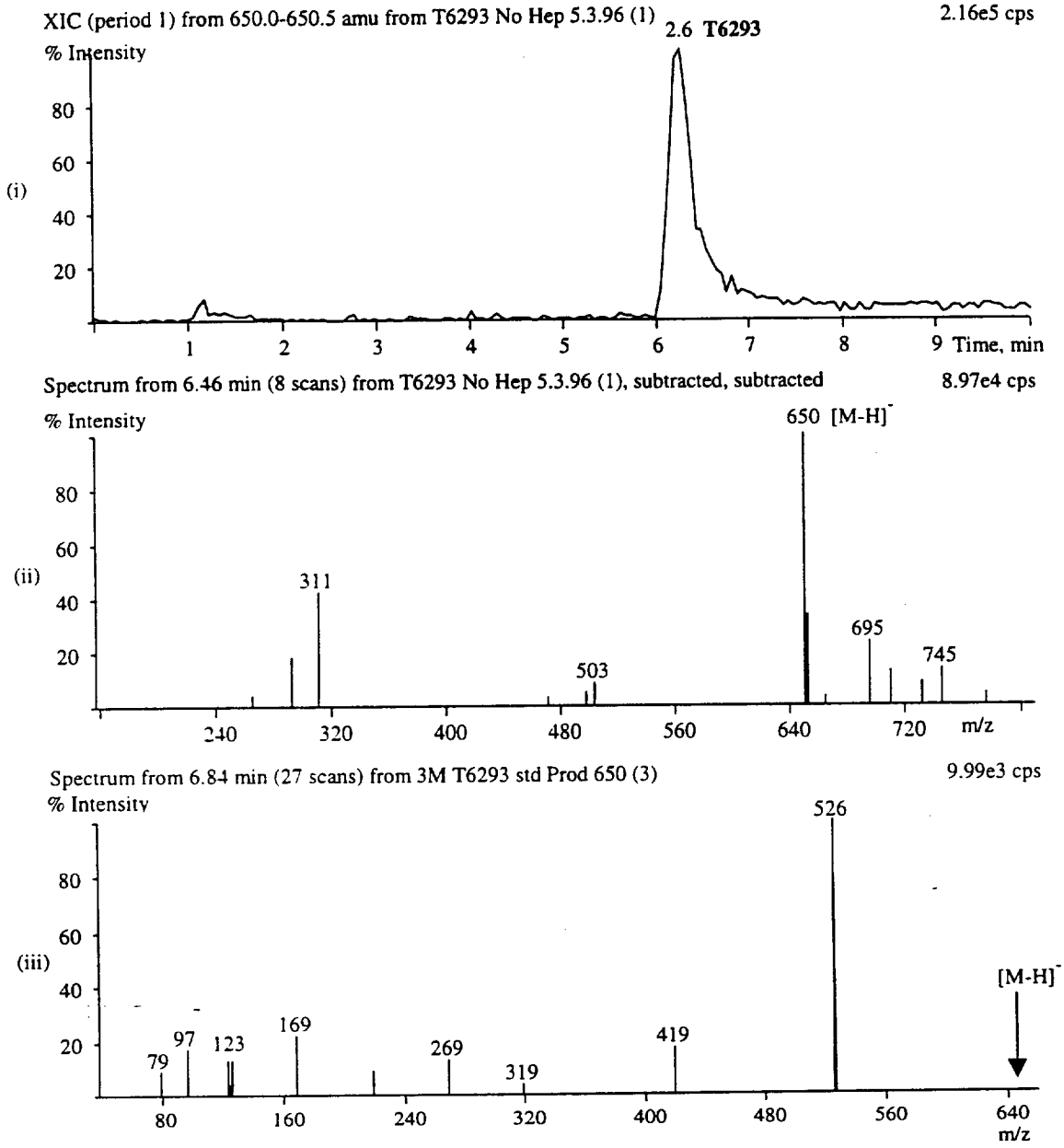


Figure 2. Reconstructed ion chromatogram (i) and LC/MS/MS product-ion mass spectrum (ii) of authentic T-6294.

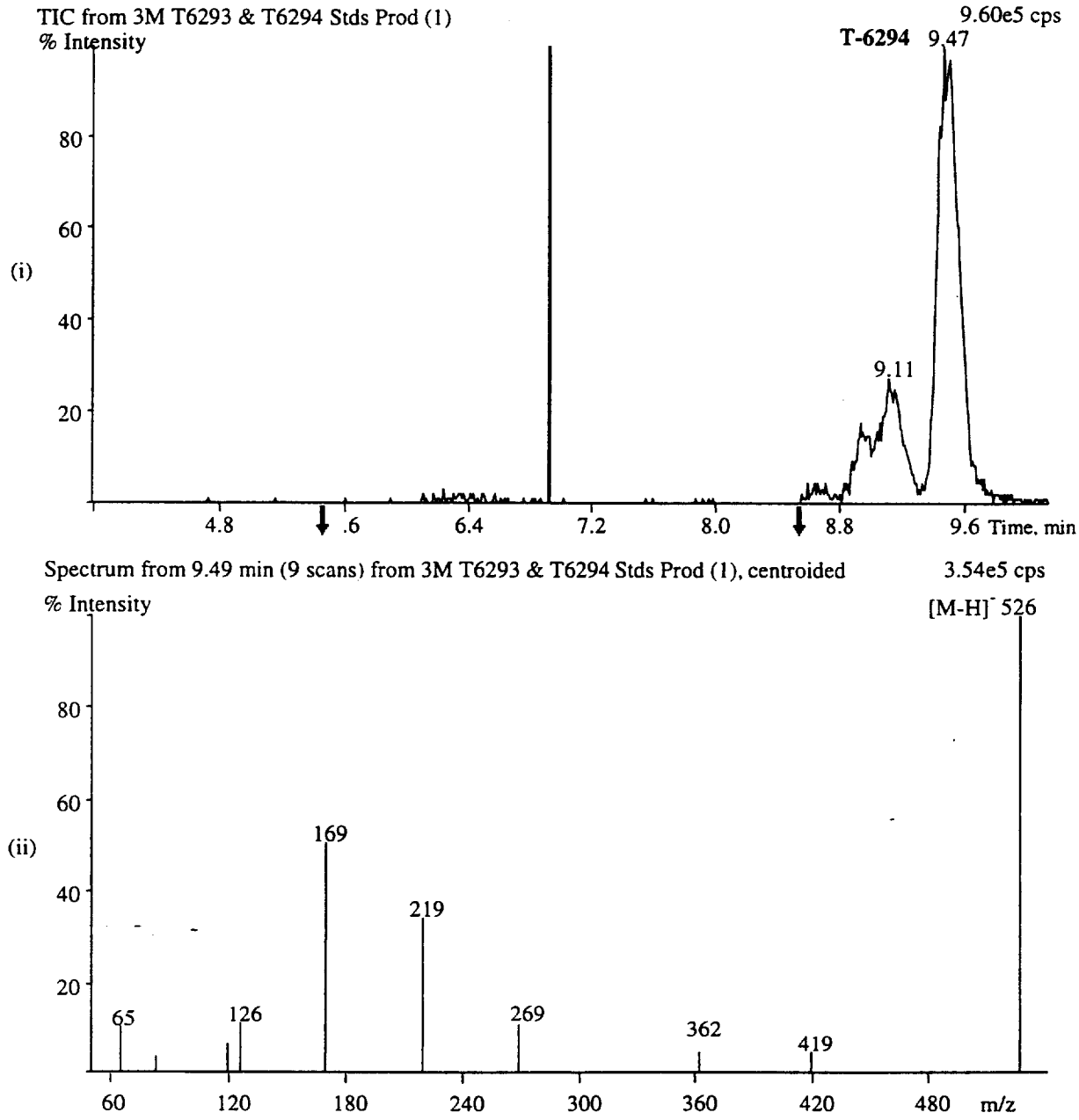
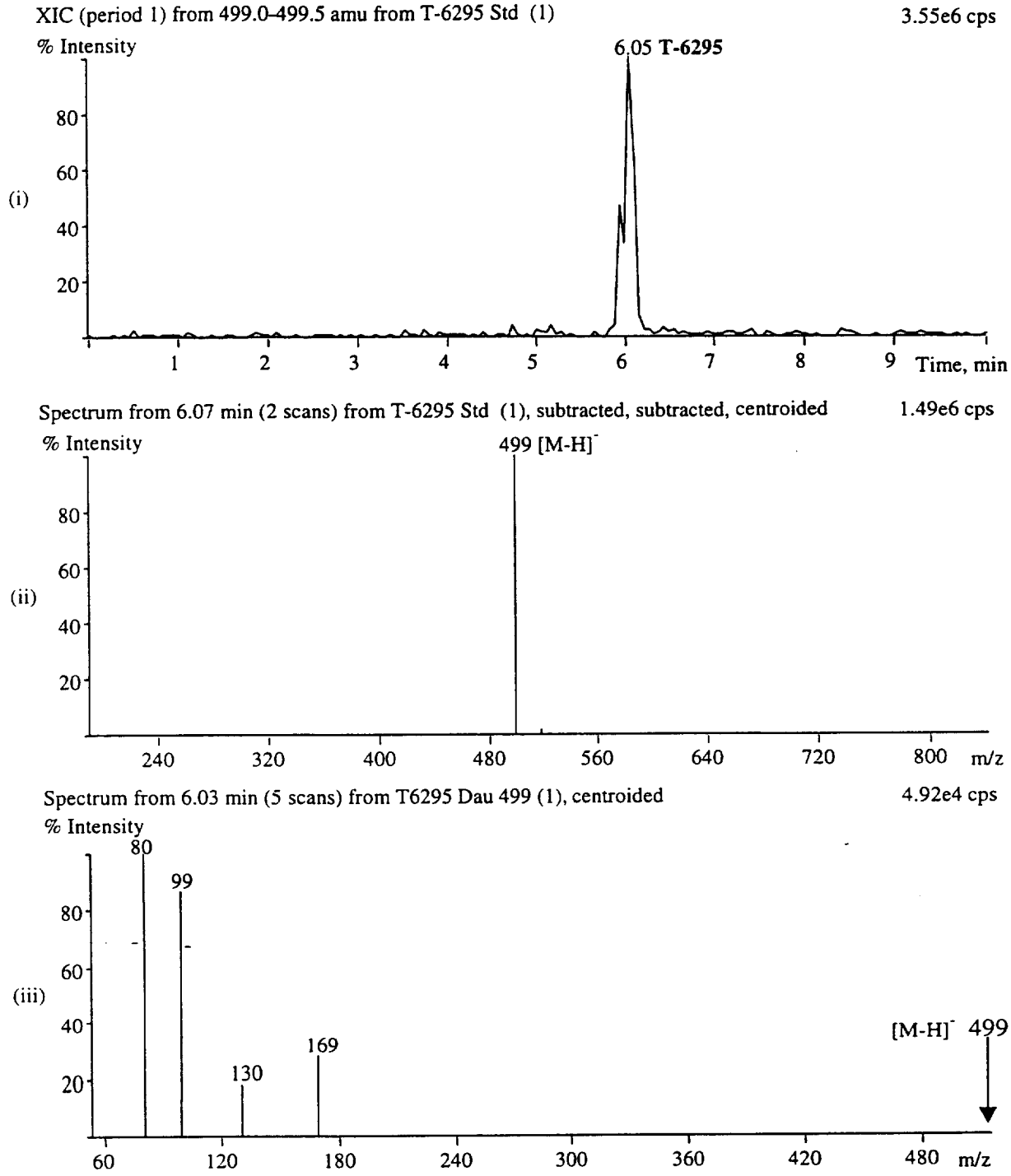
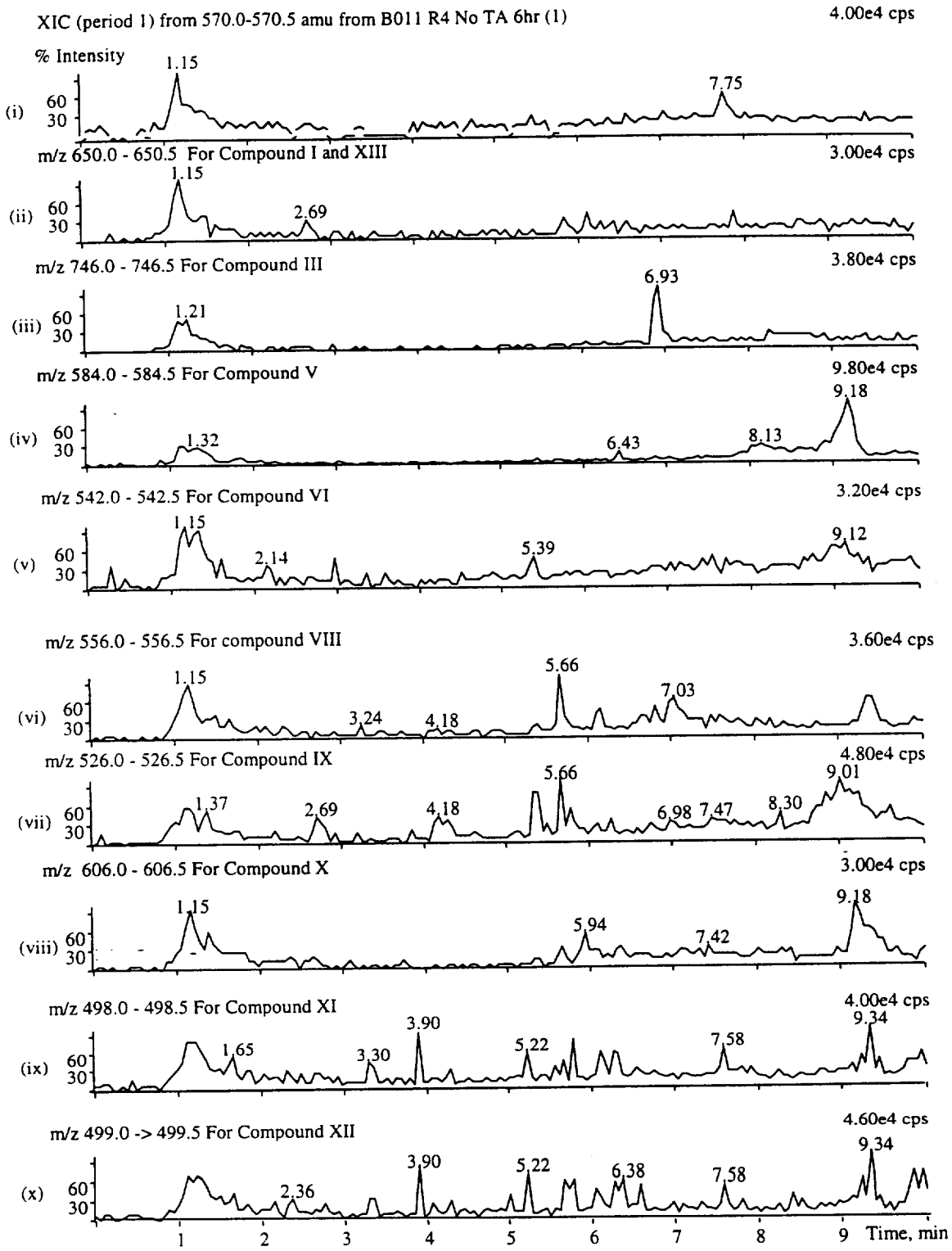


Figure 3. Reconstructed ion chromatogram (i), negative ionization mass spectrum (ii) and LC/MS/MS product-ion mass spectrum (iii) of authentic T-6295.



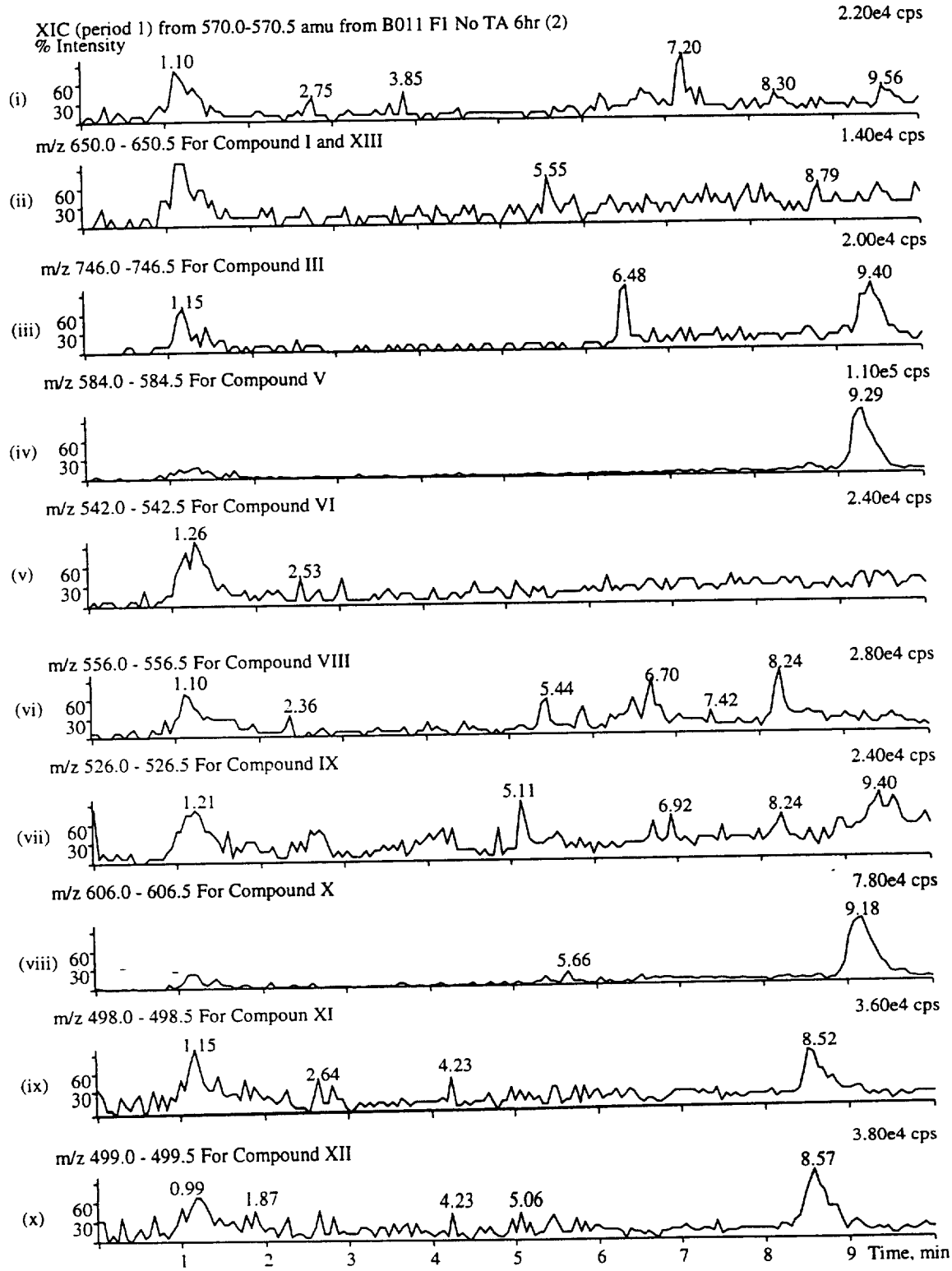
REPORT: 98AGKP01.3M

Figure 4. Full scan reconstructed ion chromatograms of control rat hepatocytes incubated for 6 hours with no test articles



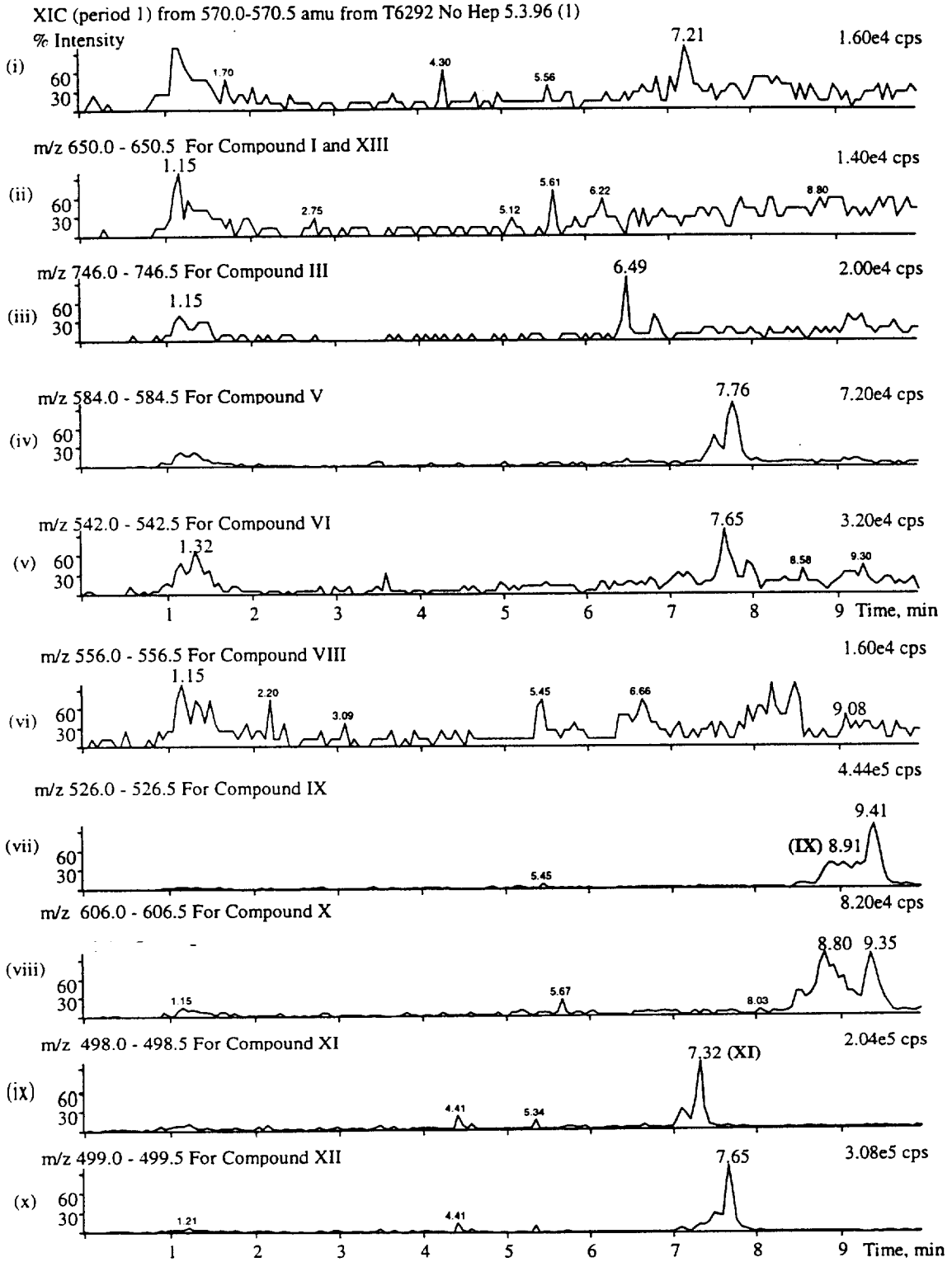
REPORT: 98AGKP01.3M

Figure 5. Full scan reconstructed ion chromatograms of control human hepatocytes incubated for 6 hours with no test articles



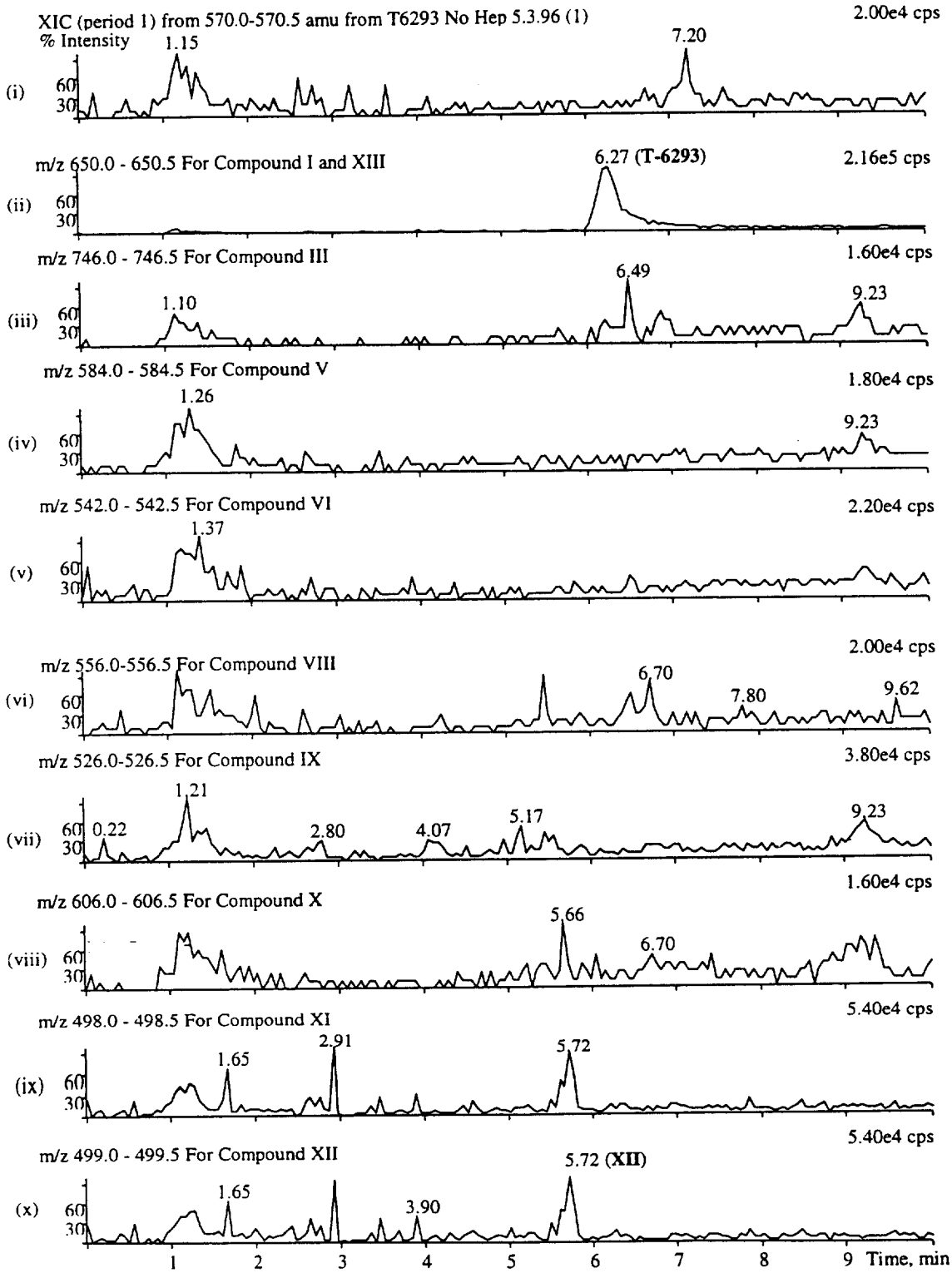
REPORT: 98AGKP01.3M

Figure 6. Full scan reconstructed ion chromatograms of T-6292 incubated for 6 hours with no hepatocytes



REPORT: 98AGKP01.3M

Figure 7. Full scan reconstructed ion chromatograms of T-6293 incubated for 6 hours with no hepatocytes



REPORT: 98AGKP01.3M

Figure 8. Full scan reconstructed ion chromatograms of T-6294 incubated for 6 hours with no hepatocytes

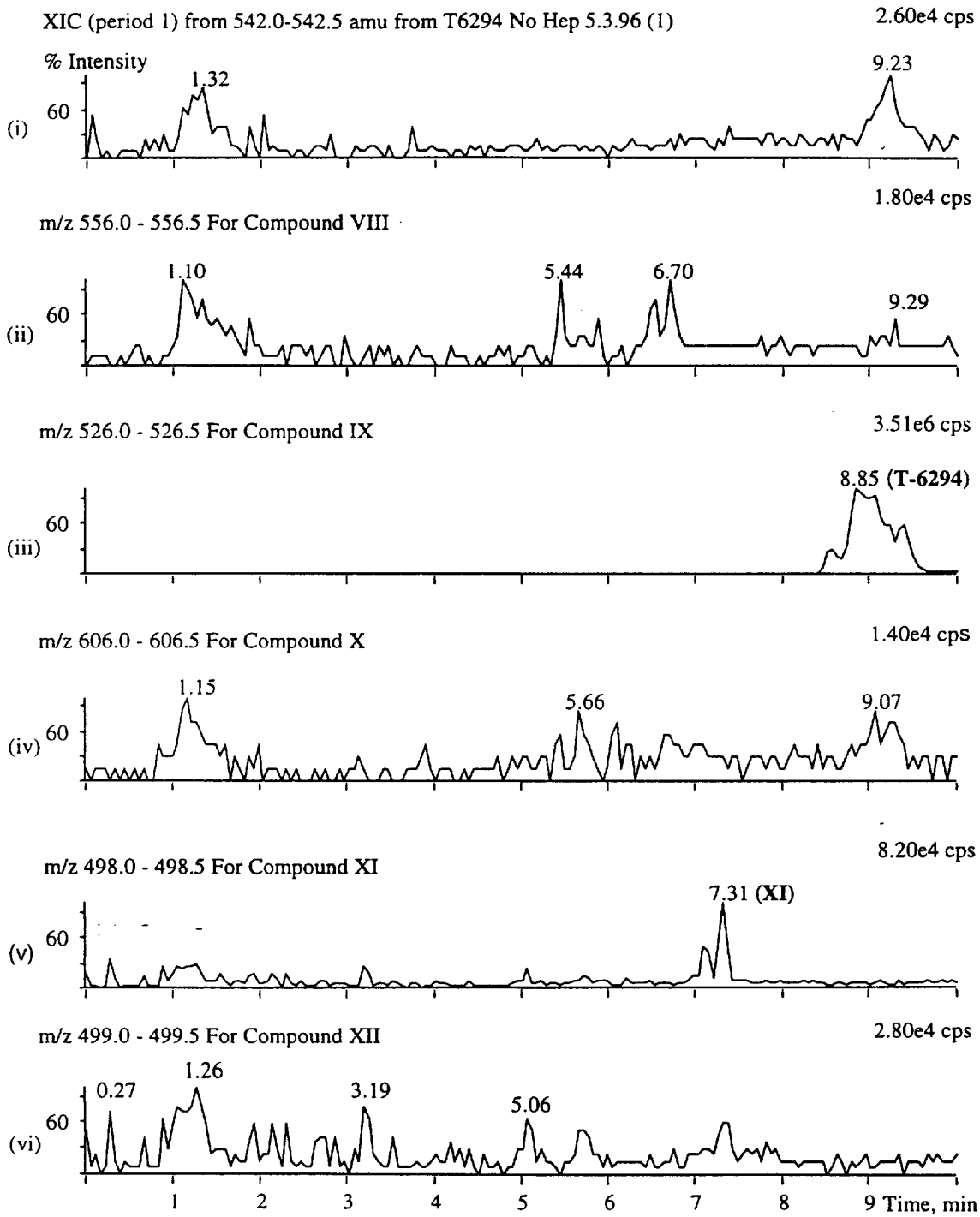
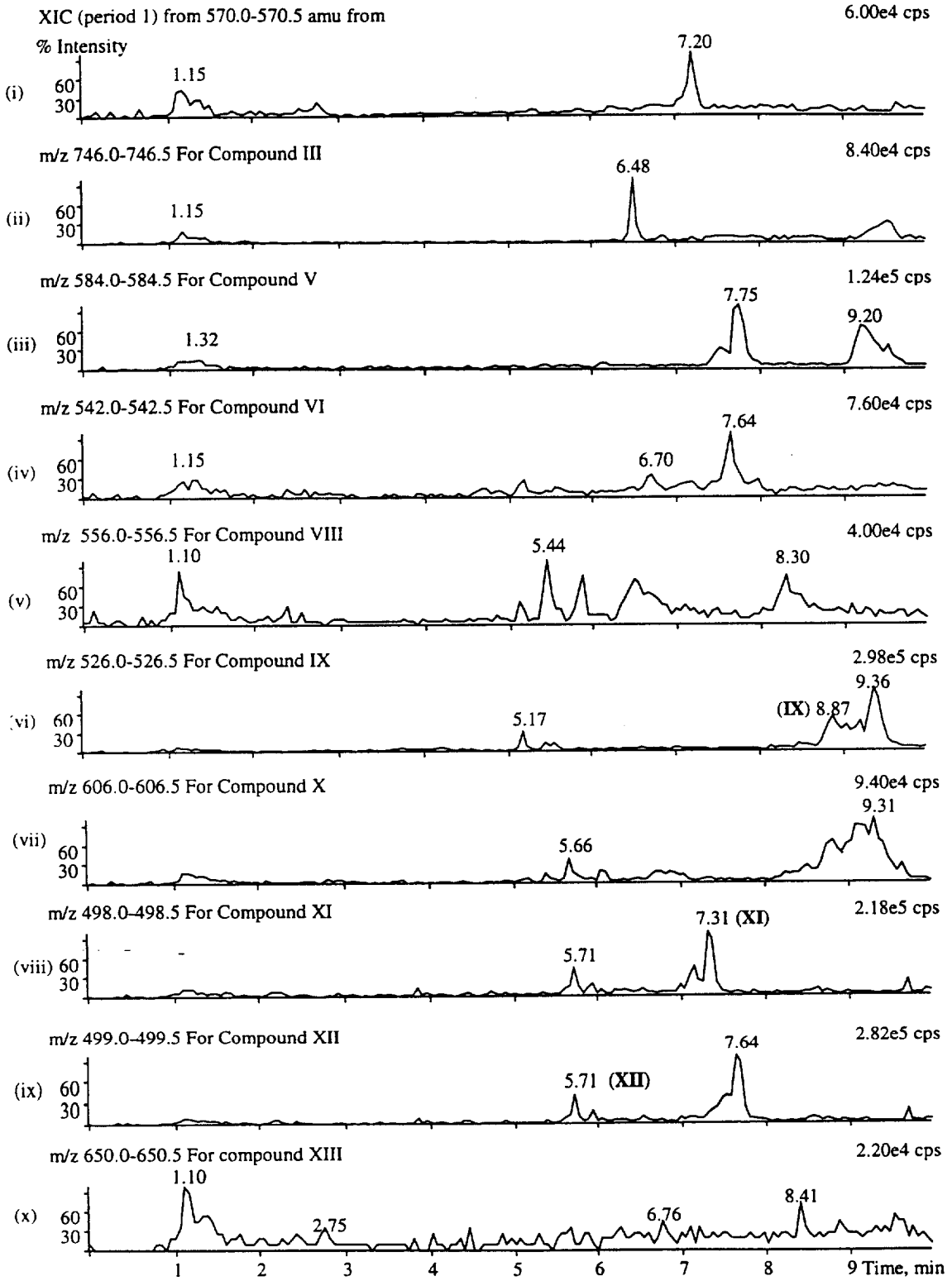
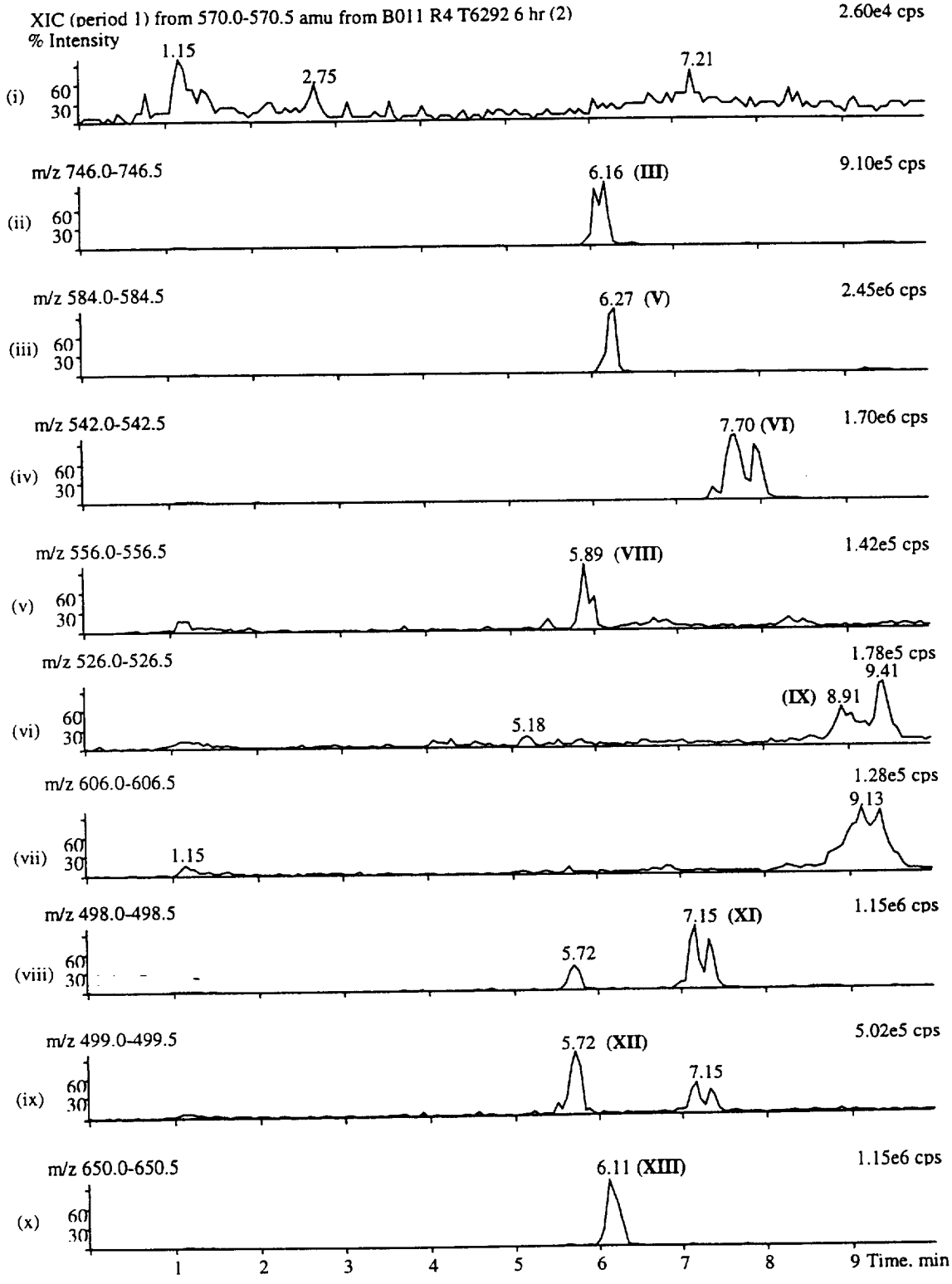


Figure 9. Full scan reconstructed ion chromatograms showing the presence of metabolites after incubating rat hepatocytes with T-6292 at 0 hour.



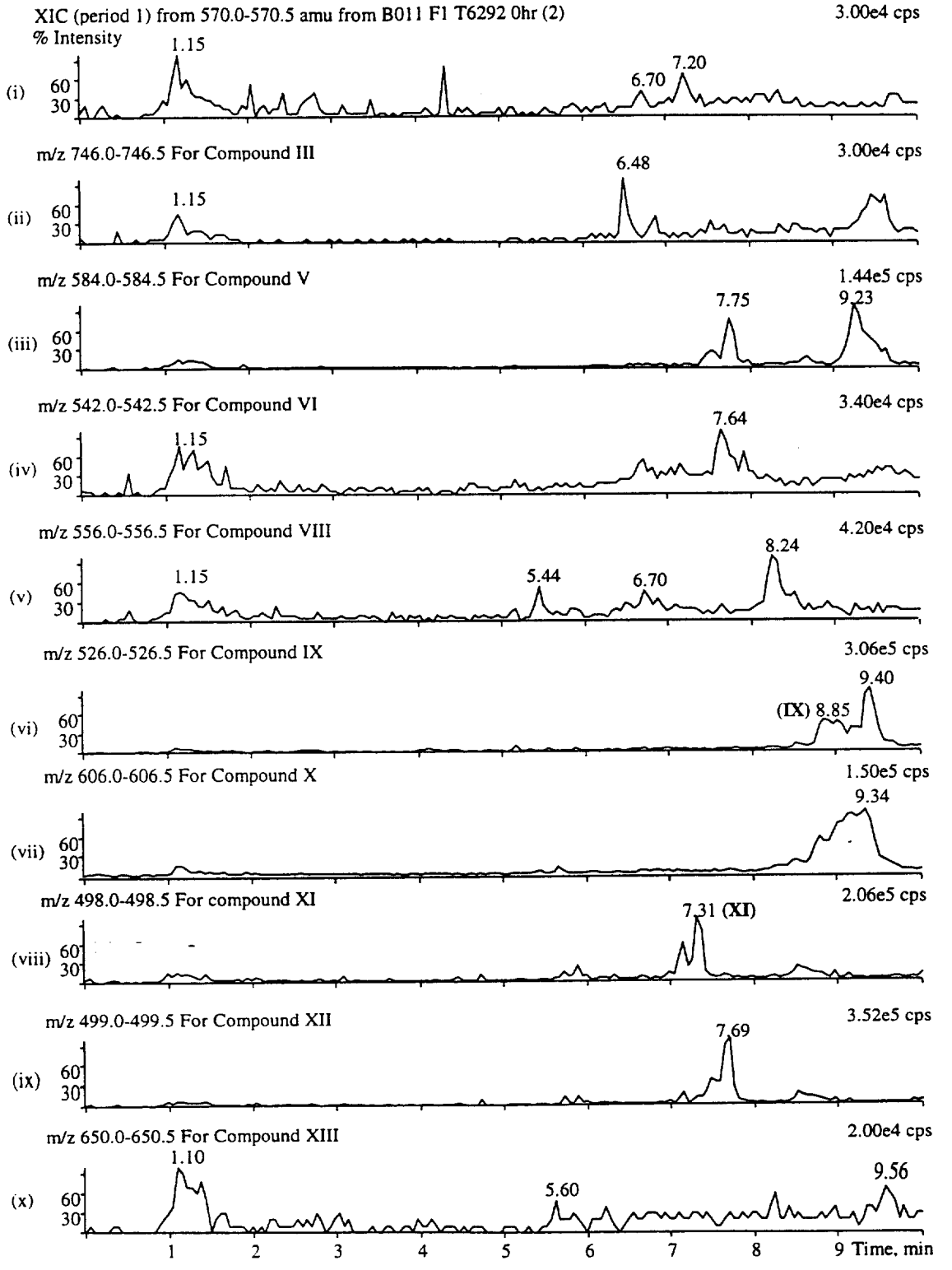
REPORT: 98AGKP01.3M

Figure 10. Full scan reconstructed ion chromatograms showing the presence of metabolites after incubating rat hepatocytes with T-6292 for 6 hour.



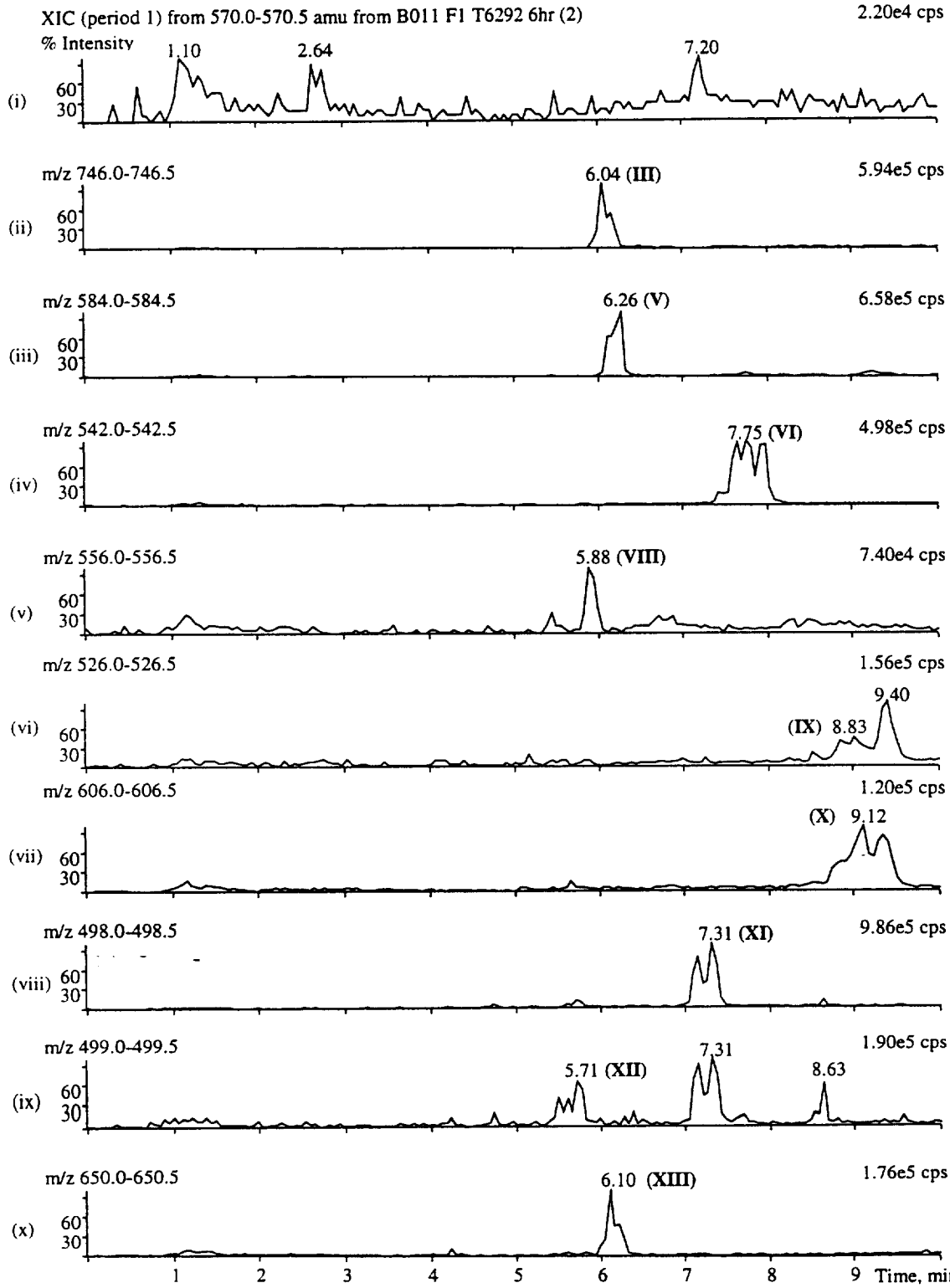
REPORT: 98AGKP01.3M

Figure 11. Full scan reconstructed ion chromatograms showing the presence of metabolites after incubating human hepatocytes with T-6292 at 0 hr.



REPORT: 98AGKP01.3M

Figure 12. Full scan reconstructed ion chromatograms showing the presence of metabolites after incubating human hepatocytes with T-6292 for 6 hour.



REPORT: 98AGKP01.3M

Figure 13. (i) SRM chromatograms of rat hepatocytes incubated with no test articles; (ii) SRM chromatograms of human hepatocytes incubated with no test articles; (iii) SRM chromatograms of human hepatocytes incubated with no test articles.

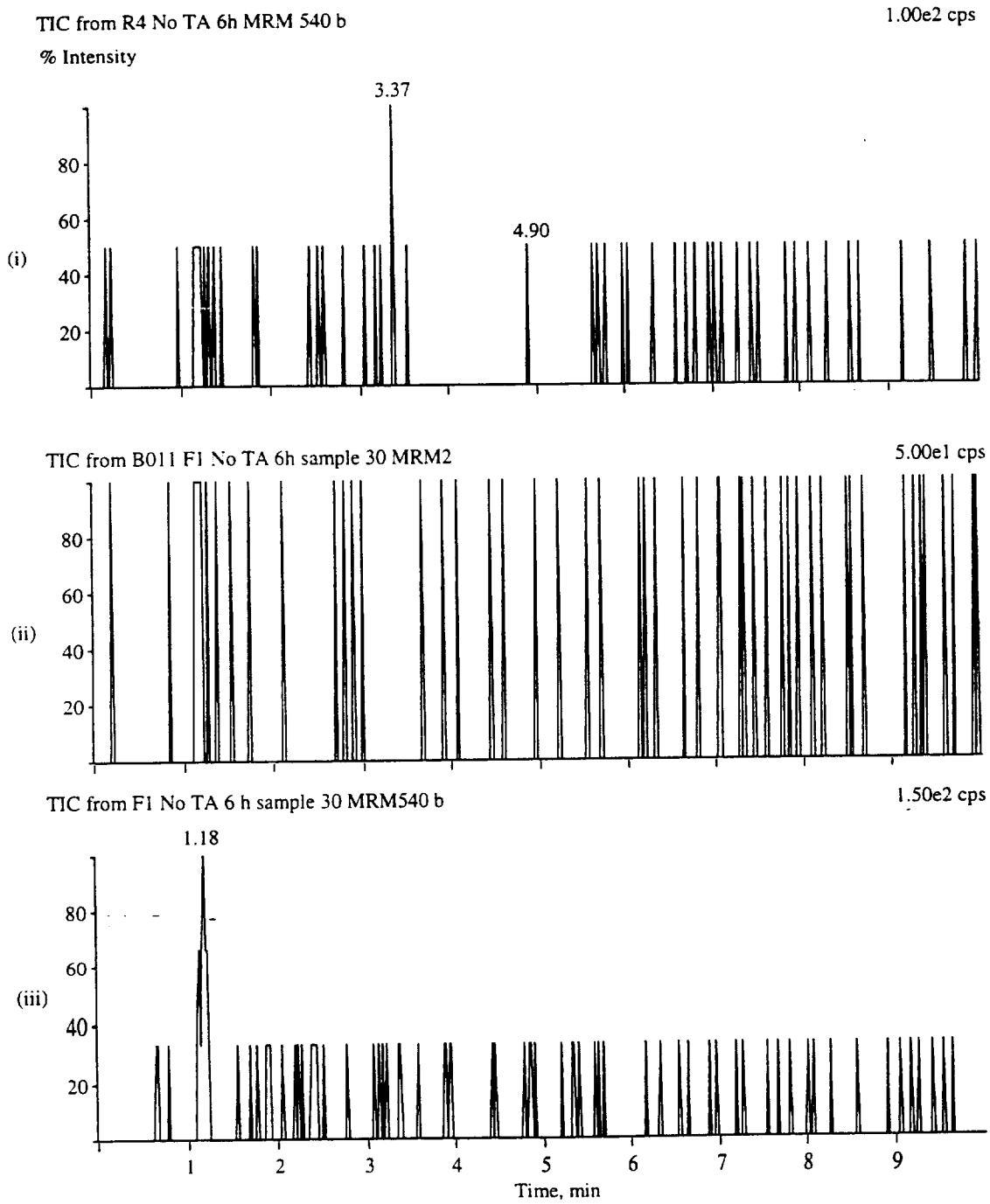


Figure 14. SRM chromatograms showing the absence of metabolite IV in (i) rat hepatocytes incubated with T-6292 at 0 hour; (ii) rat hepatocytes incubated with T-6292 for 6 hour; (iii) human hepatocytes incubated with T-6292 at 0 hour and (iv) human hepatocytes incubated with T-6292 for 6 hour.

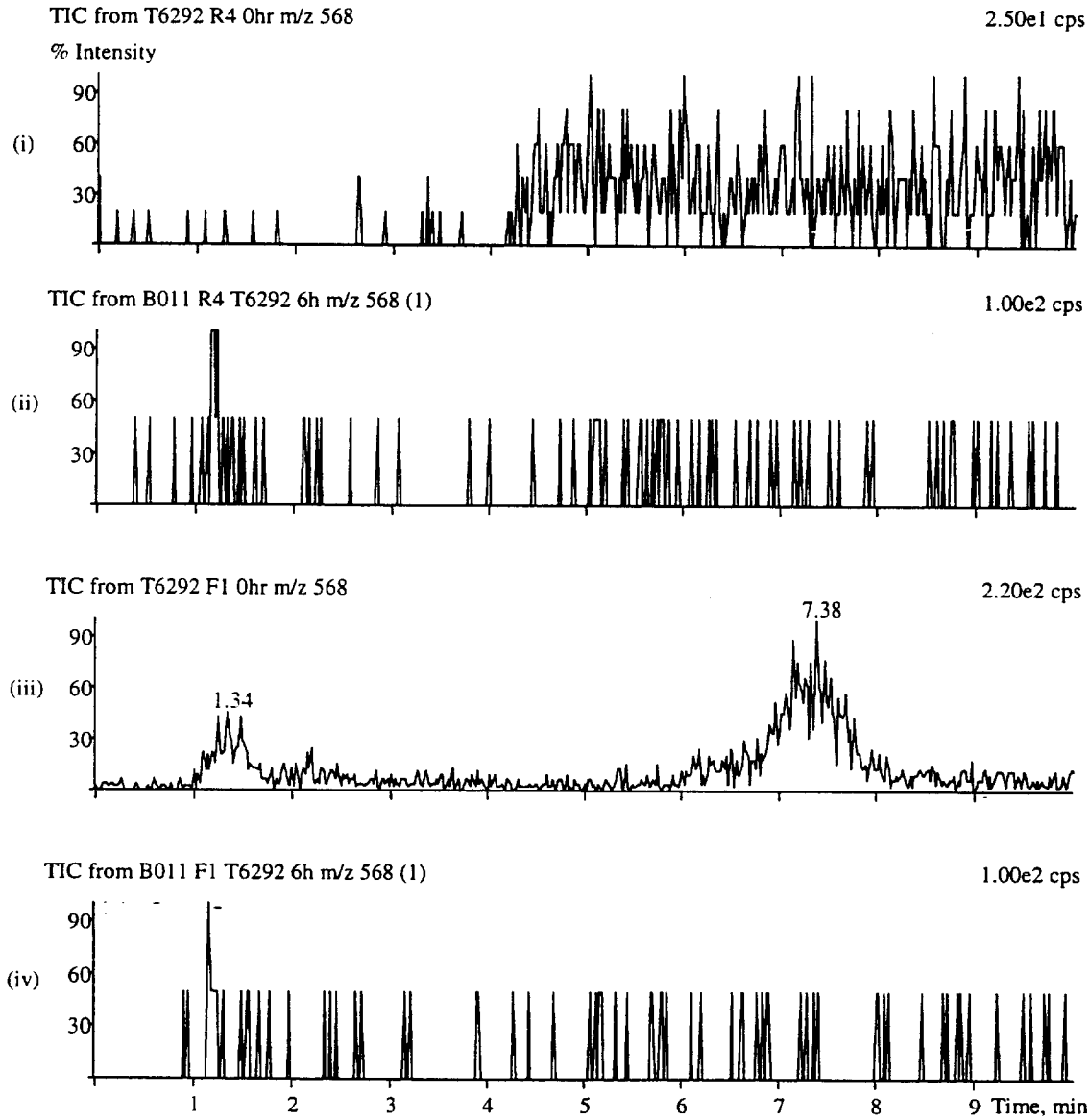


Figure 15. SRM total ion chromatograms showing the presence of metabolite VII in (i) T-6292 standard solution; (ii) incubation of T-6292 with rat hepatocytes at 0 hour; (iii) incubation of T-6292 with rat hepatocytes at 6 hour; (iv) incubation of T-6292 with human hepatocytes at 0 hour and (v) incubation of T-6292 with human hepatocytes at 6 hour.

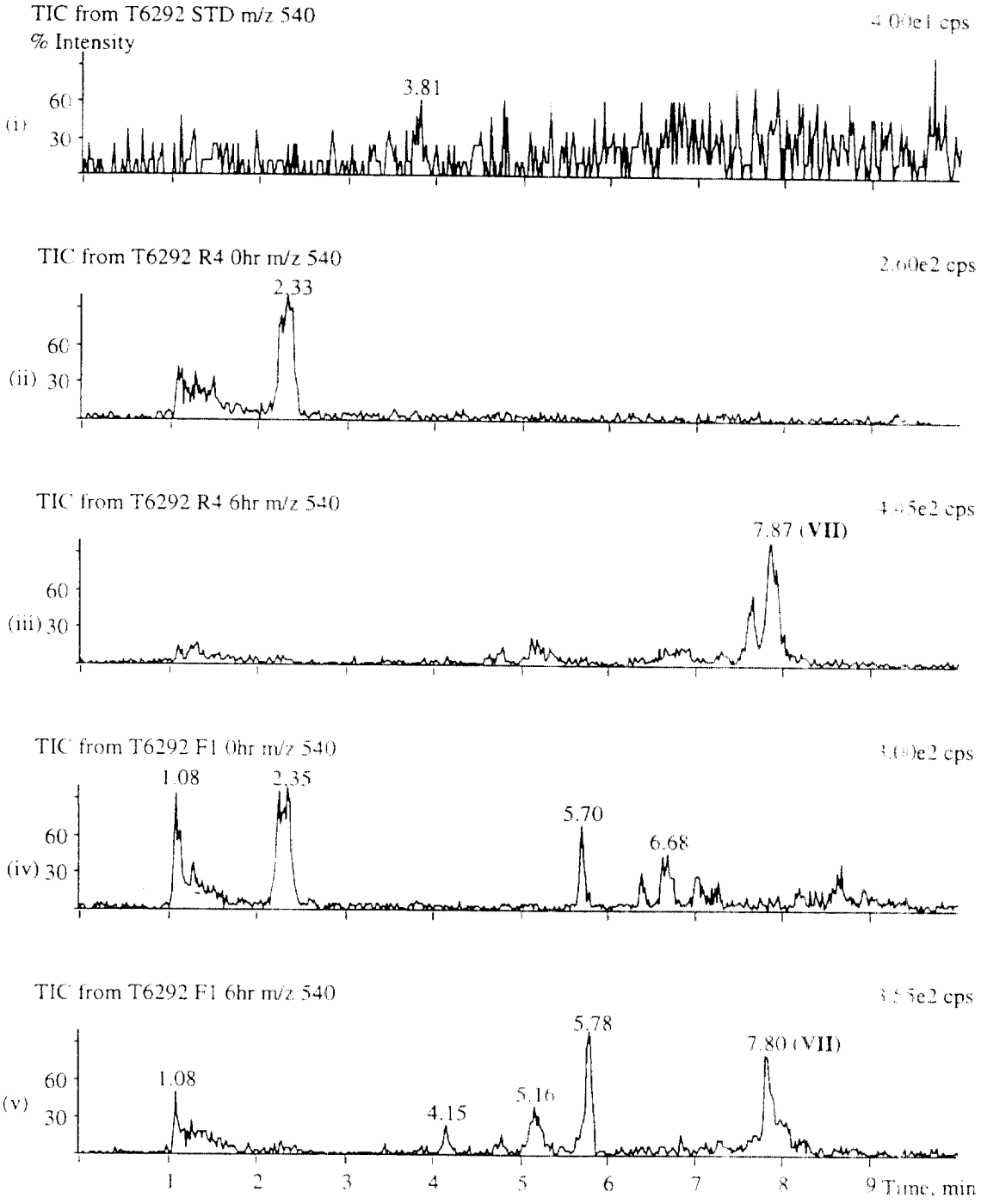
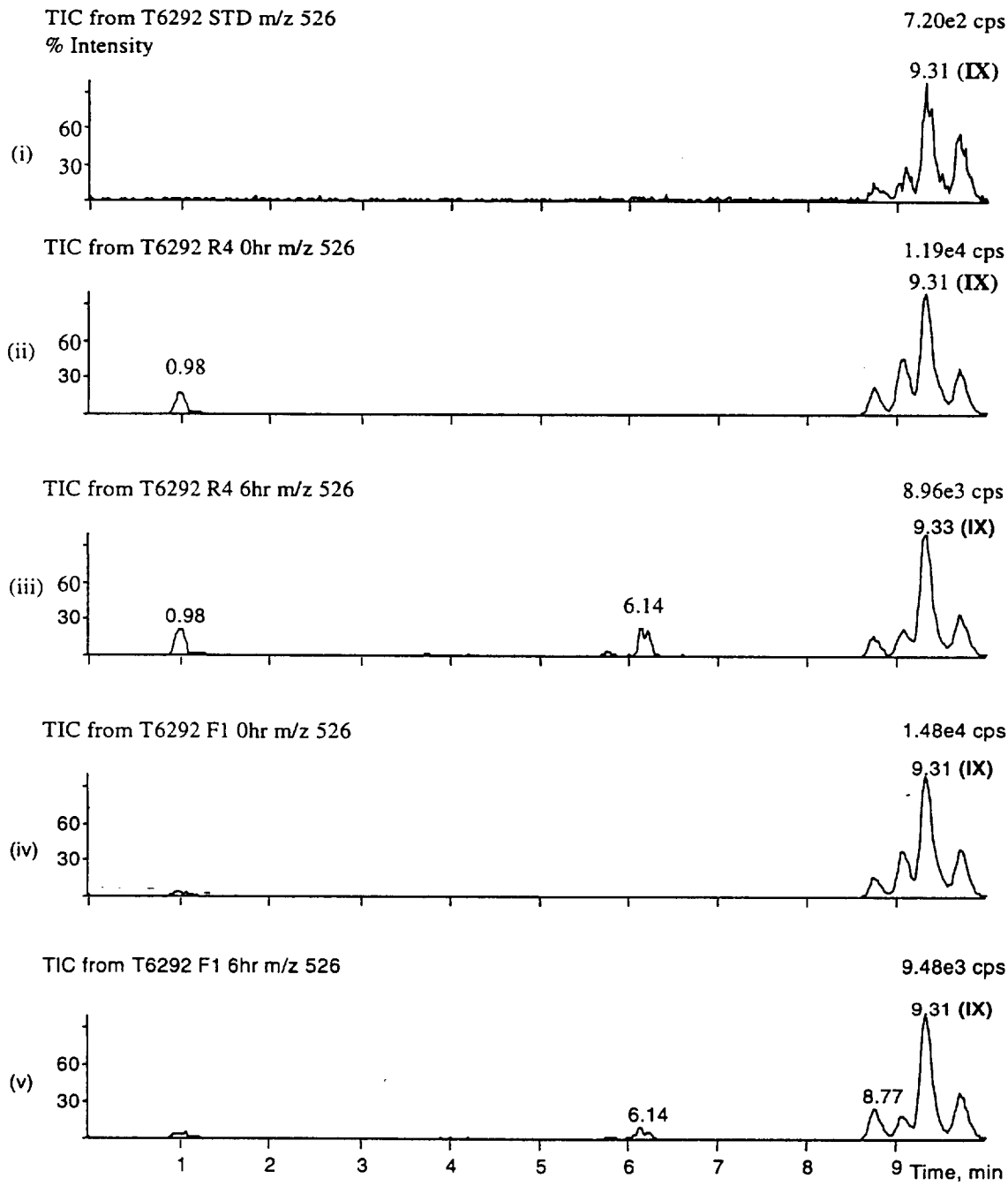
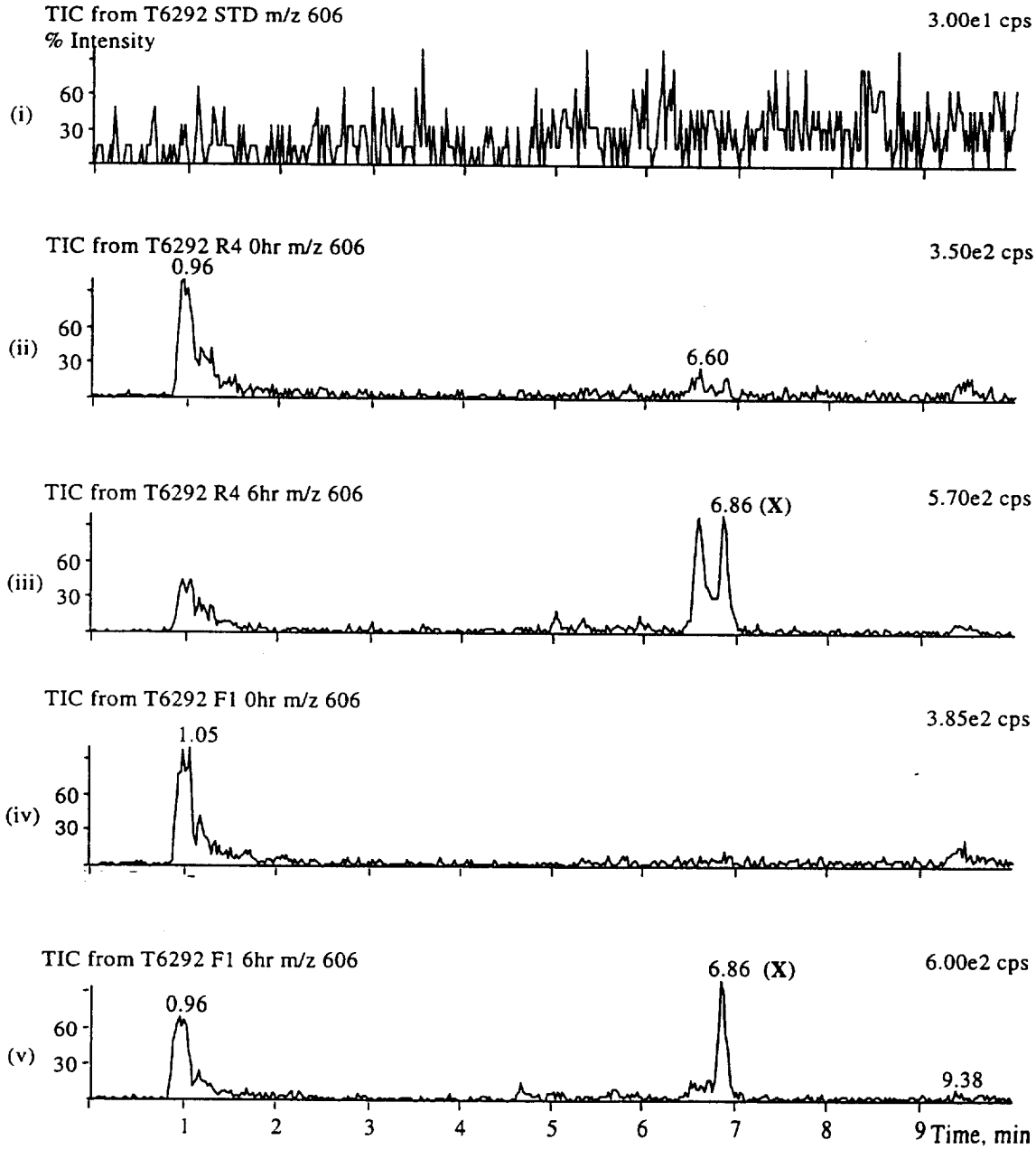


Figure 16. SRM total ion chromatograms showing the presence of metabolite IX in (i) T-6292 standard solution; (ii) incubation of T-6292 with rat hepatocytes at 0 hour; (iii) incubation of T-6292 with rat hepatocytes at 6 hour; (iv) incubation of T-6292 with human hepatocytes at 0 hour and (v) incubation of T-6292 with human hepatocytes at 6 hour.



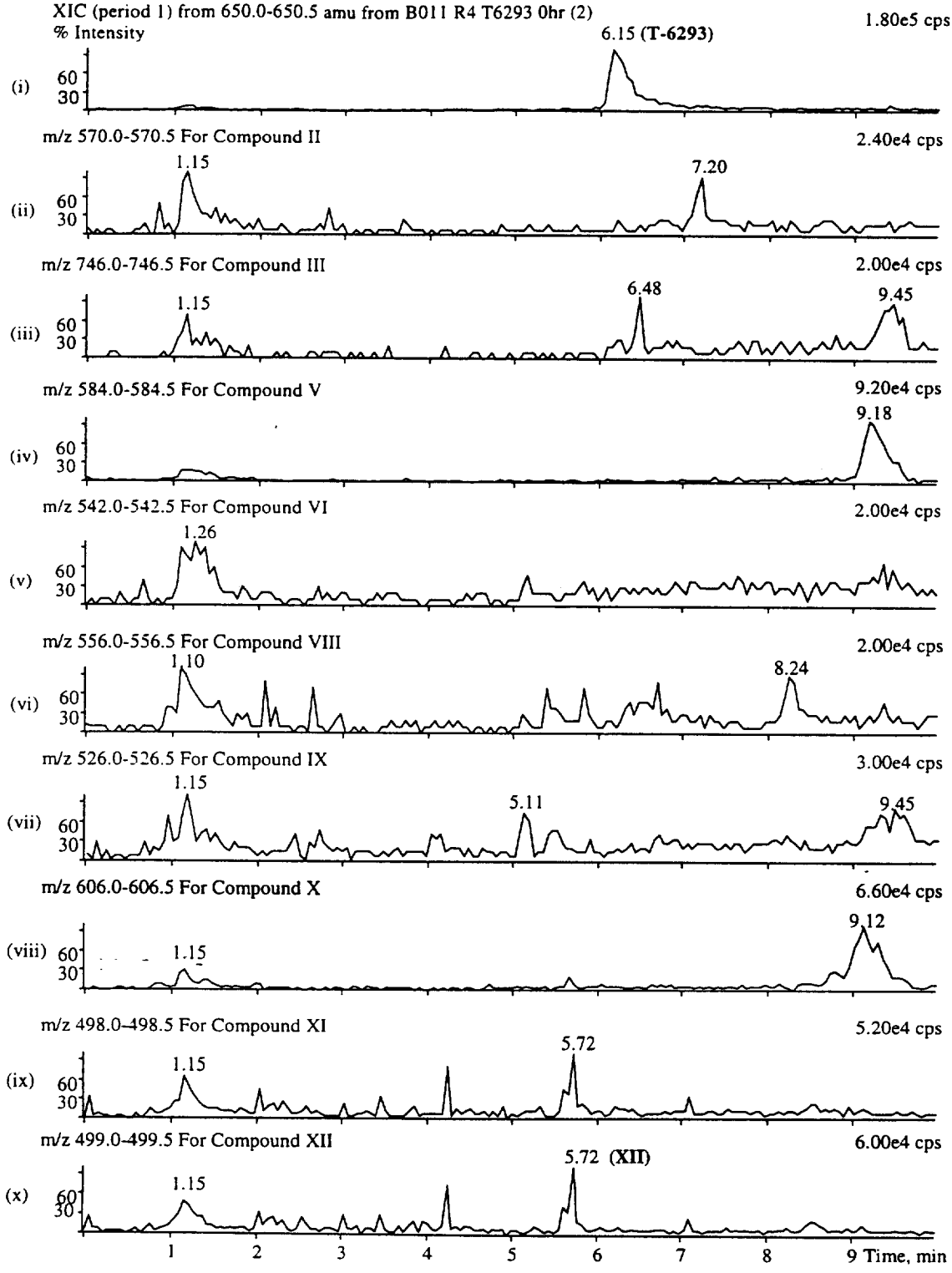
REPORT: 98AGKP01.3M

Figure 17. **SRM total ion chromatograms showing the presence of metabolite X in (i) T-6292 standard solution; (ii) incubation of T-629 with rat hepatocytes at 0 hour; (iii) incubation of T-6292 with rat hepatocytes at 6 hour; (iv) incubation of T-6292 with human hepatocytes at 0 hour and (v) incubation of T-6292 with human hepatocytes at 6 hour.**



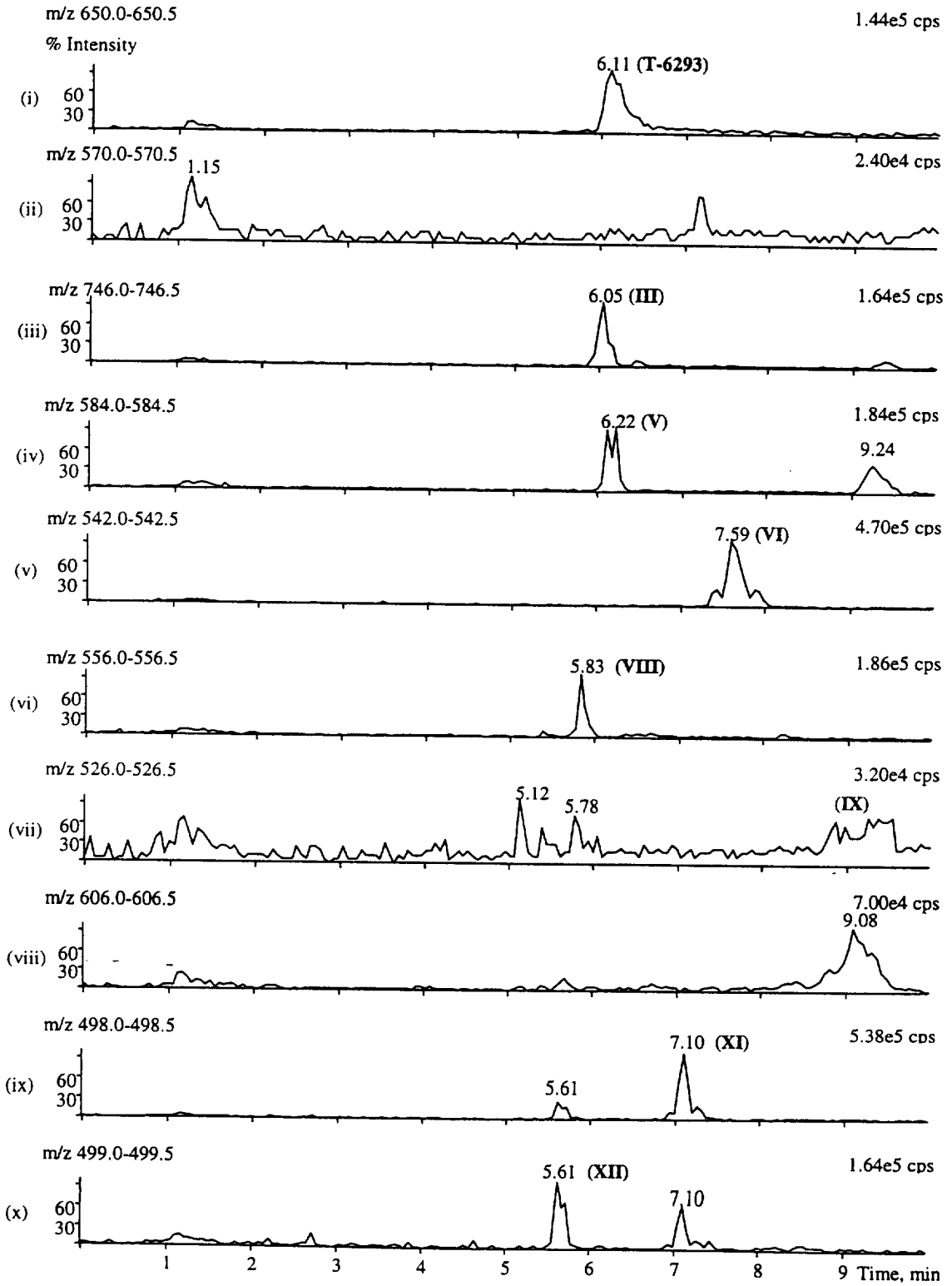
REPORT: 98AGKP01.3M

Figure 18. Full scan reconstructed ion chromatograms showing the presence of metabolites after incubating rat hepatocytes with T-6293 for 0 hour.



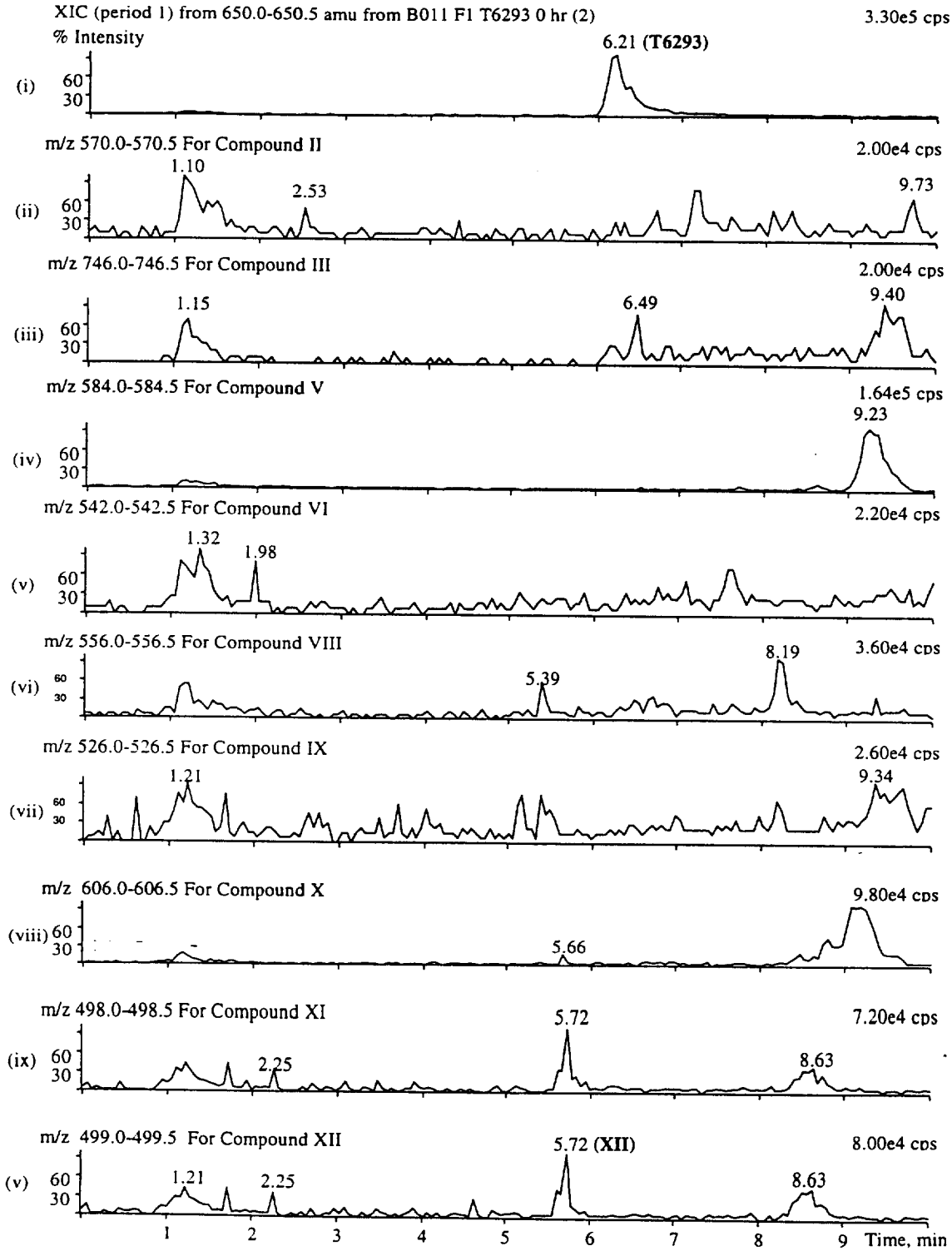
REPORT: 98AGKP01.3M

Figure 19. Full scan reconstructed ion chromatograms showing the presence of metabolites after incubating rat hepatocytes with T-6293 for 6 hour.



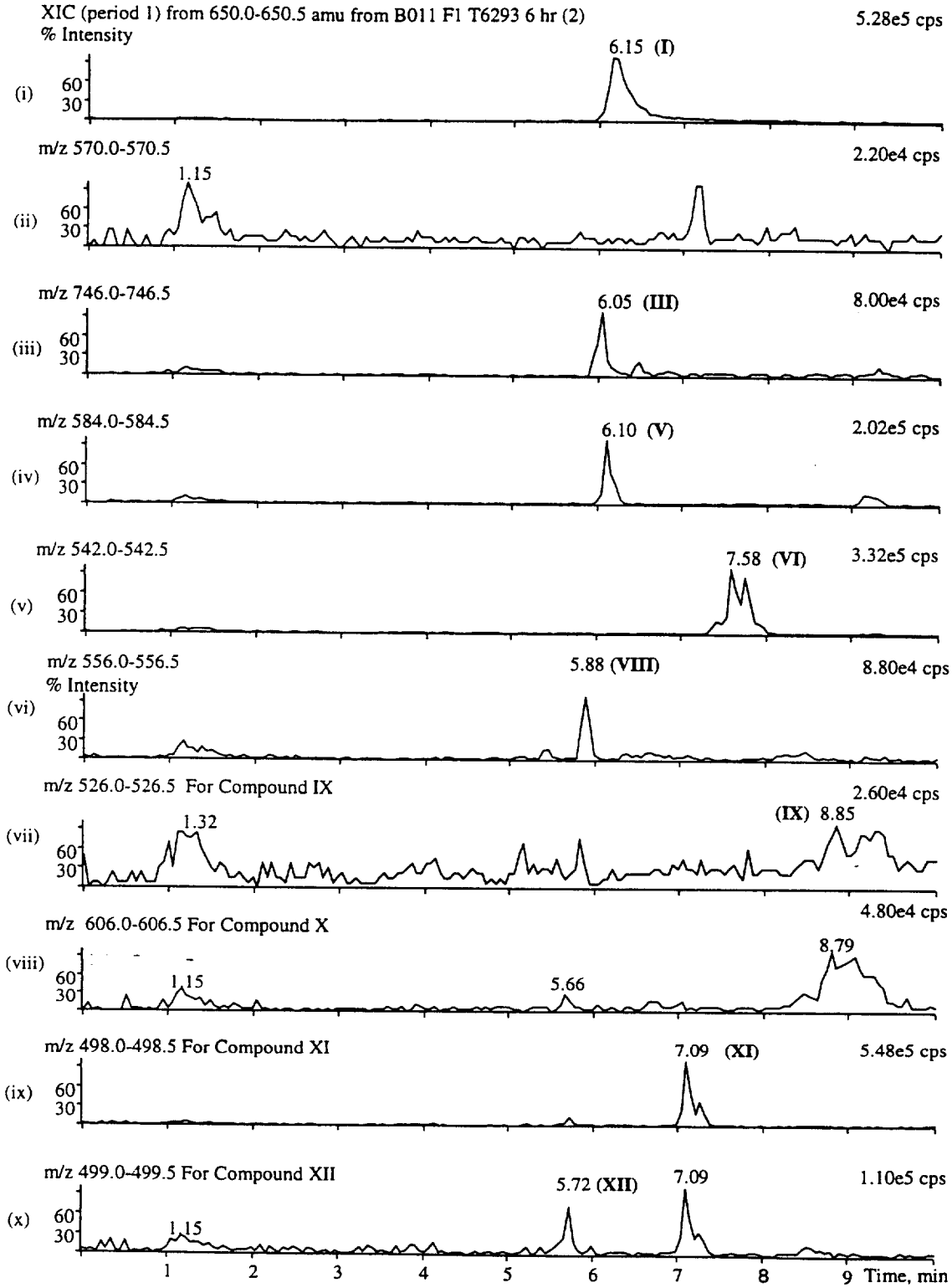
REPORT: 98AGKP01.3M

Figure 20. Full scan reconstructed ion chromatograms showing the presence of metabolites after incubating human hepatocytes with T-6293 for 0 hour.



REPORT: 98AGKP01.3M

Figure 21. Full scan reconstructed ion chromatograms showing the presence of metabolites after incubating human hepatocytes with T-6293 for 6 hour.



REPORT: 98AGKP01.3M

Figure 22. SRM total ion chromatograms showing the presence of metabolite IV in (i) incubation of T-6293 with rat hepatocytes at 0 hour; (ii) incubation of T-6293 with rat hepatocytes at 6 hour; (iii) incubation of T-6293 with human hepatocytes at 0 hour and (iv) incubation of T-6293 with human hepatocytes at 6 hour.

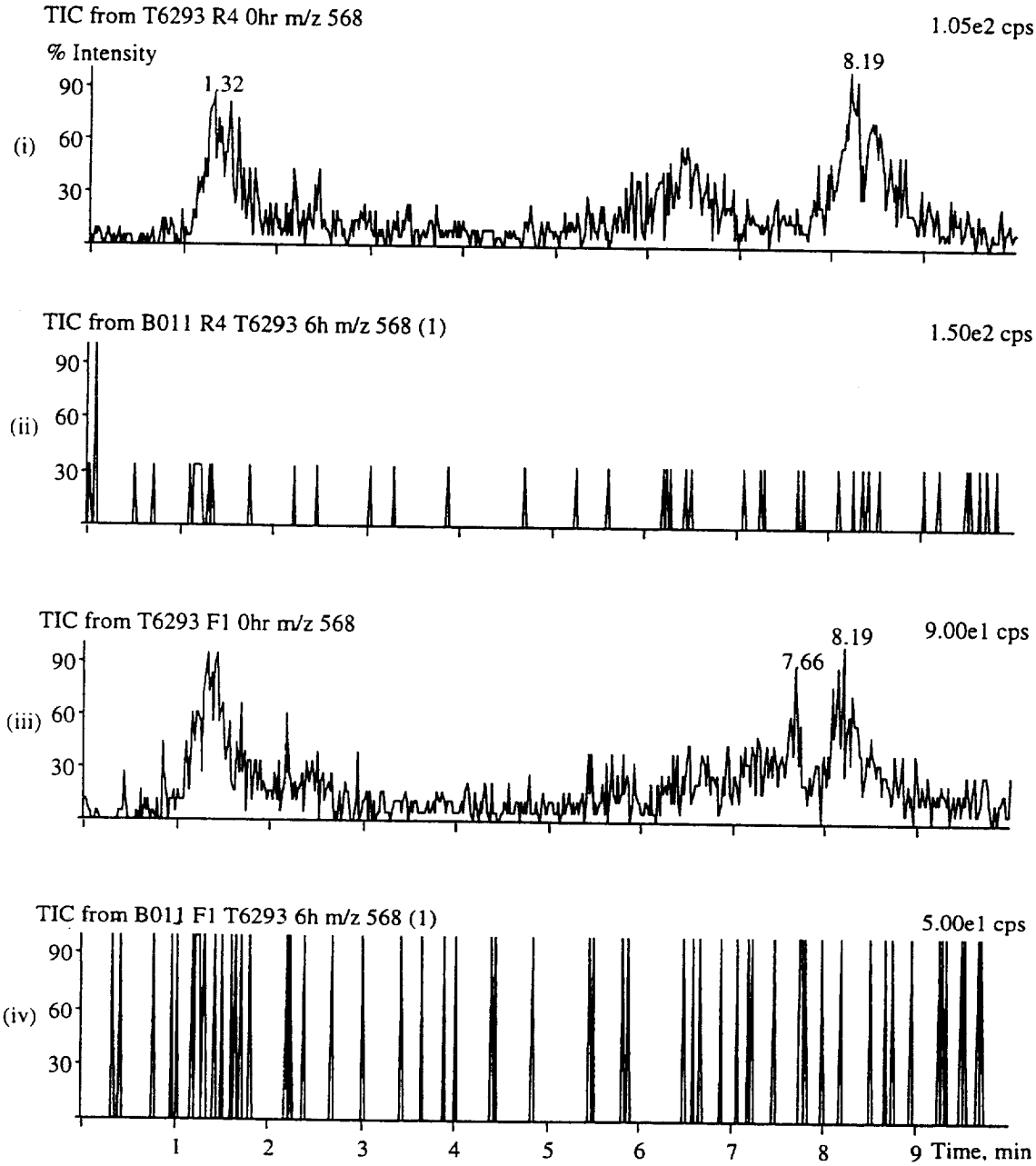


Figure 23. SRM total ion chromatograms showing the presence of metabolite VII in (i) T-6293 standard solution; (ii) incubation of T-6293 with rat hepatocytes at 0 hour; (iii) incubation of T-6293 with rat hepatocytes at 6 hour; (iv) incubation of T-6293 with human hepatocytes at 0 hour and (v) incubation of T-6293 with human hepatocytes at 6 hour.

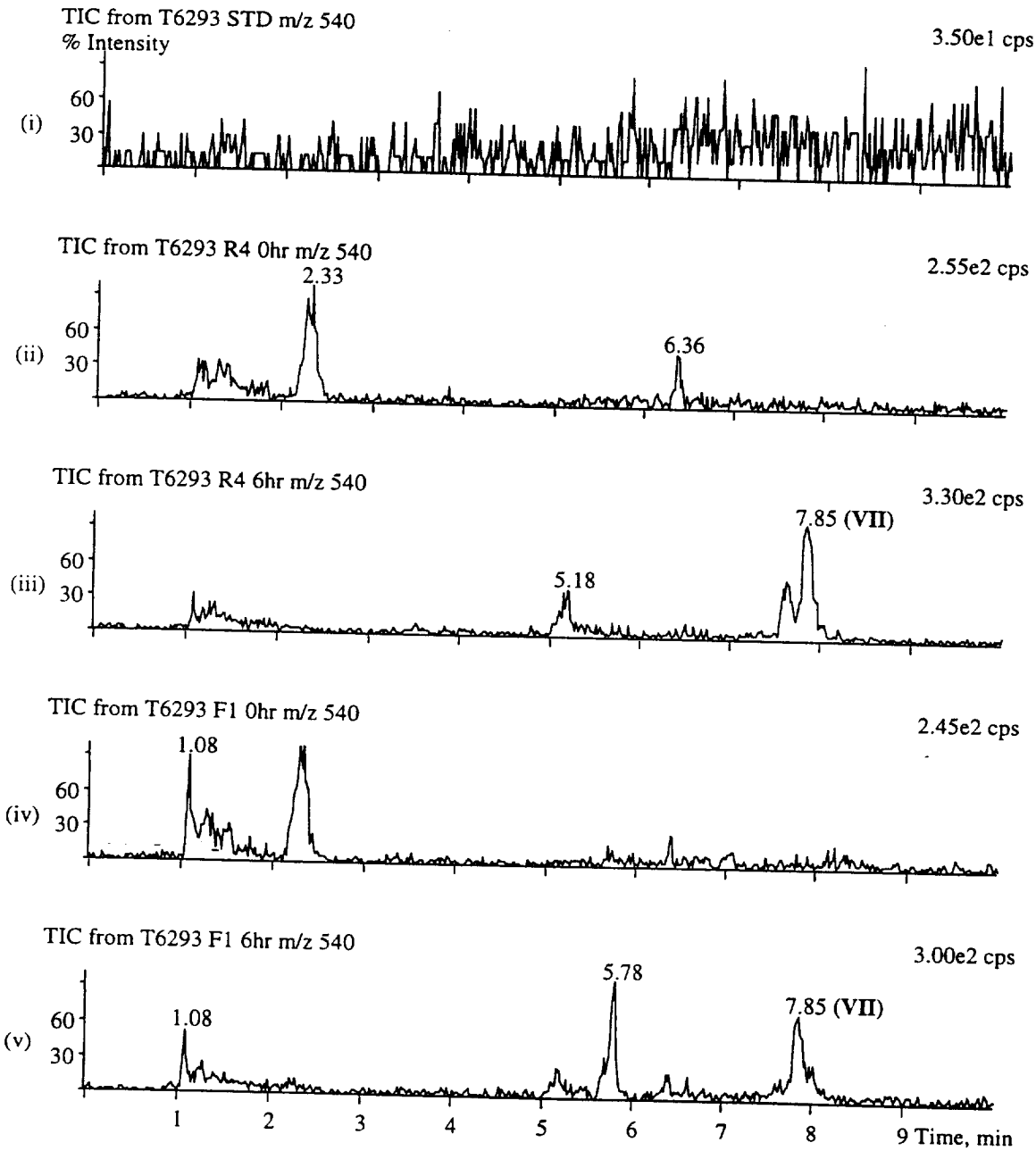


Figure 24. SRM total ion chromatograms showing the presence of metabolite IX in (i) T-6293 standard solution; (ii) incubation of T-6293 with rat hepatocytes at 0 hour; (iii) incubation of T-6293 with rat hepatocytes at 6 hour; (iv) incubation of T-6293 with human hepatocytes at 0 hour and (v) incubation of T-6293 with human hepatocytes at 6 hour.

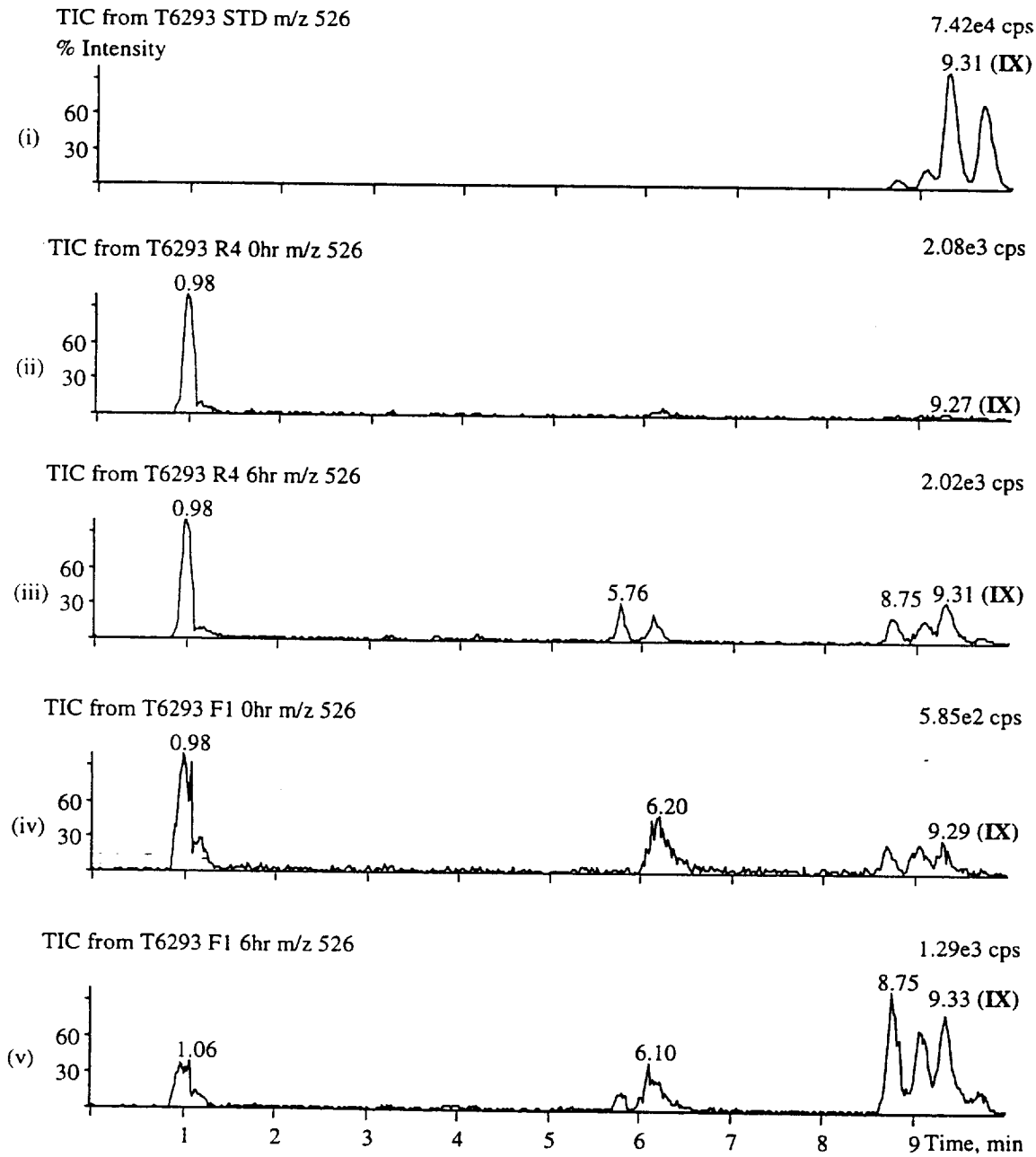


Figure 25. SRM total ion chromatograms showing the presence of metabolite X in (i) T-6293 standard solution; (ii) incubation of T-6293 with rat hepatocytes at 0 hour; (iii) incubation of T-6293 with rat hepatocytes at 6 hour; (iv) incubation of T-6293 with human hepatocytes at 0 hour and (v) incubation of T-6293 with human hepatocytes at 6 hour.

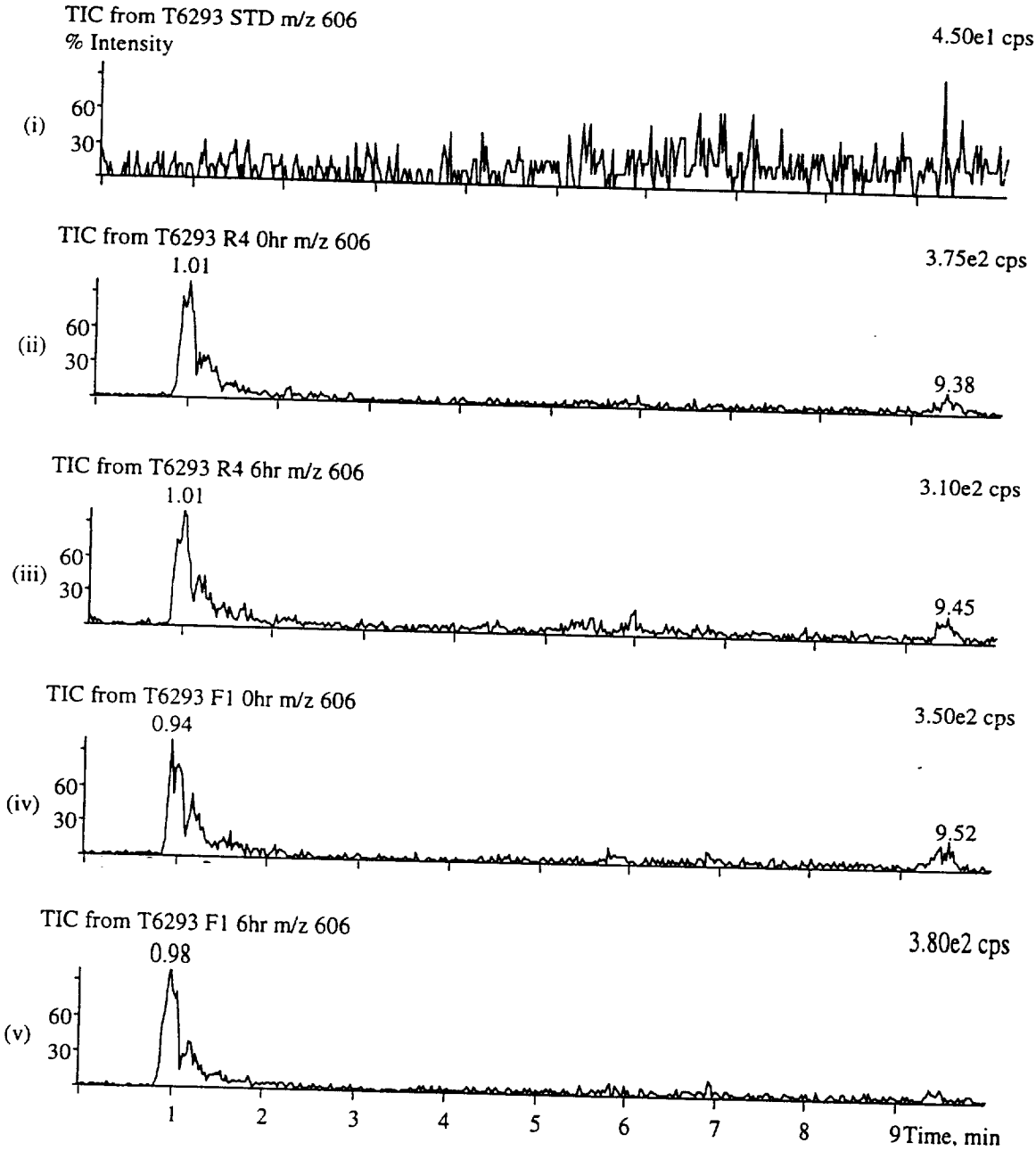


Figure 26. Reconstructed ion chromatograms showing the presence of metabolites after incubating rat hepatocytes with T-6294 for 0 hour.

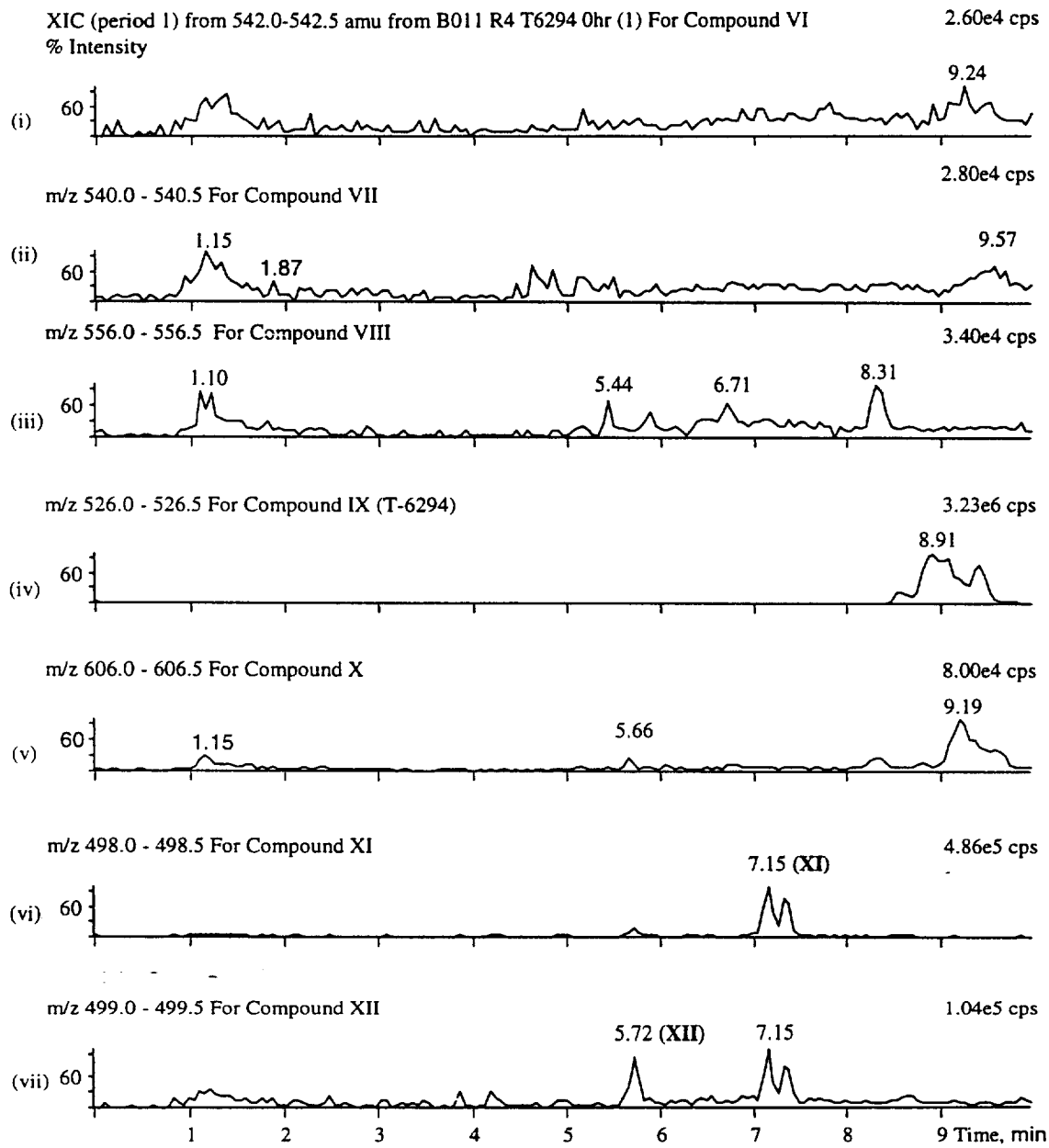


Figure 27. Reconstructed ion chromatograms showing the presence of metabolites after incubating rat hepatocytes with T-6294 for 6 hour.

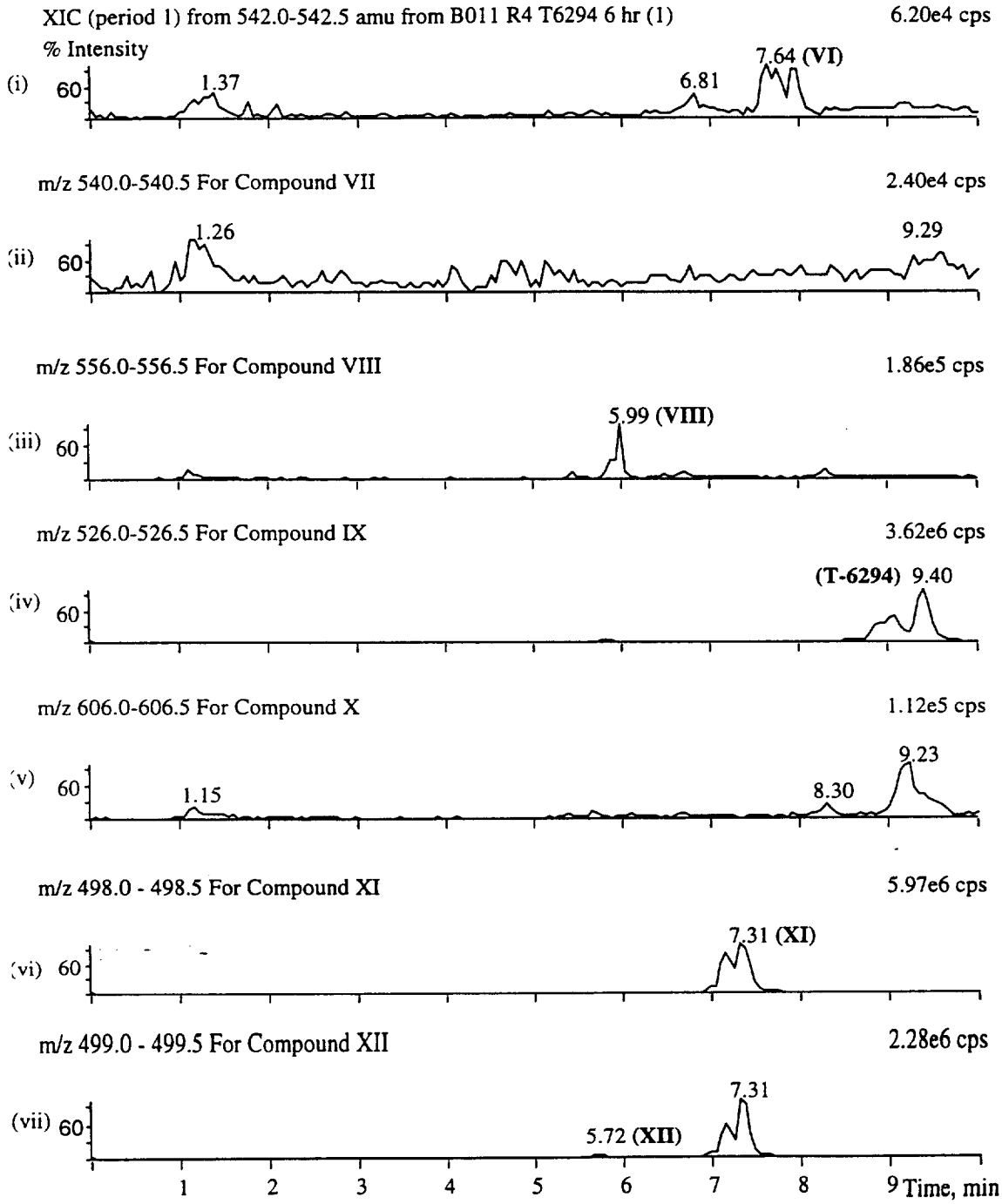
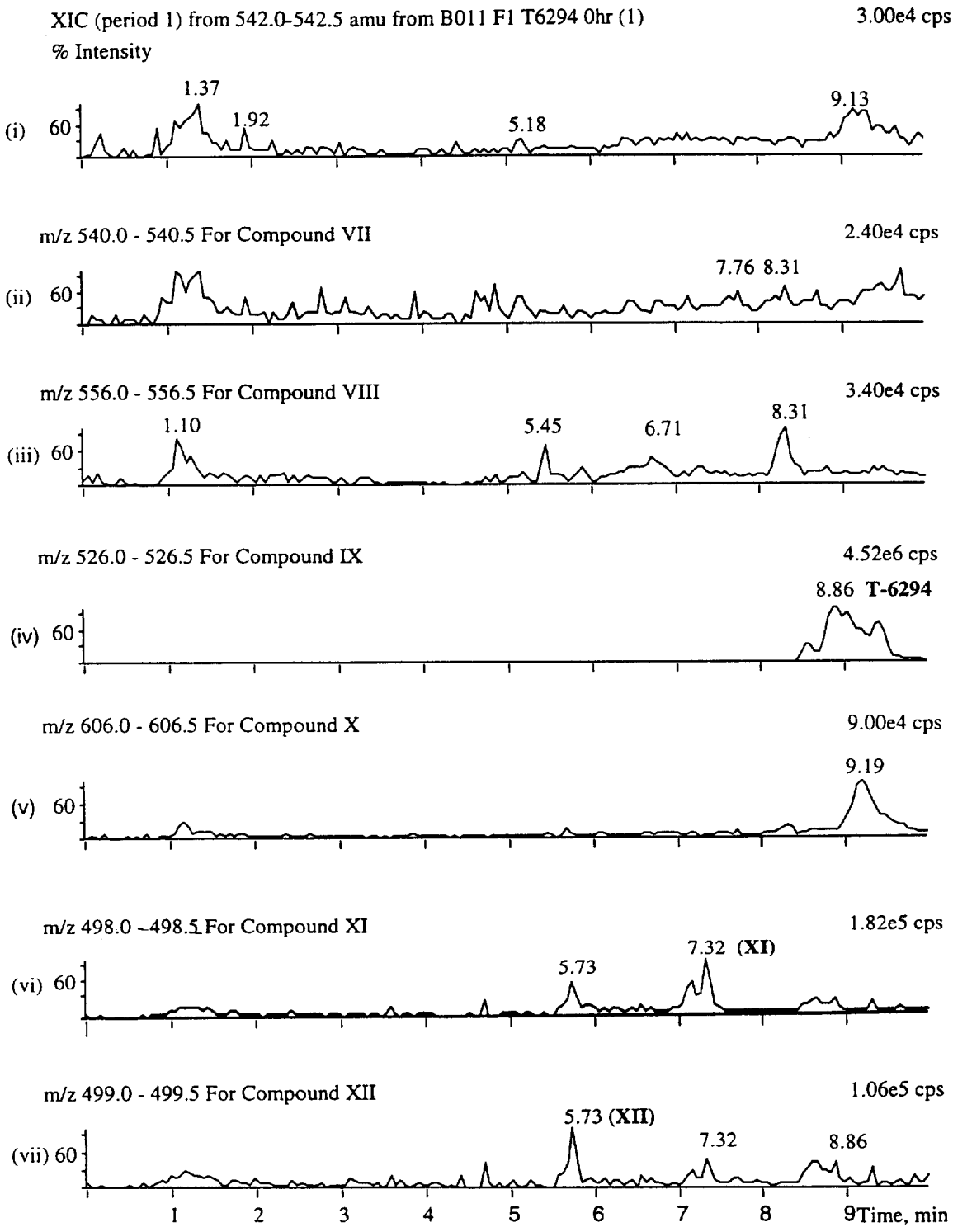
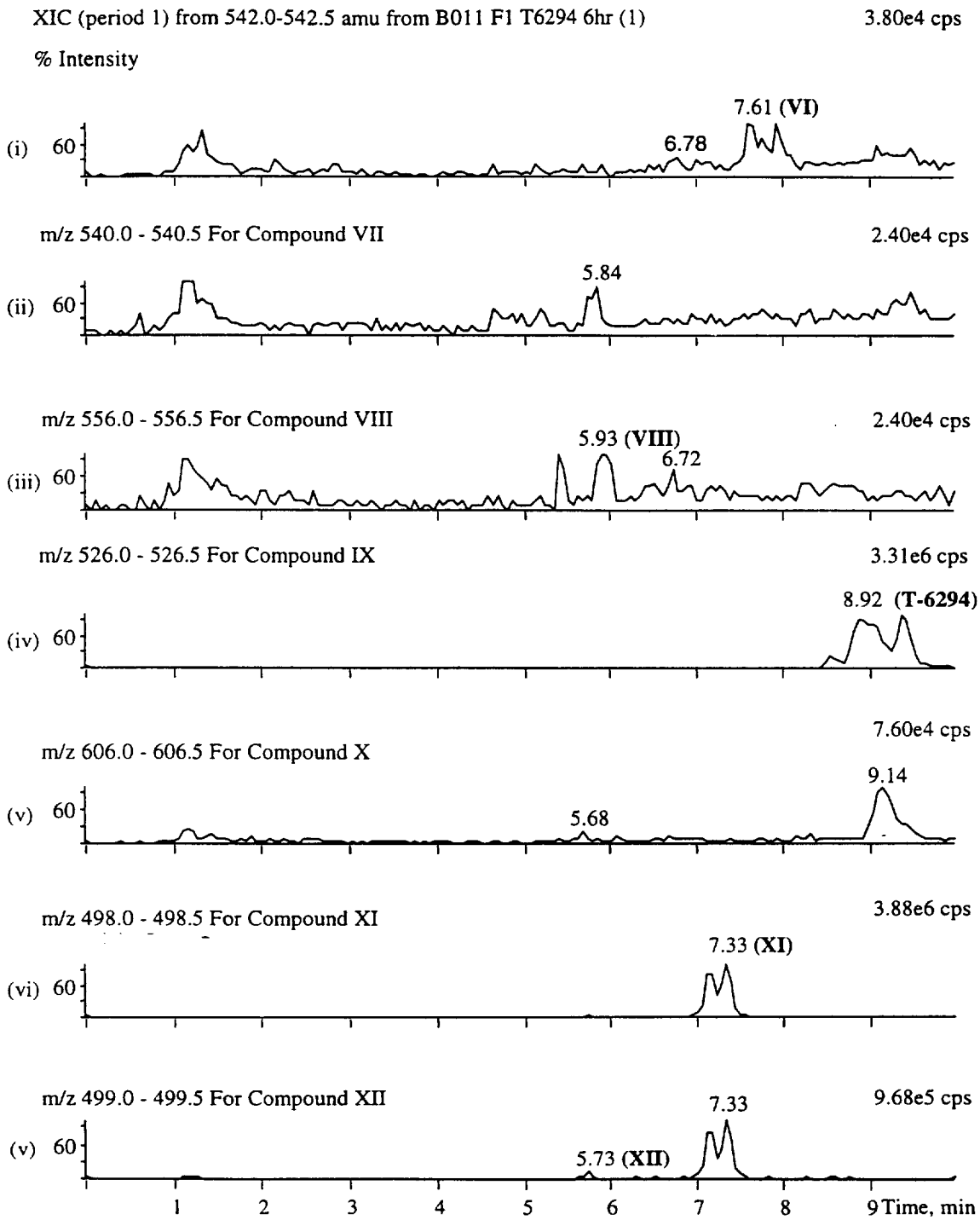


Figure 28. Reconstructed ion chromatograms showing the presence of metabolites after incubating human hepatocytes with T-6294 for 0 hour.



REPORT: 98AGKP01.3M

Figure 29. Reconstructed ion chromatograms showing the presence of metabolites after incubating human hepatocytes with T-6294 for 6 hour.



REPORT: 98AGKP01.3M

Figure 30. **SRM total ion chromatograms showing the presence of metabolite VII in (i) T-6294 standard solution; (ii) incubation of T-6294 with rat hepatocytes at 0 hour; (iii) incubation of T-6294 with rat hepatocytes at 6 hour; (iv) incubation of T-6294 with human hepatocytes at 0 hour and (v) incubation of T-6294 with human hepatocytes at 6 hour.**

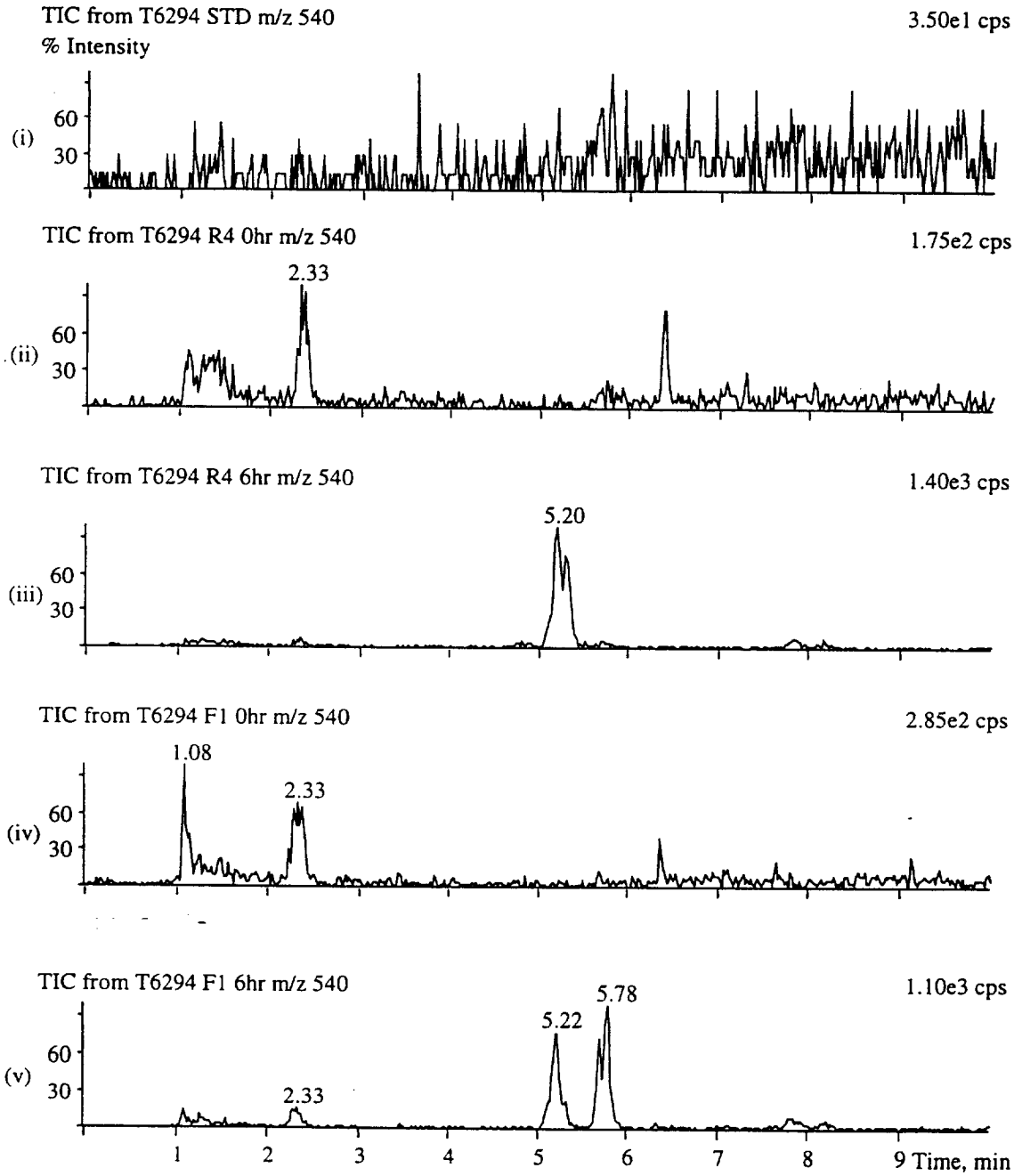


Figure 31. SRM total ion chromatograms showing the presence of metabolite X in (i) T-6294 standard solution; (ii) incubation of T-6294 with rat hepatocytes at 0 hour; (iii) incubation of T-6294 with rat hepatocytes at 6 hour; (iv) incubation of T-6294 with human hepatocytes at 0 hour and (v) incubation of T-6294 with human hepatocytes at 6 hour.

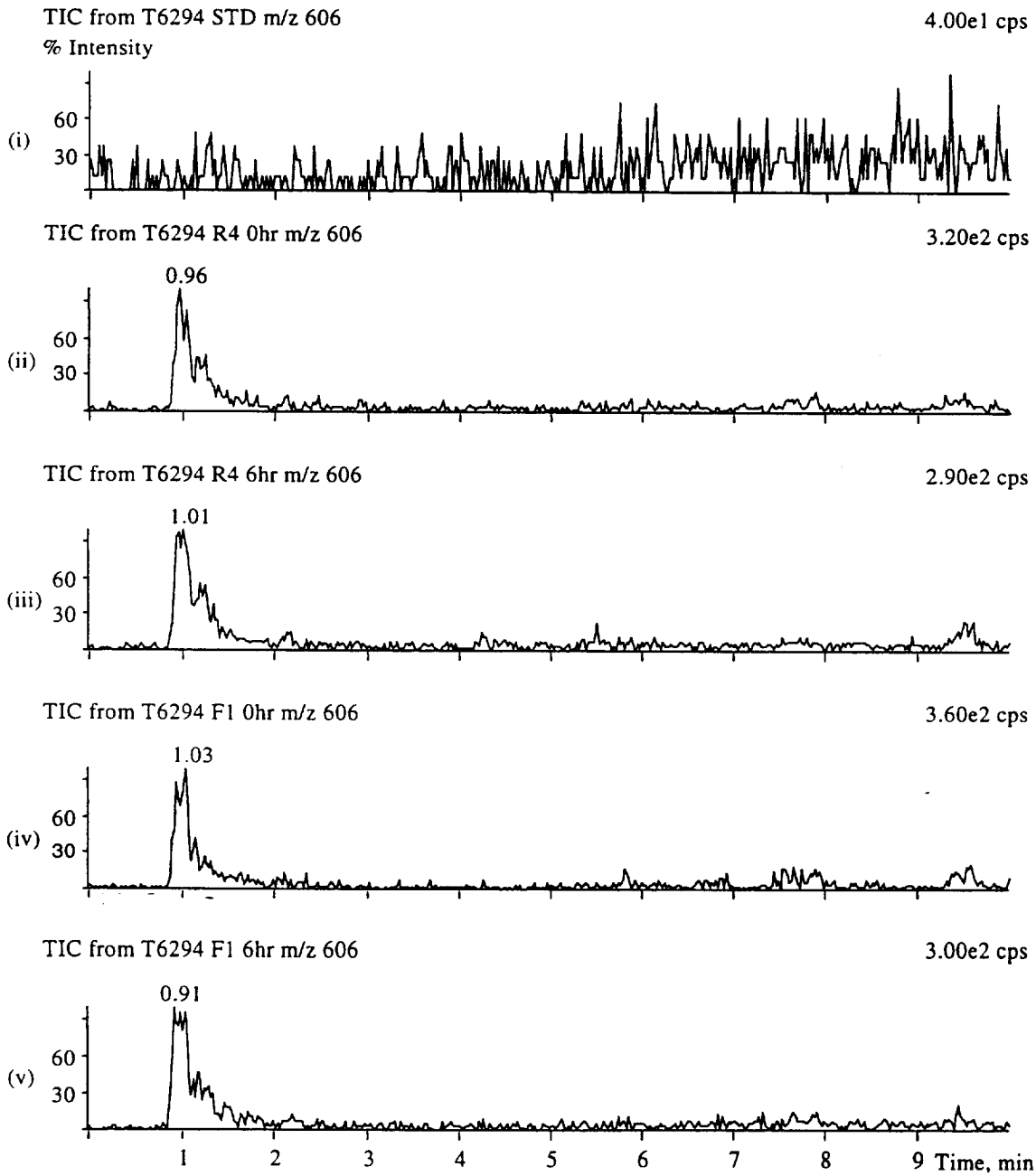


Figure 32. Negative ionization product-ion mass spectra of (i) metabolite III; (ii) metabolite VI and (iii) metabolite VIII.

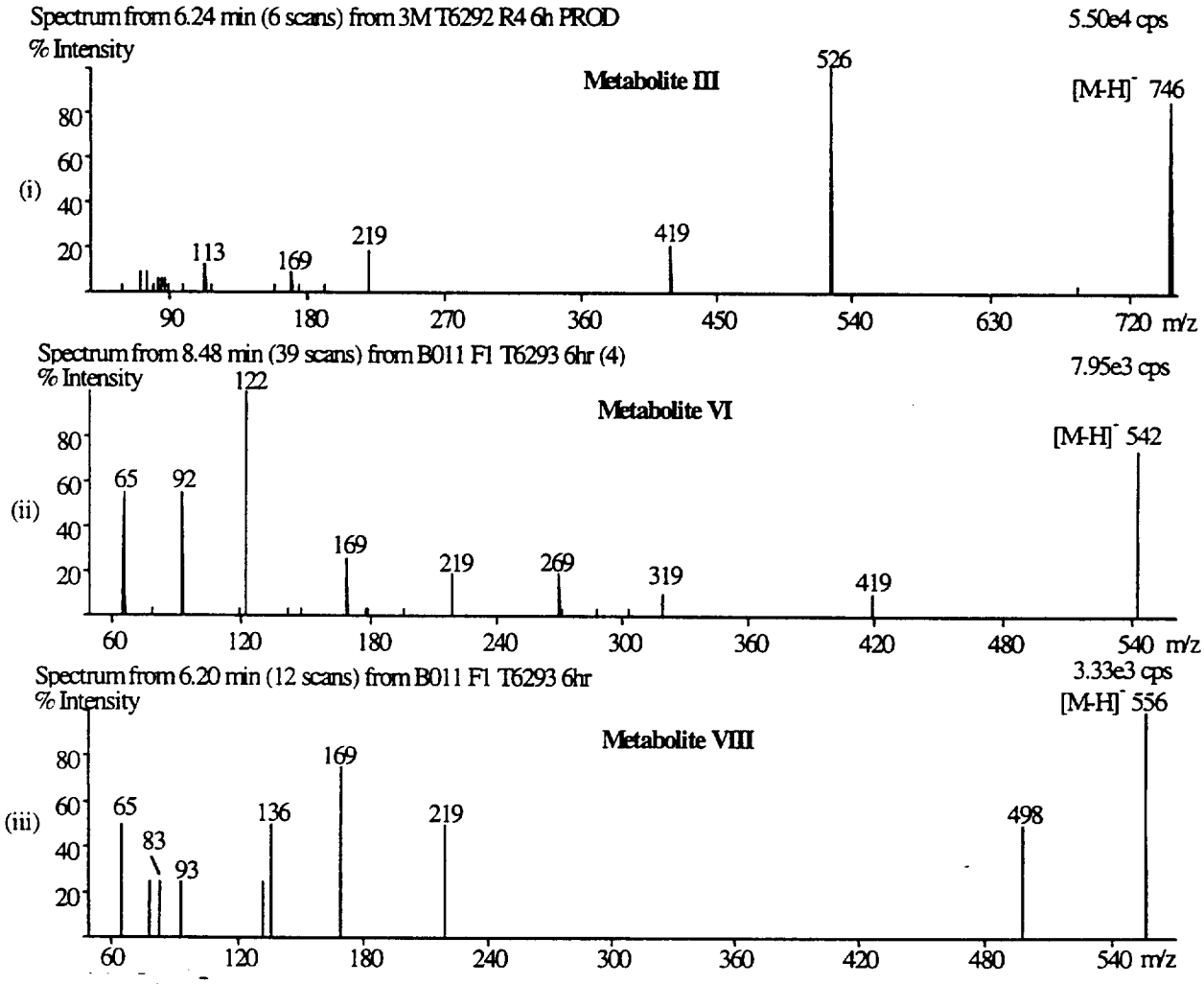


Figure 33. Product-ion mass spectra of (i) Metabolite XIII and (ii) metabolite XI

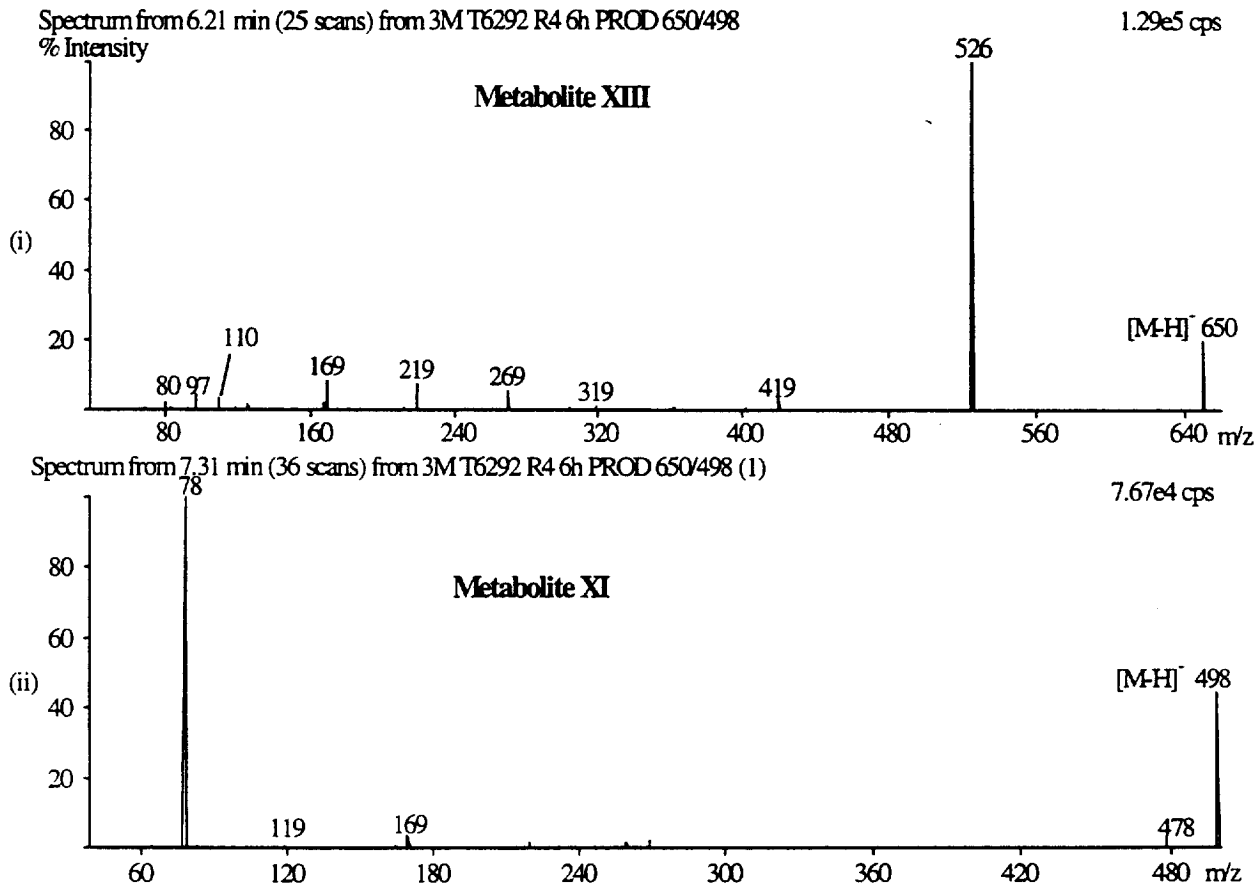


Figure 34. Selected Reaction Monitoring Chromatograms from LC/MS/MS of a Calibration Standard Hepatocyte Extract (2 ng/mL T-6295)