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Simultaneous determination of JTT-501 and its main metabolite in human plasma by liquid chromatography–ionspray mass spectrometry

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Abstract

An LC–MS–MS analytical method was developed for the determination of a new antidiabetic agent, JTT-501 and its main metabolite (JTP-20604) in human plasma. The compounds were isolated from plasma by protein precipitation before analysis by HPLC with atmospheric pressure positive ionisation MS–MS detection. An isotopically labelled analog of JTT-501 was used as the internal standard. Linearity was demonstrated over the calibration range of about 5–10 000 ng/ml for both compounds. The assay was validated with respect to accuracy, precision and analyte stability. This method was used for the determination of plasma concentrations for the two compounds in a clinical tolerability study. A cross-validation exercise between two different mass spectrometers, used for the determination of clinical samples, is also reported.

Keywords: JTT-501; JTP-20604

1. Introduction

Type 2 (noninsulin-dependent) diabetes mellitus is characterised by insulin resistance in insulin-sensitive peripheral tissue, particularly in the skeletal muscle [1]. The thiazolidinediones including troglitazone [2], rosiglitazone [3] and pioglitazone [4] comprise a new class of oral antidiabetic agents, which reduce plasma glucose concentrations in Type 2 diabetes mellitus models due to enhancement of insulin sensitivity in peripheral tissues.

JTT-501, 4-[4-[2-(5-methyl-2-phenyl-4-oxazolyl)etoxy]benzyl]-3,5-isoxazolidone (Fig. 1), is a novel insulin-sensitizing agent [5] under clinical evaluation for treatment of Type II diabetes mellitus. This isoxazolidinedione derivative, structurally distinct from the thiazolidinediones, is effective in lowering glucose, insulin and triglyceride concentrations in Type 2 diabetes mellitus models [6].

The main metabolite in humans was identified as a malonic amide (Fig. 1) created by reductive cleavage of the isoxazolidine ring [7] and is denoted as JTP-20604. The in vitro insulin-sensitizing effect of JTP-20604 was as potent as that of JTT-501 [7].

The objective of this study was to develop and validate a LC–MS method for the determination of

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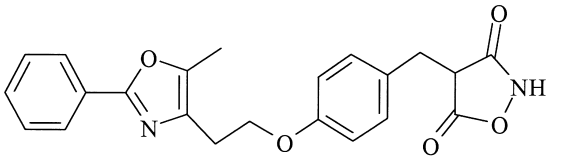
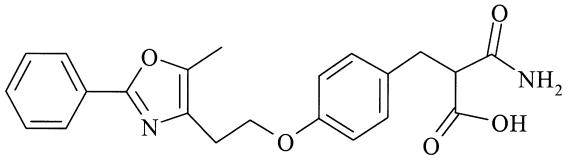
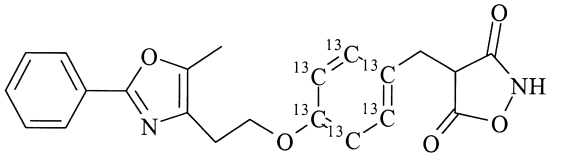


JTT-501



JTP-20604



$^{13}C_6$-JTT-501

Fig. 1. Structures of JTT-501, JTP-20604 and $^{13}C_6$-JTT-501.

JTT-501 and its metabolite (JTP-20604) in human plasma.

2. Experimental

2.1. Materials

JTT-501 and JTP-20604 were supplied by Japan Tobacco. The stable label internal standard (I.S.) $^{13}C_6$-JTT-501 (Fig. 1), was synthesised in the Global Drug Metabolism Department, Pharmacia (Nerviano, Italy) [8]. All other chemicals and solvents were of analytical grade from Carlo Erba (Milan, Italy).

Water was prepared in house using a Milli-Q Plus 185 system (Millipore, Vimodrone, Italy).

2.2. Preparation of standard solutions

For preparation of calibration standards, stock solutions of JTT-501 and JTP-20604 were prepared at concentration of 1 mg/ml in methanol. Working standard solutions were prepared by dilution of the stock solutions, and aliquots (10 or 20 μl) were brought to a final volume of 1 ml with heparinised human plasma to provide plasma concentrations of JTT-501 and its metabolite equivalent to 10 000, 5000, 2000, 700, 150, 30 and 5 ng/ml. For the preparation of QC samples, two different stock solutions were prepared at a concentration of 0.9 mg/ml in methanol. Aliquots of three working solutions obtained from these stock solutions were added to 10-ml volumes of human plasma to generate QC samples at the concentrations of about 17, 440, 8800 and 22 000 ng/ml for both compounds.

2.3. Sample preparation

Unknown and standard samples were prepared by adding 50 μl of human plasma into labelled Eppendorf microcentrifuge tubes after ensuring that all samples were thoroughly defrosted and mixed. Acetonitrile (200 μl) containing $^{13}C_6$-JTT-501 (300 ng/ml) was added to each tube. This mixture was vortex mixed for 10 s and centrifuged for 3 min at 21 000 *g*. An aliquot (150 μl) of the resulting supernatant was diluted with an equal amount of 5 m*M* ammonium formate, pH 3.5, and 40 μl of each extract were injected into the LC system.

For samples containing high concentrations of either analyte, plasma samples (20 μl) were diluted with 180 μl of blank human plasma and, after mixing, a 50-μl aliquot was analysed as described above.

2.4. HPLC conditions

The liquid chromatograph consisting of a HP-1100 solvent-delivery system (Agilent, Cernusco, Italy) equipped with a PE-200 autosampler (Perkin-Elmer, Monza, Italy). Separations were effected using a 150×4.6 mm I.D. Validated C_{18} column, 5 μm, (Perkin-Elmer) maintained at 45 °C. The compounds were eluted using a mobile phase containing acetonitrile and 5 m*M* ammonium formate at pH 3.5 (65:35,

v/v) with a flow-rate of 1 ml/min. The LC flow was split so that 0.1 ml was directed towards the MS interface.

During analysis, the autosampler and HPLC system were controlled by a Macintosh computer running Sciex's RAD (routine acquisition and display) software (version 2.6-FPU).

2.5. MS conditions

LC–MS–MS was performed on a PE-Sciex (Applied Biosystems, Monza, Italy) Model API III+ triple–quadrupole mass spectrometer. The instrument was operated utilising the PE-Sciex ion spray interface. Curtain gas (nitrogen) was 1.6 l/min, while the nebulizer gas (air) pressure was set at 4.105 Pa. Multiple reaction monitoring (MRM) in the positive ion mode was used. The mass spectrometer was programmed to admit pseudomolecular ions $(M+H)^+$ at m/z 393, 395 and 399 for JTT-501, JTP-20604 and internal standard, respectively, via the first quadrupole mass filter (Q_1) followed by collision-induced fragmentation in Q_2 (collision gas argon at density of $300 \cdot 10^{12}$ molecules cm^{-2}) and monitoring via Q_3 of the product ions at m/z 186 (JTT-501 and internal standard) and 334 (JTP-20604). The orifice potential and the collision energy were set at 45 V and 22 eV, respectively.

The dwell time for each transition was 200 ms. Peak area ratios obtained from the MRM chromatograms of the parent compound (m/z 393→186), the metabolite (m/z 395→334) and the internal standard (m/z 399→186) were used for quantitation. All calculations were performed using Sciex's MACQUAN software (version 1.6).

2.6. Assay validation experiments

Calibration curves were constructed using weighted linear least-squares regression and concentrations of the compounds in the samples were calculated by interpolation from the calibration curve.

Inter-assay variation, accuracy and linearity were assessed from the analysis of three separate analytical batches including calibration and QC samples. Intra-assay variation for JTT-501 and its metabolite was determined by analysis of five plasma samples in one analytical run. QC samples with analyte concentrations above the calibration range were analysed after dilution (1:10) with blank human plasma. Precision and accuracy at the lower limit of quantitation (LLOQ) at 5.44 and 5.66 ng/ml for JTT-501 and JTP-20604, respectively, were assessed by analysis of six spiked plasma samples in one analytical run. Stability of organic (methanol) standard solutions at three concentrations, stored at +4 °C was assessed by repeated injections over a period of 1 month. Stability of both analytes in plasma was determined by analysis of QC samples after they had been left for 24 h at room temperature, after three freeze–thaw cycles, for 6 months at −20 °C, and also for 24 h in an autosampler in the final extract.

Ion suppression effects were checked by injections of pure standard solutions and samples spiked with both compounds into extracts of blank human plasma.

The mean extraction recoveries of JTT-501 and JTP-20604 were evaluated comparing the RF (response factor = ratio between the area of the analyte and its concentration) of extracted samples ($n=3$) and the mean RF of unextracted samples (using methanol instead of plasma, $n=3$).

2.7. Cross-validation experiment

During analysis of clinical study samples it was necessary to change from an API III+ to a PE-Sciex API 3000 instrument. For this reason, a cross-validation experiment between these instruments was performed. Six pooled samples were prepared from human plasma samples of subjects treated with 120 mg of JTT-501. For each pool, ten aliquots were extracted and then analysed with both the API III+ and API 3000 instruments. In all experiments, single extractions were prepared and then injected into both instruments.

2.8. Statistical analysis of data

Statistical comparison of data resulting from cross-validation experiments was performed using descriptive statistics and regression analysis routines supplied in the statistical analysis package of EXCEL, version 6.0 (Microsoft, Redmond, WA, USA). Fixed and proportional errors were determined from the

95% confidence limits around the slope and intercept functions of the linear regression.

3. Results and discussion

A Q1 scan of JTT-501, JTP-20604 and I.S. revealed the protonated parent molecule $(M+H)^+$ to be in abundance with mass-to-charge ratios (m/z) of 393, 395 and 399, respectively. The product ion spectrum of JTT-501 (Fig. 2a) and I.S. (Fig. 2c), using collision energy of 22 eV resulted in a major fragment at m/z 186 for both compounds. No evidence of crosstalk due to the use of the same fragment for the parent compound and I.S., was observed. Under the same conditions, the product ion spectra of the metabolite generates a similar fragment ion to the parent compound but a major fragment ion at 334 m/z was also seen (Fig. 2b) and was chosen for quantitation. The proposed fragment ions are shown in Fig. 2.

Under the chromatographic conditions utilised in this study, the retention time was about 2 min for JTT-501 and I.S. and 2.5 min for JTP-20604 (Fig. 3). The compounds were eluted with low k' values (0.3 and 0.6), very close to the solvent front and this could increase the potential for ion suppression. However, in the method described in this paper similar responses were seen following injections of standard solutions compared with identical concentrations of both compounds spiked into extracts of blank human plasma indicating that control extracts do not significantly suppress responses.

No interfering endogenous peaks were detected in any extracts of control human plasma and no interfering peaks were noted in predose samples from clinical studies.

The calibration curves for JTT-501 were linear over the range of 5.44–10 300 ng/ml, and over the range of 5.66–10 600 ng/ml for JTP-20604. The linear correlation coefficients (r^2) ranged from 0.9944 to 0.9964 for JTT-501 and from 0.9926 to 0.9947 for JTP-20604, using linear regression with $1/x^2$ weighting. The weighting factor was chosen to minimise deviation of backcalculated values from theoretical concentrations. The backcalculated calibration standard points showed a RSD ranging from 3.1 to 7.9% for JTT-501 and from 5.5 to 10.1% for JTP-20604. The percent difference between the standard theoretical concentrations and those derived from the calibration curve ranging from −7.8 to 8.7 for JTT-501 and from −11.4 to 10 for JTP-20604.

A lower limit of quantitation (LLOQ) of 5.44 ng/ml (JTT-501) and of 5.66 ng/ml (JTP-20604) was achieved. This represents approximately 20 pg on column for both compounds. The accuracy at these levels was better than ±5% and the intra-day RSD was <10%.

The results of intra- and inter-day precision and accuracy are described in Table 1.

Stock solutions, stored in the dark at +4 °C, were stable for at least 1 month. There was no evidence of degradation of JTT-501 and JTP-20604 after storage in plasma at room temperature for 24 h, after 6 months at −20 °C, after three freeze–thaw cycles and at room temperature for 24 h after extraction (Table 2).

Recovery from plasma samples ranged from 71.7 to 105.8% for JTT-501 and from 67.3 to 104.0% for JTP-20604 (Table 3). As shown in this table, the recovery varied with concentration however good precision and accuracy was obtained with independent QCs at all concentrations tested for both compounds, indicating the accuracy of the method is not effected.

Comparison of individual results for the cross-validation between the API 3000 and the API III+ (Table 4) showed that the two instruments gave similar results for both drug and metabolite, and no differences >15% were seen for individual samples. Concentrations of the parent drug measured using API 3000 were on average 7% higher than those measured with the API III+. Whilst a statistically significant difference was indicated using the confidence intervals approach, the actual differences in concentrations were small and unlikely to be relevant in a bioanalytical context. For the metabolite, values obtained with the API 3000 were about 2% lower than those obtained with the API III+, which was not statistically significant. Similar results were obtained using regression analysis to compare the two instruments (Table 4). During this comparison an improved S/N (about three times greater than the API III+) was observed with API-3000, which is consistent with the claim reported by Applied Biosystems of greater sensitivity with this instrument.

The current method was applied to the determi-

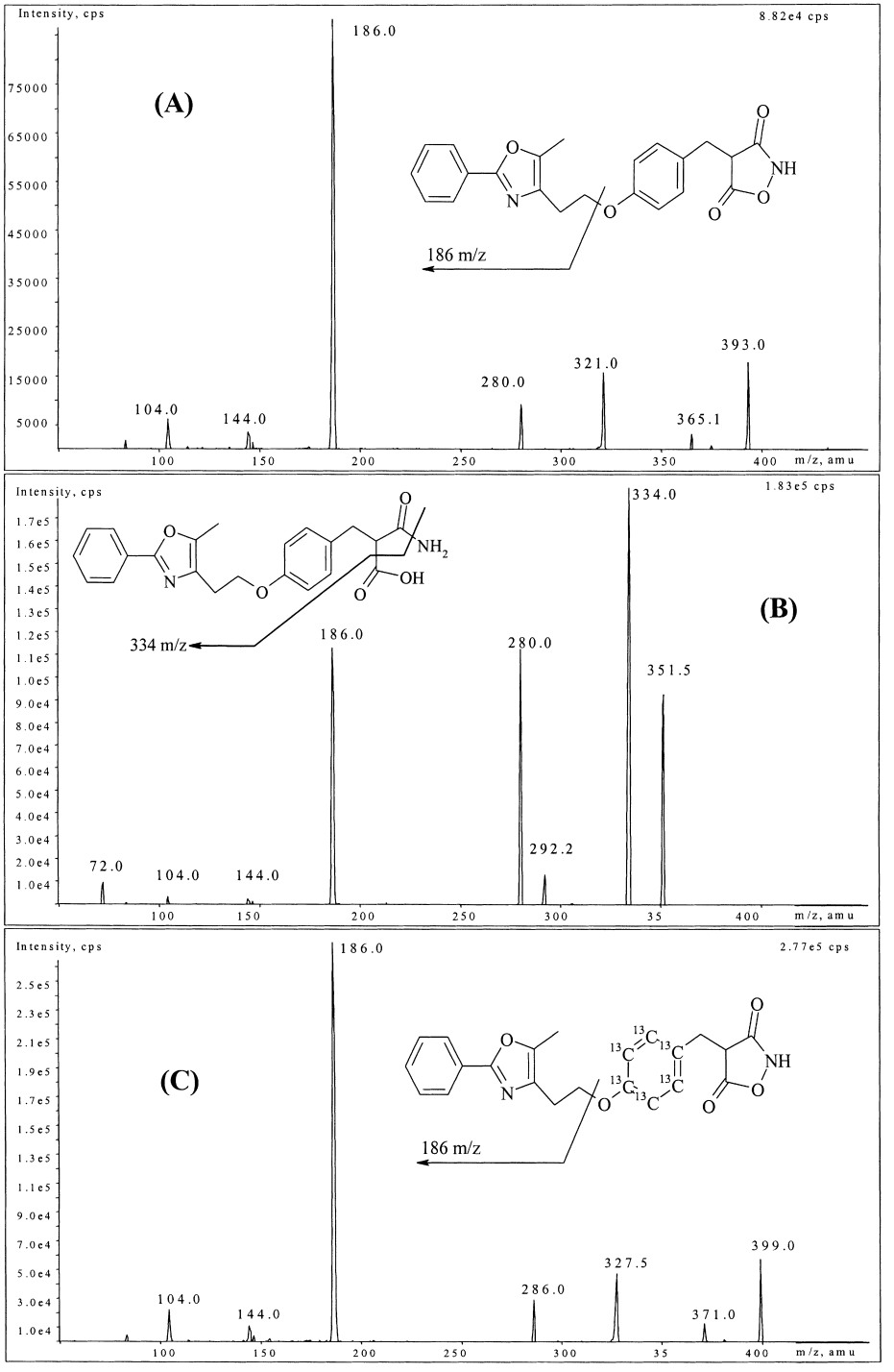


Fig. 2. Positive product ion mass spectra of the molecular ions of (A) JTT-501, (B) JTP-20604 and (C) $^{13}C_6$-JTT-501.

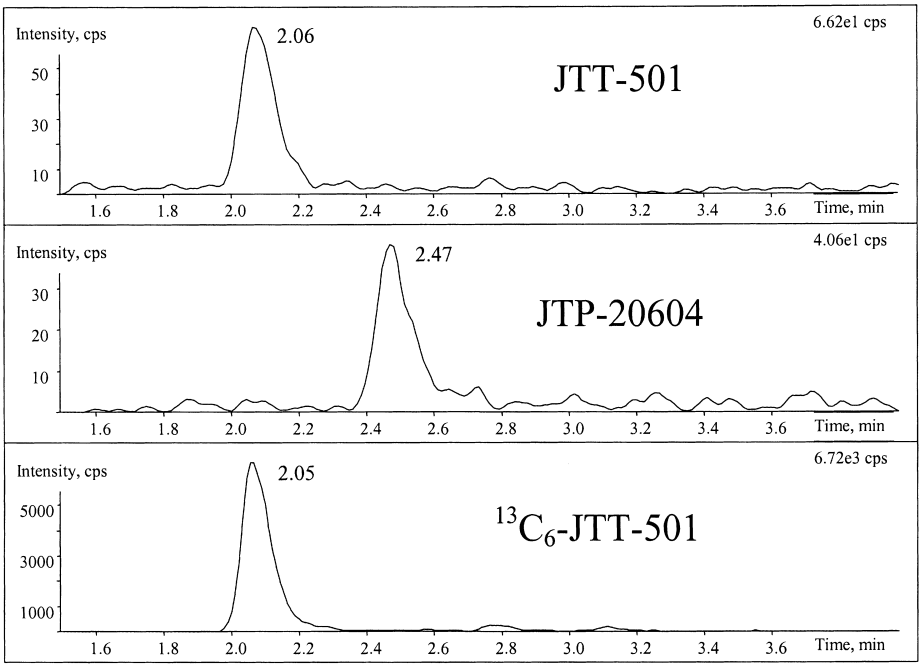


Fig. 3. Extract ion chromatograms of human plasma spiked with 5.44 ng/ml of JTT-501 and 5.66 ng/ml of JTP-20604.

nation of JTT-501 and JTP-20604 concentrations in plasma from healthy volunteers in a clinical tolerability study. Typical chromatograms are shown in Fig. 4.

4. Conclusions

A simple and reliable LC–MS–MS method for the simultaneous determination of JTT-501 and its metabolite (JTP-20604) in human plasma was developed and validated. This method was used to analyse over 2000 samples from clinical studies demonstrating its robustness and its reproducibility. The procedure is suitable for measuring therapeutic levels of JTT-501 and its metabolite in patients.

The results of the cross-validation experiment indicated good correlation between the data obtained

Table 1
Accuracy and precision of JTT-501 and JTP-20604 determination in human plasma

Compound	Concentration (ng/ml)	Intra-assay precision (RSD, %, $n=5$)	Inter-assay precision (RSD, %, $n=15$)	Intra-assay Accuracy (Bias, %, $n=5$)	Inter-assay Accuracy (Bias, %, $n=15$)
JTT-501	17.6	4.0	3.9	−5.1	−7.8
	441	1.3	6.0	−2.7	−5.1
	8810	3.5	6.1	−3.8	−4.3
	22 000	4.0	–	−5.2	–
JTP-20604	17.4	6.1	6.1	−5.8	−3.1
	436	3.0	3.0	+8.9	+6.4
	8720	4.5	4.5	+5.7	+1.0
	21 800	3.8	–	+2.6	–

Table 2
Stability of JTT-501 and JTP-20604 in human plasma

Storage conditions	JTT-501		JTP-20604	
	Concentration (ng/ml)	Bias (%)	Concentration (ng/ml)	Bias (%)
24 h at room temperature	17.6	−8.7	17.4	2.9
	441	−15.0	436	14.4
	8810	−10.9	8720	−8.7
24 h in the final extract	17.6	−10.8	17.4	10.3
	441	−12.4	436	14.6
	8810	−13.4	8720	−5.2
After 3 freeze–thaw cycles	17.6	−8.1	17.4	6.3
	441	−2.2	436	11.3
	8810	−2.0	8720	5.2
After 6 months at −20 °C	17.6	8.3	17.4	−10
	441	6.0	436	−7.9
	8810	4.9	8720	−0.1

Table 3
Recovery of JTT-501 and JTP-20604 from human plasma

Compound	Concentration (ng/ml)	*n*	Mean recovery (%)	SD	RSD (%)
JTT-501	17.6	3	71.7	3.7	5.1
	441	3	90.8	1.0	1.1
	8810	3	105.8	0.4	0.4
JTP-20604	17.4	3	67.3	1.2	1.8
	436	3	96.7	1.2	1.2
	8720	3	104.0	1.4	1.5

Table 4
Descriptive statistics and linear regression analysis performed between the analysis obtained with API III+ and API 3000 instruments

	JTT-501	JTP-20604
n	60	60
% ratio mean±S.E.M.	107.1±0.91	98.6±1.47
95% interval of mean	105.3–109.0	95.6–101.5
R	0.9980	0.9788
Slope±S.E.M.	1.01±0.01	0.96±0.02
95% interval of slope	1.00–1.02	0.93–1.00
Intercept±S.E.M.	19.5±9.83	3.71±1.89
95% interval of intercept	−0.14–39.2	−0.07–7.50

using API III+ and API 3000. Thus, these instruments can be regarded as interchangeable in bioanalysis of JTT-501 and its metabolite in man. However, API 3000 is the preferred choice of instrument due to its higher sensitivity and the possibility of near continuous working without the recycle period, which is needed for API III+.

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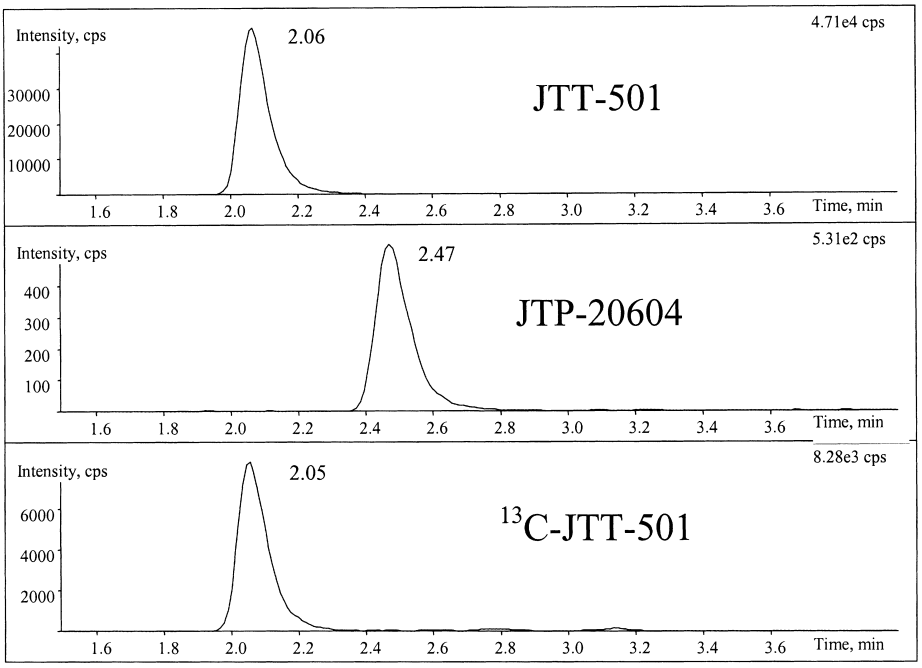

Fig. 4. Extract ion chromatograms of a sample obtained from a healthy volunteer 3 h (3340 ng/ml of JTT-501 and 65.8 ng/ml of JTP-20604) after a 120 mg oral dose of JTT-501.

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