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Determination of 13-*O*-demethyl tacrolimus in human liver microsomal incubates using liquid chromatography–mass spectrometric assay (LC–MS)

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Abstract

A simple, sensitive and specific liquid chromatography coupled electrospray ionization mass spectrometric (LC/ESI/MS) method for the determination of 13-*O*-demethylated metabolite (MI), one of the major metabolites of tacrolimus has been developed. The assay uses 32-demethoxyrapamycin (IS) as the internal standard; ethyl acetate as extraction solvent; a Hypersil-Keystone Beta Basic-18 reversed-phase column; and a gradient mobile phase of consisting 0.1% formic acid in water and methanol–acetonitrile (3:49, v/v). Mass detection is performed on a single quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface and operated in a positive ionization mode. MI in the microsomal incubates was quantitated by computing the peak area ratio (MI/IS) analyzed in single ion monitoring (SIM) mode (*m*/*z*: 804 and *m*/*z*: 901 for MI and IS, respectively). Precision of the assay was determined by calculating the intra-run and inter-run variation at three concentrations (15, 25, 80 ng/ml); the intra run relative standard deviation (R.S.D.) was less than 10% and ranged from 5.0 to 8.3%; and the inter-run R.S.D. was less than 10% and ranged from 4.6 to 9.6%. The limits of detection was 2 ng/ml. This assay has been used to evaluate the effect of three human immunodeficiency virus (HIV) protease inhibitors on the metabolism of tacrolimus in human liver microsomes.

Keywords: Tacrolimus; 13-*O*-demethyl tacrolimus; LC/ESI/MS; Human liver microsome; Protease inhibitors

1. Introduction

Liver transplantation is a life-saving therapy for patients with end-stage liver disease. Tacrolimus (FK506), a macrolide lactone produced by the fungus *Streptomyces tsukubaensis*, is the primary immunosuppressive drug used in liver transplant patients. Tacrolimus is at least 100 times more potent than cyclosporine [1] and has significantly reduced the incidence of acute rejection in organ transplant patients [2].

Tacrolimus is primarily metabolized by the cytochrome P450 superfamily of mono-oxygenases, specifically by CYP 3A4/5 isoform in the liver and small intestine [3–5]. The primary metabolites formed by CYP3A4/5 are the *O*-demethylated products of tacrolimus [3,6–10] (as depicted in Fig. 1a) [11]. Several CYP3A inducers [12,13] and inhibitors [13] have been shown to alter the metabolism of tacrolimus. The metabolism and the metabolic drug interactions of tacrolimus could be evaluated using human liver microsomes in vitro [3]. In order to characterize the mechanism(s) of inhibition of CYP mediated metabolism of tacrolimus, a specific and sensitive assay to determine its metabolite(s) must be established.

Quantitation of tacrolimus and its metabolites has been challenging due to the lack of significant UV absorption and low levels of the metabolites in biological fluids. High pressure liquid chromatographic (HPLC) methods [6–8]

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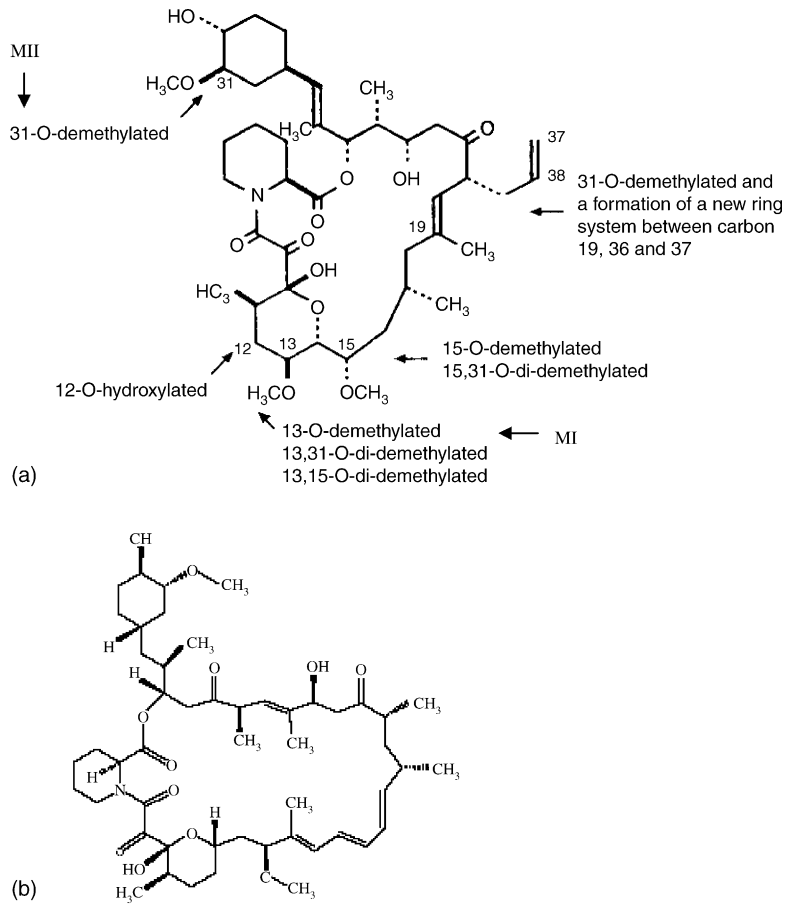

Fig. 1. Structure of tacrolimus and its metabolites (a) and internal standard (IS) (b).

generally require long analysis time for each sample to achieve appropriate separation of the various metabolites and tacrolimus. Large amount of microsome (not less than 1.0 mg in the incubation system) with high concentration of tacrolimus (25 μM) is also needed to produce enough metabolites for detection by HPLC. LC–MS methods will increase the selectivity and sensitivity of detection of tacrolimus and its metabolites. Lensmeyer [14] reported an optimal and stable LC–MS method for quantitation of tacrolimus in blood without determination of its metabolites. Zhang [15] applied LC–MS–MS method for the determination of tacrolimus concentration in whole blood but did not quantify specific metabolites. Other reports on the measurement of tacrolimus metabolites by LC–MS–MS assay[16–19] and fast-atom bombardment mass spectrometry (FAB-MS) assay [8] haven't provided information on quantitation of metabolites. One method that quantitated the metabolite [20] in blood sample required a sophisticated column switching procedure with gradient system, which is not readily available in many clinical laboratories. A specific and sensitive methodology for quantification of the major metabolite(s) of tacrolimus is not currently available. The purpose of this study was to develop a simple quantitative method for determination of 13-*O*-demethyl tacrolimus (MI) in human liver microsomal incubates and apply this method to study the effect of HIV protease inhibitors on the metabolism of tacrolimus.

2. Experimental

2.1. Reagent and chemicals

Tacrolimus (Fig. 1) (MW: 803.5), MI, and 31-*O*-demethyl tacrolimus (MII) (MW: 789.5) were obtained from Fujisawa Inc (Japan). The 32-demethoxyrapamycin used as the internal standard (IS) (Fig. 1) (MW: 900.6) was obtained from Wyeth (Marietta, PA, USA). NADPH was purchased from Sigma (St. Louis, MO, USA). Methanol, acetonitrile and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was from Sigma, and was of reagent grade. Human liver sample was obtained form Hepatocyte Transplantation Laboratory at the University of Pittsburgh. Liver microsomes were prepared by a standard differential centrifugation procedure [21].

2.2. LC–MS system

The LC–MS system was from Thermo Finnigan including the Surveyor autosampler and MS pump (Thermo-Finnigan, San Jose, CA, USA). A reversed-phase, Thermo-Hypersil-Keystone Beta Basic-18, 5 μm, 150 mm × 3 mm i.d (Thermo-Hypersil, Bellefonte, PA, USA) column was used for all chromatographic separation at 50 °C. The mobile phase was a linear gradient consisting of water phase (A): 0.1% formic acid in water and organic phase (B): methanol–acetonitrile (3:49, v/v) that was pumped at a flow rate of 0.4 ml/min with the following program:

Time (min)	Flow rate (μl/min)	A (%)	B (%)
0.00	400	40	60
7.50	400	5	95
14.50	400	5	95
15.00	400	40	60

The injection volume was 20 μl. The total run time for the analysis was 15 min. Column eluates were analyzed with a Thermo Finnigan MSQ mass spectrometer. The mass spectrometer was operated in a positive electrospray ionization mode with a probe voltage of 3.0 kV at a temperature of 225 °C.

The response of MI and IS were measured in the positive mode with a Cone voltage of 40 V (Volts). In the single ion monitoring (SIM) experiments, the ion selected for MI was $[M + H^+]$ (*m*/*z* 804) and that for IS was $[M + H^+]$ (*m*/*z* 901), both ions had a dwell time of 500 ms per ion. The data acquisition and analysis were performed with Xcalibur software version 1.2.

2.3. Stock solution

A 100 μg/ml solution of IS was prepared in methanol and was further diluted with methanol to 1 μg/ml. For calibration standards, a 1.5 mg/ml of stock solution of MI was prepared in methanol and was further diluted with methanol to obtain desired concentrations to spike into mircrosmal samples. The stock solutions were kept refrigerated and were discarded one month after preparation.

2.4. Microsomal incubations

Optimal conditions for the evaluation of the metabolism of tacrolimus were selected by varying the time of incubation and the microsomal protein concentrations. Tacrolimus was incubated with human liver microsome (80 μg/ml, final protein concentration) and $MgCl_2$ (10 mM) in 0.1 mM phosphate buffer (pH 7.4). The final volume of 0.5 ml was allowed to equilibrate in a shaking water bath for 2 min at 37 °C. The reaction was initiated with the addition of 20 μl NADPH (1 mM). After 10 min of incubation, the reaction was stopped by placing the samples on ice and immediately adding 10 ng (10 μl of 1.0 μg/ml) of IS and 5 ml of cold ethyl acetate. After vortexing for 4 min, and centrifugation at 3500 g for 10 min at 4 °C, the organic layer was transferred to a 10 ml tube and evaporated to dryness. One hundred microlitres of methanol was added to the dried extract, vortex mixed for 60 s and a 20 μl aliquot was injected into the LC–MS.

2.5. Calibration and linearity

Calibration curves were obtained daily for 3 days using seven different concentrations of MI (5, 10, 20, 60, 200, 600 and 1200 ng/ml) in incubation buffer containing all the components for microsomal incubation, except NADPH. The ratio of the peak areas of MI to the peak areas of IS was calculated. The calibration curves were constructed by weighted ($1/y$) least-squares linear regression analysis of the peak area ratios of MI/IS versus the concentrations of MI. Calibration curve equations were used to calculate the concentrations of MI in the microsomal samples and QC samples from their peak area ratios.

2.6. Precision and accuracy

Three levels of quality control (QCs) at 15.0, 25.0 and 80.0 ng/ml (low, medium and high) were prepared in incubation buffer containing all the constituents except NADPH. These concentrations were selected based on the concentration of MI metabolite observed in the microsomal incubation mixture. The intra-run and inter-run precision and accuracy were determined by analyzing a set of QC samples ($n = 6$) at 15.0, 25.0 and 80.0 ng/ml.

2.7. Recovery

Microsomal samples (incubation buffer containing all incubation constituents except NADPH) were spiked with MI at 15.0, 25.0 and 60.0 ng/ml and extracted according to the method described above ($n = 6$ at each level). Then 10 ng (10 μl of 1.0 μg/ml) of IS was added to the extracts and the sample was injected into the LC–MS. The ratio of area for MI to IS was calculated. Standard mixtures of MI and IS equivalent to the concentration in the microsomal samples were directly injected into the LC–MS and the ratio of area for MI to IS was calculated. The recovery of MI was evaluated by comparing the peak area ratios of MI/IS in the microsomal extracts to that of the standard mixtures.

2.8. HIV protease inhibitors on tacrolimus metabolism

We examined the effect of the three HIV protease inhibitors (ritonavir (RT), indinavir (IND) and nelfinavir (NE)) on the formation of MI from tacrolimus. Tacrolimus (600 ng/ml) was incubated as described in 2.4. Each protease inhibitors (1 μM) was added 3 min before incubation. The formation of MI was measured in the absence and in the presence of each protease inhibitor.

3. Results and discussion

3.1. Determination of MS settings

In order to minimize undesirable fragmentation and achieve the highest response, various fragmentor voltages from 20 to 50 V were tested. The highest response was obtained using a value of 40 V. The protonated molecule of MI $[M + H^+]$ (*m*/*z* 790) and the sodium adduct of MI $[M + Na^+]$ (*m*/*z* 813) were tested at 40 V. Because the former was more sensitive than the latter, MI $[M + H^+]$ (*m*/*z* 790) was selected for further studies. The *m*/*z* value for tacrolimus was $[M + H^+]$ (*m*/*z* 804), MII was $[M + H^+]$ (*m*/*z* 790) and IS was $[M + H^+]$ (*m*/*z* 901).

3.2. Separation and specificity

We also evaluated a high pressure liquid chromatography-ultraviolet detection (HPLC-UV) method for analysis of the microsomal samples. In order to achieve good separation of all the components, a run time of 48 min was necessary. The HPLC chromatogram is shown in Fig. 2. The HPLC condition and the results are similar to previously published reports and are provided in the figure legend [7,8]. The retention time for MI and tacrolimus were 8.3 and 42.3 min, respectively. Ion chromatograms for MI and IS from blank microsomal sample (Fig. 3a), microsomal sample spiked with MI, MII and IS (Fig. 3b) and microsomal sample incubated with tacrolimus and IND (Fig. 3c) are shown in Fig. 3. The retention time for MI, MII, tacrolimus and IS are 3.5, 4.2, 6.7 and 7.5 min, respectively. The total run time was less than 15 min.

3.3. Linearity, precision and accuracy

Calibration curves were plotted as the peak area ratio (MI/IS) versus MI concentrations. Results for the calibration curve ($n = 6$) showed good linearity ($r = 0.999$) over the concentration range of 5–1200 ng/ml, with an equation of $y = 0.0093x - 0.0743$, where x = MI concentration in ng/ml and y = MI area/IS area.

The precision of the assay was determined by calculating the intra-run and inter-run variation at three concentrations of MI (15, 25, 80.0 ng/ml) in six replicates. As shown in Table 1, the intra-run R.S.D.% was less than 10% and ranged from 5.0 to 8.3% respectively. As shown in Table 2, the inter-run R.S.D.% was less than 10% and ranged from 4.6 to 9.6%. These results indicate that the method was reliable within the analytical ranges and the use of the IS was very effective in obtaining reproducibility by LC–MS. The mean overall accuracy of the method was 98% (Table 1). The lower limit of detection, defined as a signal-to-noise ratio of 3, was 2 ng/ml, using 20 μl of the sample.

3.4. Recovery

The mean recovery of MI from human microsome was 100.3 ± 10.2%. The recovery data reported here is the average for the three QC standards shown in Table 3.

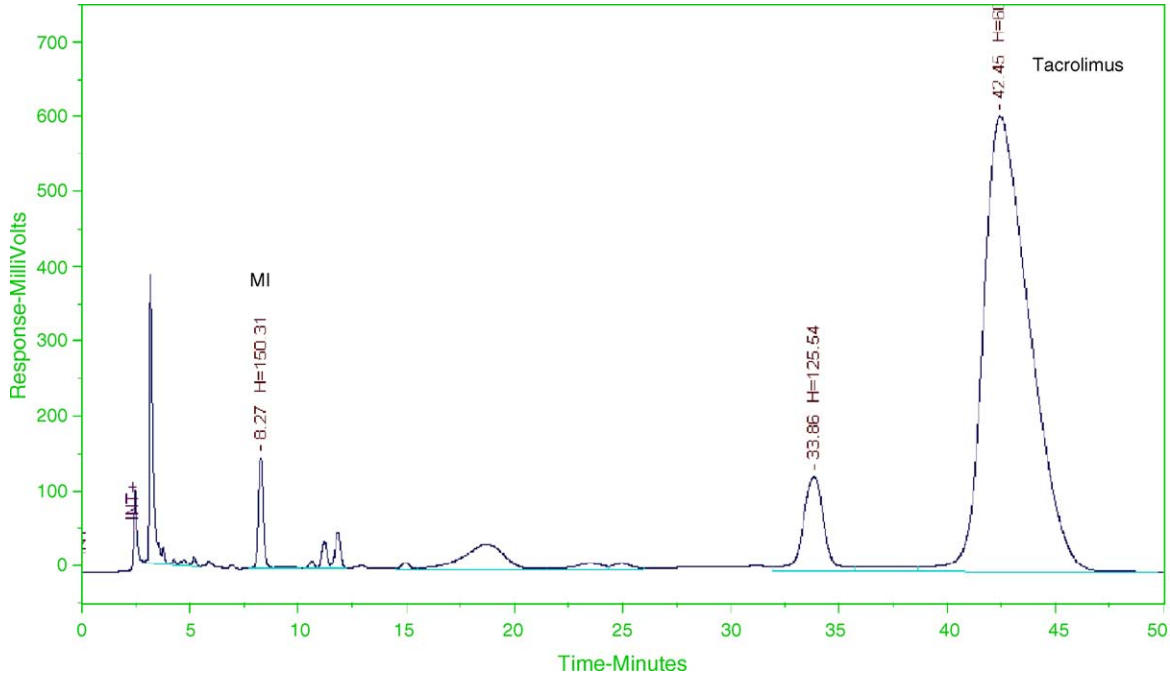

Fig. 2. HPLC tracing of the liver microsomal incubate. Liver microsomes were incubated with tacrolimus for 10 min. Incubation samples were injected on to a reverse phase, Hypersil-Keystone Beta Basic-C 18 column (5 μM, 250 mm × 4.6 mm i.d.), maintained at 60 °C. The mobile phase consisted of acetonitrile: methanol: Orthophosphoric acid (pH 3.0): 49:3:48. The eluents were monitored at 214 nm. Tacrolimus elutes at 42.45 min and metabolite MI elutes at 8.27 min. The peak at 33.8 min is a contaminant in tacrolimus and corresponds to less than 5% of tacrolimus.

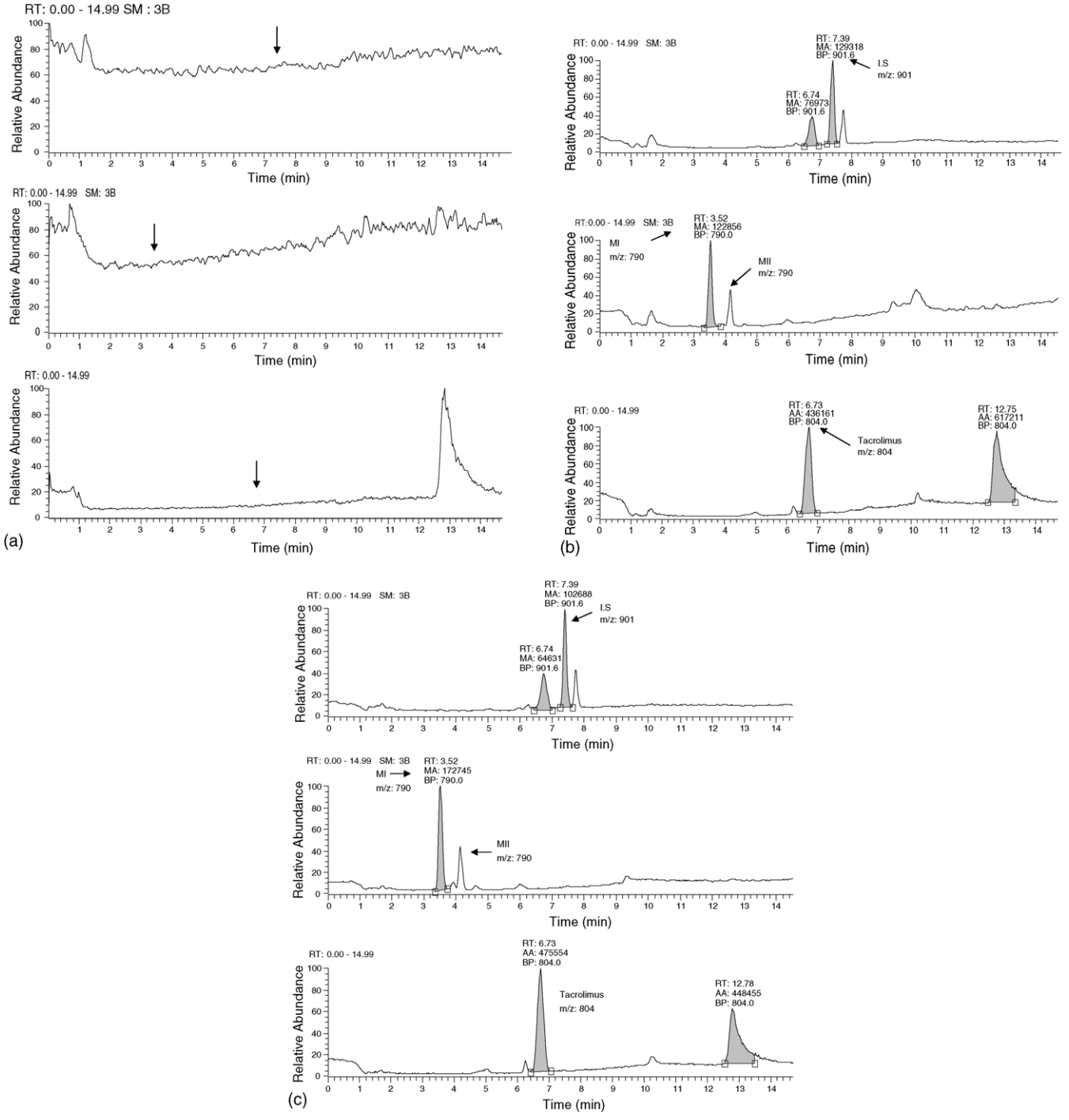

Fig. 3. Representative MS chromatograms of tacrolimus and its metabolites in the human liver microsomal incubate: Top panel (panel 1) corresponds to the IS; (peaks other than IS were due to impurities in IS) middle panel (panel 2) corresponds to metabolites, MI and MII; bottom panel (panel 3) corresponds to tacrolimus. (a) Blank microsomal sample. Arrows point to the time when peaks of interest elute. (b) Microsomal sample spiked with tacrolimus, MI, MII and 32-Desmethoxyrapamycin (IS). (c) Microsomal sample incubated with tacrolimus (600 ng/ml) and indinavir (IND) (1 μM) for 10 min.

Table 1
Intra-run precision and accuracy for MI ($n = 6$)

Normal concentration (ng/ml)	Calculated concentration (ng/ml) (mean ± S.D.)	R.S.D. (%)	Accuracy (%)
15	15.83 ± 0.39	5.0	105
25	22.40 ± 0.84	5.8	90
80	78.76 ± 5.88	8.3	99

Table 2
Inter-run precision and accuracy for MI ($n = 6$)

Normal concentration (ng/ml)	Calculated concentration (ng/ml) (mean ± S.D.)	R.S.D. (%)	Accuracy (%)
15	16.10 ± 0.77	4.6	107
25	21.59 ± 0.73	5.4	86
80	79.55 ± 3.32	9.6	99

Table 3
Extraction efficiency of MI ($n = 6$)

Added concentration (ng)	Recovery (%)	R.S.D. (%)
15.83	99.6	17.9
22.40	101.2	5.2
62.87	100.2	7.6

All concentrations were calculated from calibration curve.

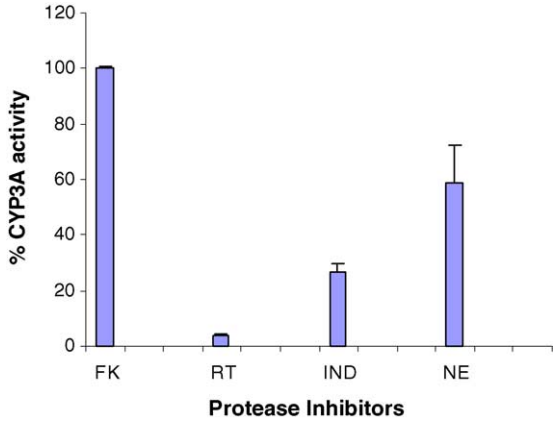

Fig. 4. Inhibition of tacrolimus metabolism by protease inhibitors: *Y* axis represents CYP3A activity as measured by formation of MI metabolite from tacrolimus in the presence of ritonavir (RT; 1 μM), indinavir (IND; 1 μM) and nelfinavir (NE; 1 μM). FK represents control samples with out any protease inhibitors and is assigned 100%. Results are means of three estimates.

3.5. Drug–drug interaction study using human liver microsomes

The effect of the three HIV protease inhibitors (RT, IND and NE) on the metabolism of tacrolimus in human liver microsomes is shown in Fig. 4. The rank order of inhibition is: RT > IND > NE. The result is similar to a previous published report [22] on the inhibition of the formation of the 6β-hydroxyltestosterone from testosterone (a measure of CYP3A activity) by these HIV protease inhibitors, with RT being the most potent inhibitor of tacrolimus metabolism.

4. Conclusions

This publication describes a simple, rapid and sensitive LC/ESI/MS method for direct quantitation of MI in the concentration range 5–1200 ng/ml in human liver microsomal incubates. HPLC has several advantages, e.g., very selective separations, comparatively short analysis times, and simple pretreatment of the sample. Electrospray ionization mass spectrometry (ESI-MS) has the advantage that it can easily be coupled to HPLC as the ion source is at atmospheric pressure and the detection is very sensitive and specific. Due to the technique of pneumatic-assisted electrospray and low flow rate (MS) pump, the flow rate was set at 0.4 ml/min. Under such conditions, the peaks are symmetrical and sharp, which is suitable for quantitative analysis. Only 15 min is need for the analysis of each sample. The retention time for tacrolimus, MI and IS were 6.8, 3.5, 7.5 min, respectively. Another advantage of this study is that by optimizing the incubation condition, we can use low amount of microsomal protein (40 μg) in the incubation system to generate MI. This will dramatically decrease the contamination of the MS system.

In conclusion, the use of LC–MS allows for accurate, precise and reliable measurement of the major metabolite (MI) of tacrolimus in human microsomal samples.

The assay has proven to be rapid and simple, with each sample requiring no more than 15 min of analysis time. The assay method is specific due to the inherent selectivity of mass spectrometry. The major advantages of this method are a simple extraction procedure, the rapidity of separation and high recovery. MI was the major metabolite produced through the primary phase I reaction in human liver microsomes. Measurement of the formation of MI has been used to evaluate interaction between tacrolimus and protease inhibitors. Of the various protease inhibitors tested RT is the most potent inhibitor of tacrolimus metabolism.

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