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Journal of Pharmaceutical and Biomedical Analysis
15 (1996) 349–357

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

In-vitro metabolic studies of tacrolimus using precision-cut rat and human liver slices

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Received for review 18 January 1996; revised manuscript received 23 April 1996

Abstract

The objective of this study was to investigate the in-vitro metabolism of tacrolimus in liver slices from rats and humans. [^{14}C]Tacrolimus (2 or 20 μM) was incubated with precision-cut human and rat liver slices in 12-well plates for up to 12 h. Concentrations of tacrolimus and metabolites were determined by high-performance liquid chromatography (HPLC) radiochromatography. The 13-*O*-demethylated tacrolimus metabolite (M-I) was the major oxidative metabolite in both rat and human liver slices. The other primary metabolites of tacrolimus (M-II, M-III, and M-IV) were not seen in either species. Unidentified peaks, which eluted early in the HPLC system, were probably due to secondary or conjugated metabolites. The eluate had no pharmacological activity. The finding that M-I was the major tacrolimus metabolite in both human and rat liver slice preparations is consistent with previous studies of rat and human liver microsomes.

Keywords: FK506; Human; Liver in-vitro metabolism; Rat; Tacrolimus

1. Introduction

Tacrolimus (FK506), a 21-membered macrolide produced by *Streptomyces tsukubaensis* [1,2], is a potent immunosuppressive agent that is being clinically utilized to prevent graft rejection following organ transplantation [3,4]. Tacrolimus undergoes extensive hepatic metabolism via cytochrome P450 isozymes of the P450 3A subfamily [5]. Metabolic studies of tacrolimus using rat and human liver microsomes indicate that *O*-demethylation and hydroxylation are the major metabolic pathways in liver microsomes. At least eight metabolites have been isolated and identified from liver microsomes [5–8].

The technique of precision-cut liver slices was recently developed as a new in-vitro tool in the screening of the metabolic profile of drugs in various animal species and humans [9]. With this technique, slices of uniform thickness are prepared from standardized diameter blocks of the liver (or other tissues) and maintained in a suitable culture system. The preparation of liver slices

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PII S0731-7085(96)01863-8

appears to be less complicated than the isolation of hepatocytes, especially from human livers. Thus liver slices are of great potential value for drug metabolism studies. Liver slices can be used to study the biotransformation reaction of a drug in both Phase I (oxidative) and Phase II (conjugated) metabolisms.

This study was designed to determine the metabolic profiles of tacrolimus using rat and human liver slices. Human liver slices from two donors (male and female) were investigated.

Materials and methods

2.1. Chemicals and reagents

Tacrolimus and [^{14}C]tacrolimus were biosynthesized at the Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan) [6,8]. [^{14}C]Tacrolimus was prepared by fermentation, using [2,6-^{14}C]pipecolic acid as a precursor [8]. The compound was further purified at the authors' laboratory using high-performance liquid chromatography (HPLC) as described below. The radiochemical purity of [^{14}C]tacrolimus used in this study was >97% and the specific activity was 15.7 μCi mg^{-1}. The purified materials were stored at −80°C until use.

Tacrolimus metabolites, 13-*O*-demethyl tacrolimus (M-I), 31-*O*-demethyl tacrolimus (M-II), 15-*O*-demethyl tacrolimus (M-III), and 12-hydroxylated tacrolimus (M-IV), were obtained from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). The isolation and identification of these metabolites were described previously [6]. HPLC grade acetonitrile and methanol were obtained from Baxter Scientific (Deerfield, IL). Krebs–Henseleit buffer medium was obtained from Sigma Chemical Company (St. Louis, MO). The medium was supplemented with heptanoic acid, gentamicin sulfate, amikacin sulfate, and ITS Premix and the pH was adjusted to 7.3 with sodium carbonte (Sigma). All other chemicals were obtained from local commerical sources.

2.2. Rat liver slices

Male Sprague–Dawley rats (220–250 g) were fed a standard diet and had free access to drinking water. Rat liver slices were prepared according to the method described previously using a Krunkieck Tissue Slicer (Alabama Research and Development Corp., Munford, AL) [9]. The instrument was adjusted to prepare slices from an 8 mm diameter core with a thickness of approximately 300 μm. The slices were prepared in Waymouth medium (Gibco, Grand Island, NY) and transferred immediately to the Krebs–Henseleit buffer medium in a 12-well microtiter plate. The slices were incubated for 1 h on an orbital shaker housed within a 37°C (95% air, 5% CO_2) incubator (Cedco, Model 1400, Portland, OR) along with a slice-free medium control. Following this, the [^{14}C]tactolimus solutions were added and the slices were incubated for varying times as discussed below.

2.3. Human liver slices

Human liver slices were obtained from the International Institute for the Advancement of Medicine (IIAM; Philadelphia, PA). Livers were perfused upon removal with ice-cold University of Wisconsin solution and slabs approximately 8 mm in diameter were precision-cut into approximately 300 μm thick slices using the Krunkieck Tissue Slicer. The slices were shipped cold by overnight mail. Slices were obtained from two donors: one was a 62-year-old mentally retarded, Caucasian female, with no history of drinking or smoking who died of anoxia, and the other was an 18-year-old Caucasian male with a history of drug and alcohol use who died of cranial cervical dissociation.

Slices were transferred into wells containing the Krebs–Henseleit buffer supplemented with heptanoic acid, insulin, amikacin sulfate, and gentamicin sulfate (pH 7.3 adjusted with $NaHCO_3$) immediately upon arrival at this laboratory. After 30 min, a methanol solution of [^{14}C]tacrolimus was added to the medium containing the human liver slices. The final concentration of tacrolimus in each well was 2 or 20 μM, while methanol concentrations were less than 0.5%.

At the end of the incubation period, the slices were removed from the wells and the medium in each well was centrifuged for 5 min at 10 000 rev

min $^{-1}$. The medium was injected into an HPLC system for the determination of tacrolimus and metabolites or stored at −80°C until analysis.

2.4. HPLC procedure

The HPLC system consisted of a Hitachi L-6200A pump, a Hitachi AS-2000 autoinjector, a Merck T6300 column heater, an Applied Biosystems 785A UV detector set as 220 nm, and a Packard Flo-One Beta detector. An Altima C18 (4.6 mm × 250 mm) column (Alltech) heated to 50°C was used in this study. The pump flow rate was set at 1.0 ml min^{-1}. A gradient mobile phase system was used to separate FK506 and its metabolites. Mobile phase A was 100% acetonitrile, phase B was 80% acetonitrile, phase C was 100% methanol. A linear gradient elution was performed during the first 35 min. The mobile phase consists of 100% phase B at 0 min, 63% phase A and 37% phase B between 35 and 40 min, and 100% phase A from 45 to 50 min. The column was then flushed with phase C for 6 min.

2.5. Other methods

The protein content of the slices tested was measured by the modified Lowery method [10] for the determination of protein. Sample protein concentrations were extrapolated from a bovine serum albumin standard curve by linear-regression analysis.

7-Ethoxycoumarin (7EC) was used for the determination of the Phase I and Phase II drug-metabolizing capacities of the liver slices. 7EC (75 μM) was incubated with a single liver slice for 4 h, followed by measurement of the conversion of 7EC to 7-hydroxycoumarin (7HC; oxidation) and/or 7HC-glucuronide and 7HC-sulfate conjugates (phase II conversion). The procedure used an HPLC method with UV detection.

The viability of the liver slices was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay developed by Mosmann [11]. The assay used a colorimetric method to measure the reduction of MTT (0.5 mg ml^{-1}) by living cells to form dark blue crystals.

3. Results

The decrease in the amount of reduced dye compared with the control in the MTT assay was used as an indicator of the viability of the cells in the slices. Rat and human liver slices were viable at tacrolimus concentrations of 0–200 μM. Due to the poor solubility of tacrolimus at 200 μM, the highest concentration of tacrolimus used in this study was 20 μM.

The 7-ethoxycoumarin assay was used as a quality control of the human slices at IIAM before shipping. Upon arrival at the authors' laboratory, the 7-EC assay was used to confirm the quality control results obtained at IIAM. The formation of 7-HC glucuronide after 4 h incubation of 7-EC with one liver slice was used as an indicator of the viability of the liver slice and to assure the presence of P450 activity. Based on this assay, liver slices from the 62-year-old female had relatively higher activity compared to the slices obtained from the 18-year-old male.

3.1. Metabolism of tacrolimus in rat liver slices

When one or two rat liver slices were used, the concentration of tacrolimus metabolites produced was below the sensitivity of the HPLC radiochromatography system used. The number of liver slices was increased to four in order to obtain higher concentrations of tacrolimus metabolites. Typical radiochromatograms of tacrolimus and its metabolites incubated with four rat liver slices for 4 h are shown in Fig. 1. Under the conditions used, tacrolimus eluted at 45 min. Minor chemical decomposition products of tacrolimus in the media without liver slices eluted at 3 and 18 min (Fig. 1B). After 4 h of incubation with four liver slices, two metabolites of tacrolimus, which eluted at 4 and 32 min, were observed (Fig. 1C). The 32 min peak matched the retention time of the 13-*O*-demethylated tacrolimus metabolite (M-I). The structure of the compound which eluted at 4 min cannot be confirmed at this time, although it may be a secondary or conjugated metabolite of tacrolimus.

Table 1
Formation of tacrolimus metabolite M-I following incubation of tacrolimus for 1 h with a human liver slice

Tacrolimus initial conc. (μM)[a]	Number of slices[b]	M-I formed (pmol f^{-1} mg^{-1} protein)[c]	Conc. of tacrolimus remaining (μM)[d]
2	One	26.54 ± 7.72	1.11 ± 0.27
20	One	37.71 ± 30.84	6.55 ± 0.89

[a] Tacrolimus concentration means the initial concentration of [^{14}C]tacrolimus in the tested incubuation medium.
[b] Human liver slice is from female donor.
[c] The retention times of tacrolimus, M-I, and its chemical decomposition are 45 min, 32 min and ≈18 min respectively.
[d] These values of HPLC peak areas represent the mean ±SD. Each experiment was run in triplicate.

3.2. Metabolism of tacrolimus in human liver slices

Radiochromatograms of 20 μM tacrolimus incubated with a single human liver slice for 1 h showed three major peaks and two smaller ones. The large peaks eluting at 45, 32, and 18 min were ascribed to authentic tacrolimus, 13-*O*-demethyl tacrolimus (M-I), and a chemical decomposition product. A smaller peak at 3 min was due to a chemical decomposition product of tacrolimus. The identity of the product which eluted with a small peak at 4 min is unknown. In this study, no other oxidative metabolites, such as M-II, M-III, or M-IV, were observed with the human liver slices. The relative retention times of M-II, M-III, and M-IV in the system used were 39, 36, and 35 min respectively.

The immunosuppressive activity of the unknown peak which eluted at 4 min was measured by a concanavalin A (ConA) assay. In addition, the immuno-crossreactivity of this peak with the monoclonal antibody employed in the immunoassay for tacrolimus was determined by enzyme-linked immunsorbent assay (ELISA). ELISA and ConA assays were performed following established procedures [6]. No immuno-crossreactivity or immunosuppressive activity was measured for this peak in these assays. Also, the peak was determined not to be a glucuronide or sulfate conjugate of FK506 or its metabolites form reactivity with enzyme hydrolysis.

The human liver slices was shown to be viable in the MTT assay in the presence of tacrolimus concentrations up to 200 μM. In order to determine the concentration dependency of the drug, two tacrolimus concentrations (2 and 20 μM) were incubated with a single human liver slice for 1 h. The concentration of M-I produced from incubation of the 20 μM tacrolimus concentration was relatively higher than that from the 2 μM concentration (Table 1). Based on these mean results and the high % RSD, it cannot be concluded that the excretion of tacrolimus metabolites from the human liver slices was concentration-dependent.

Using four human slices, more than 40% of the radioactivity was recovered from the medium after 1 h incubation, and after 12 h incubation more than 80% of the radioactivity was recovered in the medium. Therefore, no attempt was made to try to extract tacrolimus from the slices.

3.3. Effect of number of human liver slices on tacrolimus metabolism

An increase was observed in M-I formation after 1 h of incubation when the number (1, 2, or 4) of liver slices used was increased (data not shown). This indicates that each slice exhibited a constant oxidative activity unrelated to the number of slices co-incubated. Similary, the uptake of tacrolimus and excretion of metabolite(s) appeared to be constant for each slice, irrespective of the number of slices co-cultured. The concentration of the chemical decomposition product of tacrolimus (peak around 18 min) decreased with an increase in the number of slices used. This decrease could be explained by the uptake of tacrolimus from the medium by the liver slices,

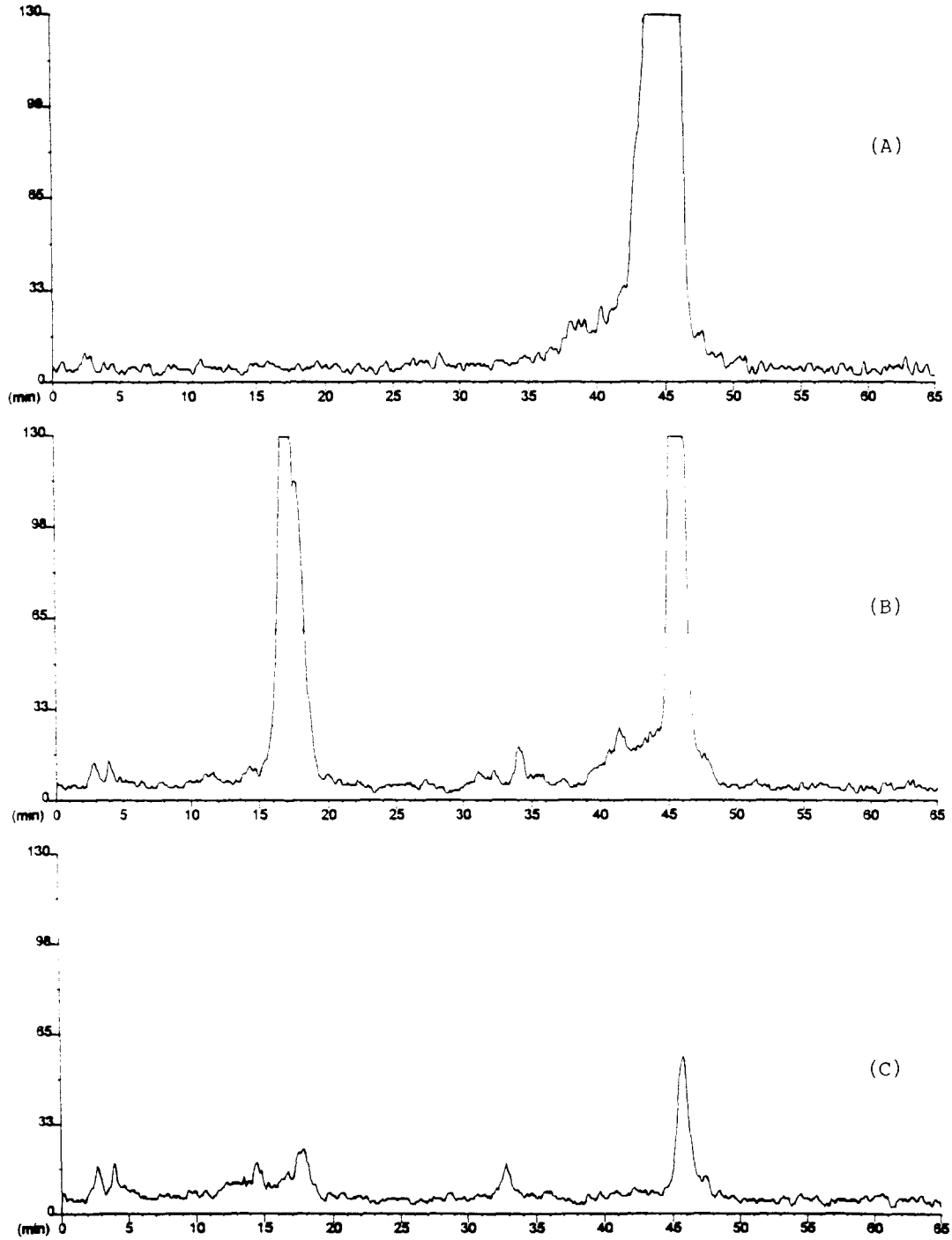

Fig. 1. Typical radiochromagtogram of 20 μM [^{14}C]tacr-olimus at (A) initial conditions in the media, (B) after 4 h incubation in the media without rat liver slices, and (C) after 4 h incubation in the media with four rat liver slices.

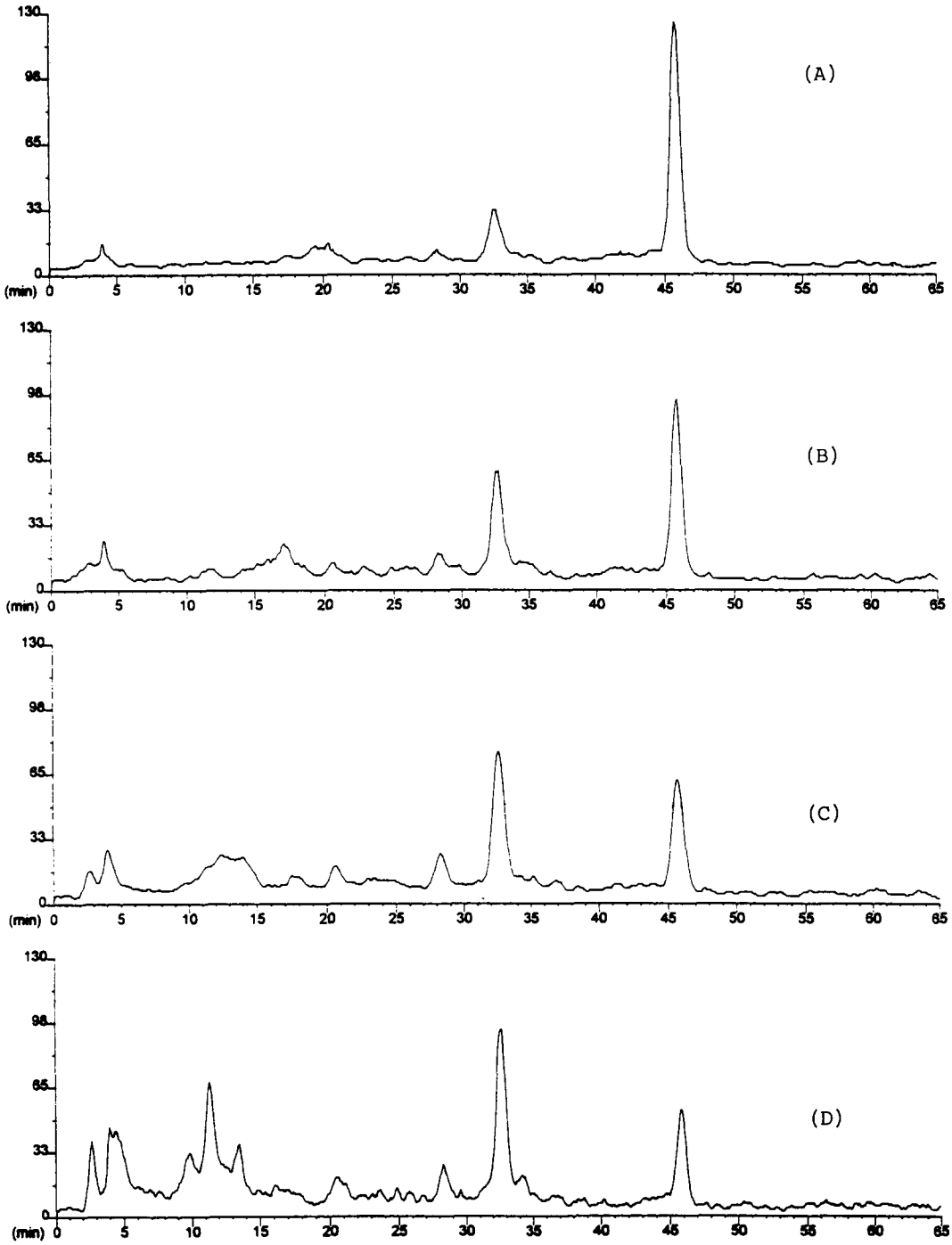

Fig. 2. Typical radiochromatograms of [^{14}C]tacrolimus (20 μM) and its metabolites after incubation with four male human liver slices and (A) 1, (B) 2, (C) 4, and (D) 8h.

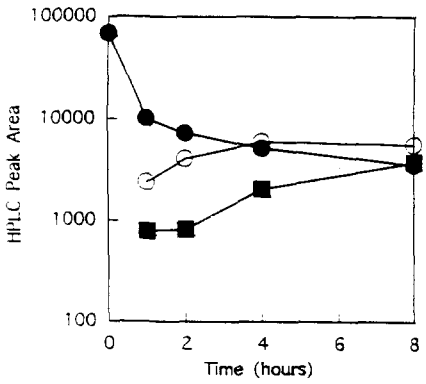

Fig. 3. The formation of metabolite M-I (○) and unknown metabolite (■) from tacrolimus (●) over time using four human liver slices. The concentration of tacrolimus was 20 μM.

resulting in a reduced concentration of tacrolimus in the medium to undergo chemical decomposition.

3.4. Time dependency of tacrolimus metabolism

The time dependency for metabolite formation from tacrolimus was investigated using four liver slices collected from a male donor. Fig. 2 shows typical HPLC radiochromatograms at various incubation times ranging from 1 to 8 h. Initially, the M-I was the only measurable metabolite; as time progressed, the size of the unidentified peak at 4 min increased. In addition, some other small peaks appeared (e.g. 12, 21, and 28 min) with longer incubations. However, M-I remained the

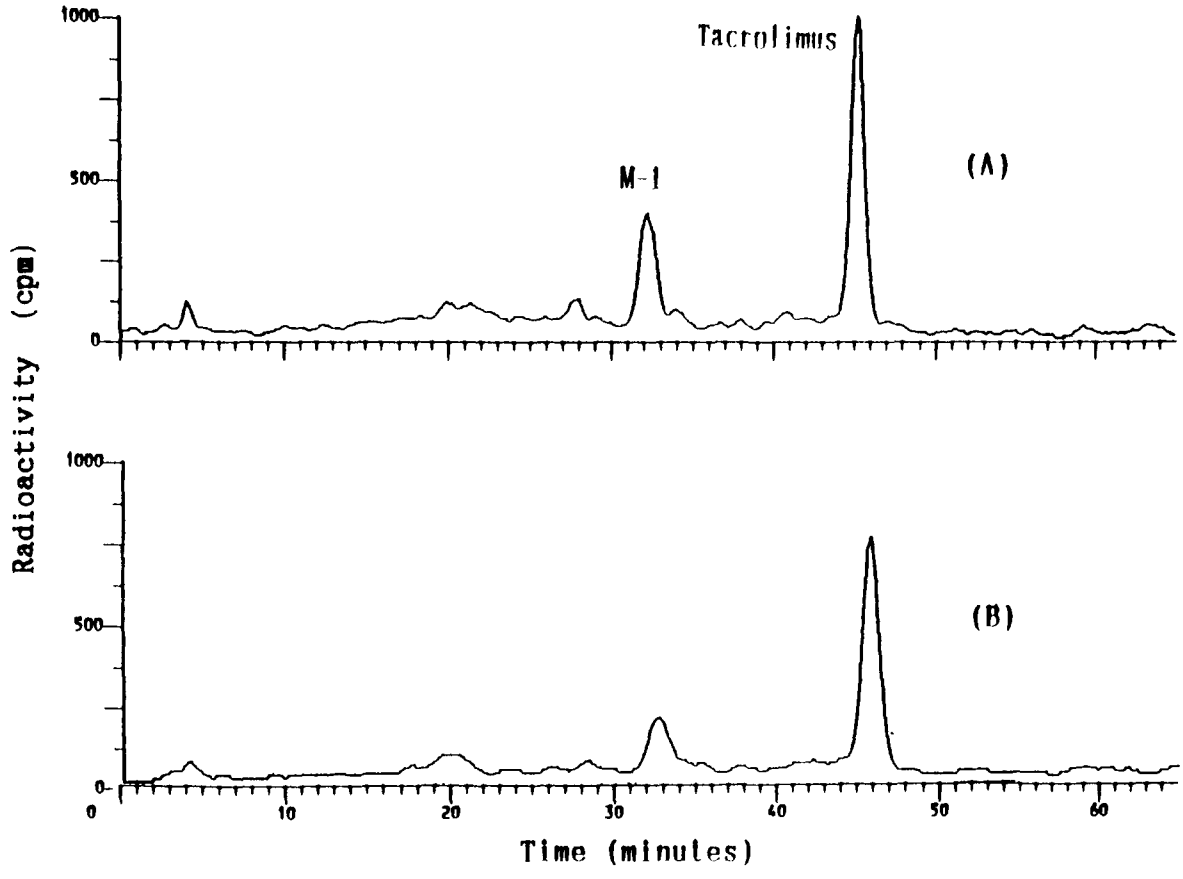

Fig. 4. Comparison of tacrolimus metabolite profiles of (A) female and (B) male human liver slices (four slices) after 1 h of incubation. Tacrolimus concentration was 20 μM.

major metabolite for tacrolimus in human liver slices.

Fig. 3 shows the time courses of the peak areas corresponding to tacrolimus, M-I, and the unknown peak which eluted at 4 min. Initially, the authentic tacrolimus peak rapidly decreased during 1 h of incubation, followed by a gradual decline. This sharp decrease in the tacrolimus peak indicates that the slice absorbed most of the tacrolimus initially. The size of the M-I peak measured by the area under the peak of the radioactive chromatogram increased over the first 4 h and then reached a plateau. The 4 min unknown peak showed a gradual increase in size in the first 4 h, and continued to increase gradually between 4 and 8 h.

3.5. Intra-individual variability of tacrolimus metabolism in human

Fig. 4 shows the radiochromatograms of tacrolimus incubated for 1 h with four liver slices from the two donors. The pattern of these chromatograms was almost the same, except that M-I formation was slighty different. M-I excretion from the liver slices obtained from the 62-year-old female donor was higher than that obtained from the 18-year-old male donor. This difference in the metabolism of tacrolimus was in line with the formation of 7-HC glucuronide by liver slices from these two individuals.

4. Discussion

Liver slices were used in the current study to examine the metabolism of tacrolimus. Of the three in-vitro methods for studying metabolism, liver slices offer some advantages over microsomes and isolated hepatocytes. Precision-cut liver slices offer the advantages of maintaining liver architecture, continuous exposure of the cells to the test substance, and no interruption of metabolite release into the medium. The technique must be applied carefully, as variables such as slice thickness and incubation conditions can affect results [9]. Microsomes are by definition homogenates of the smooth endoplasmic reticulum of hepatocytes and contain only the microsomal enzyme systems [13]. Hepatic nonmicrosomal enzyme systems are presumably contained in liver slices. Lipid solubility determines microsomal penetration and cellular and cell-to-cell transport mechanisms are lost in microsomes and isolated hepatocytes respectively [13,14]. The disadvantages of all of these methods are that they do not study the in-vivo situation, so metabolites which are normally transient can accumulate. This is more likely in an incomplete system, such as a microsome, than in liver slices. As liver slices have been used in few metabolism studies, it is important to compare the results with those using other systems.

M-I, the 13-*O*-demethyl metabolite of tacrolimus, was the major metabolite produced through the primary Phase 1 reaction in both rat and human liver slices. This present finding supports the results of other studies using liver microsomes from humans, rats, dogs, and rabbits [5,8]. No other primary metabolites of tacrolimus, such as M-II, M-III, or M-IV, were seen following the incubation of tacrolimus with human liver slices. These primary metabolites were previously shown to elute at 39 (M-II), 36 (M-III), and 35 (M-IV) min compared with 32 min for M-I in this HPLC system [12]. The absence of these metabolites in the current study was not due to the loss of the [^{14}C]label, as the labeled carbons were contained within internal carbon atoms not affected by primary metabolism.

Human liver slices showed a higher metabolic rate for M-I formation than rat liver slices in this study. One or two rat liver slices showed no detectable formation of M-I, whereas a single human liver slice did. This confirms the findings from liver microsomes that human microsomes were more active than those from rats [5]. Comparable K_m values have been reported in human and rat liver microsomes (6.2 and 6.7 μM respectively), whereas the V_{max} value was higher in human microsomes (0.38 and 0.18 nmol min^{-1} mg^{-1} of protein respectively) [8].

Apparent chemical decomposition of tacrolimus was noted in this study, both in incubation solutions containing no liver slices and in incu-

bation media of human liver slices with tacrolimus. The concentration of chemical decomposition products of tacrolimus decreased with the number of liver slices added to a single well. This decrease is explained by the uptake of tacrolimus into the liver slices, leaving less tacrolimus in the solution to undergo chemical decomposition.

In the initial studies of tacrolimus metabolism, a second metabolite was seen in both rat and human liver slice preparations. In a study of metabolite formation over time, M-I was shown to be the major metabolite initially. A second metabolite, possibly a secondary or conjugated metabolite, appeared in higher concentrations with continued incubation. This metabolite had no pharmacological activities as confirmed by ELISA and ConA assays. Also, it was not a glucuronide or sulfate conjugate of tacrolimus or its metabolites. Other unknown peaks were also observed in chromatograms following longer incubation times, but there were not examined further in the current study. The retention times of secondary metabolites (M-V through M-VIII) have not been examined in the current system, but these elute prior to tacrolimus and primary metabolites (except M-V after M-I) in a similar HPLC system [7].

Tacrolimus is reported to be metabolized primarily by the cytochrome P-450 system and especially by P-450 3A [5]. Sex differences in the metabolism of tacrolimus were reported in rats, where a higher metabolic rate was reported in males [8,15]. Female rats were reported to lack the P-450 3A enzyme, but had another enzyme which metabolized tacrolimus [15]. In this study, no apparent differences were observed in the metabolism of tacrolimus between the male and female donors. The pattern of metabolism was identical in both: M-I was the primary metabolite and an unknown peak which eluted at 4 min was the other metabolite.

Acknowledgements

This research was funded by Fujisawa USA, Inc. (Deerfield, IL), a wholly owned subsidiary of Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). The authors thank Drs. Iwaski and Terada of Fujisawa Pharmaceutical Co., Ltd., for providing the [^{14}C]FK506 and metabolites of FK506.

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