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Lipophilic benzamide and anilide derivatives as high-performance liquid chromatography internal standards: application to sirolimus (rapamycin) determination

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Abstract

Finding a suitable internal standard in reversed-phase high-performance liquid chromatography is often difficult. A general approach for selecting and synthesizing the proper internal standard is presented and applied to a validated method for quantitation of sirolimus in several biological matrices. A series of fifteen N-alkylbenzamides, N-alkyltoluamides and N-alkanoylanilines with a log *P* range of 3.51 to 6.68 were synthesized as internal standards; N-undecyl-*o*-toluamide was evaluated most extensively. Sirolimus quantitation involves a simple sample clean-up procedure followed by isocratic chromatography on a heated C_{18} analytical column with an 70% methanol–water mobile phase and ultraviolet detection at 278 nm. This method was linear from 2.5 to 200 ng with a limit of quantitation of 2.5 ng using a 1-ml blood sample. Sirolimus recovery was above 72.1%. The intra-day and inter-day coefficients of variation were less than 11.7%. Several drugs and sirolimus metabolites do not interfere with the analysis. This method was used to measure sirolimus in blood from rats, rabbits and humans. © 1997 Elsevier Science B.V.

Keywords: Internal standard; Benzamide derivatives; Anilide derivatives; Sirolimus; Rapamycin

1. Introduction

Sirolimus (formerly rapamycin) (Fig. 1) is a cyclic 31-membered macrolide compound currently under investigation in organ transplantation as an immunosuppressant in combination with cyclosporin A and prednisolone [1,2]. Sirolimus has been shown to inhibit T-lymphocyte proliferation through suppression of interleukin IL-2 and IL-4 signals [3]. As this drug has a narrow therapeutic index, it is important to develop a simple, accurate, sensitive and precise method to quantify sirolimus in biological matrices. Because sirolimus is extensively distributed in red blood cells which is independent of concentration and temperature and the level of immunosuppression appears to correlate with sirolimus blood concentrations, whole blood tends to be the preferred matrix for therapeutic drug monitoring [1].

Sirolimus is an hydrophobic, temperature-, pH- and light-sensitive compound existing in solution as a mixture of two interconvertible isomers [4,5]. Sirolimus is readily soluble in alcohols, sparingly soluble in diethyl ether, and almost insoluble in water [2]. After incubation of the drug with hepatic or intestinal microsomes from humans or rats, several hydroxylated and demethylated metabolites were

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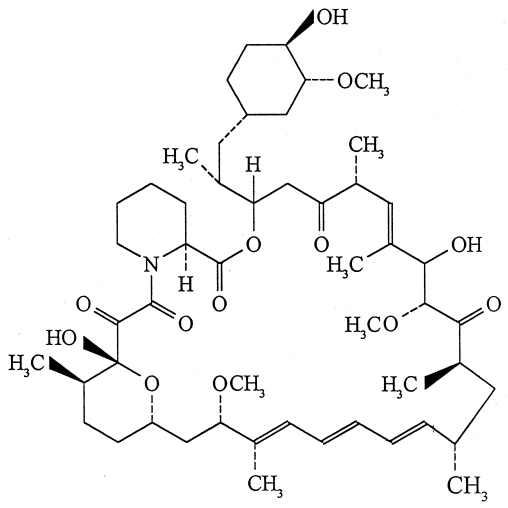

Fig. 1. Chemical structure of sirolimus ($C_{51}H_{79}NO_{13}$, M_r=914).

identified along with seco-sirolimus, the degradation product [6–8]. Taking into consideration the physicochemical and kinetic properties of sirolimus along with the nature of biological matrices (e.g. blood, microsomes), sirolimus determination is a challenge in terms of selective extraction from these matrices and specific chromatographic separation from endogenous components. In 1992, Yatscoff et al. [9] reported a simple, one-step reversed-phase HPLC method yielding low sirolimus recovery (35%) from a 2-ml rabbit blood sample. However, there were potential interferences between sirolimus metabolites and the desmethylsirolimus used as internal standard. Later, Napoli and Kahan [4,10] published a thorough, multiple-step high-performance liquid chromatography (HPLC) method not dependent on a sirolimus analog for internal standardization and achieved a 95% absolute recovery of sirolimus from 1-ml human blood samples. In 1995, a new specific method using a gradient system with a one-step extraction was reported [11] and successfully applied to several biological matrices.

The purpose of developing our method was to validate a sirolimus HPLC assay combining a simple, one-step extraction procedure yielding satisfactory recovery with a specific isocratic chromatographic separation method in which internal standardization did not depend on a sirolimus analog. Selecting the proper internal standard within the array of commercially available compounds was unsuccessful. As suggested by Verzele et al. [12] and Kikta and Stange [13], phenone or anilide derivatives can be of potential use as internal standards. Moreover, Sharma et al. [14] solved their difficulties in finding an internal standard for taxol quantitation by synthesizing several N-alkylbenzamides. This approach was extended in this paper to a broader family of amide standards, the benzamide and anilide derivatives, demonstrating a rational way of finding the proper internal standard for use in reversed-phase HPLC.

2. Experimental

2.1. Instrumentation

The HPLC instrument consisted of a Model 510 solvent delivery system, a Model 486 UV programmable multi-wavelength absorbance detector set at 278 nm and a Wisp 712 automatic sample injector (Waters Associates, Milford, MA, USA). Chromatographic separation was achieved with a Supelco LC-318 C_{18} column (250×4.6 mm I.D., 5 μm particle size) (Supelco, Bellafonte, PA, USA) equipped with a 2 cm×2 mm I.D. pre-column (Upchurch Scientific, Oak Harbor, WA, USA) packed with C_{18} pellicular media (37–53 μm particles) (Whatman, Clifton, NJ, USA). The HPLC pre-column and column temperature was maintained constant at 45°C by a Model TC-50 column heater (Eppendorf, Madison, WI, USA). Peaks and areas were recorded on a Hewlett-Packard 3392a Integrator (Avondale, PA, USA).

2.2. Chemicals and reagents

HPLC-grade *tert*-.butyl methyl ether, methanol and water used for sample extraction and mobile phase preparation were obtained from Sigma (St. Louis, MO, USA), Burdick and Jackson (Muskegon, MI, USA) and J.T. Baker (VWR Scientific, Willard, OH, USA), respectively. HPLC-grade tetrahydrofuran used for column cleaning was obtained from Burdick and Jackson. Sodium carbonate was purchased from Fisher Scientific (Fairlawn, NJ, USA). Reagents for the synthesis of the internal standards were obtained from Aldrich (Milwauke, WI, USA).

Sirolimus (rapamycin) was received as a gift from Dr. D. Hicks (Wyeth-Ayerst Research, Princeton, NJ, USA). Sirolimus and potential internal standard stock solutions were prepared in methanol at a 0.5 mg/ml concentration and stored in amber glass vials at −80°C and −20°C, respectively. In these conditions, stock solutions were stable for at least 6 months. Sirolimus solutions for the calibration curve and the quality control standards were prepared weekly by serial dilution of the methanolic stock solution and kept in amber glass vials at 4°C for a week.

2.3. Internal standards

In the search of an adequate internal standard, seven N-alkylbenzamides, five N-alkyltoluamides and three N-alkanoylanilines were synthesized and evaluated in our system (Fig. 2). The Schotten–Baumann method [15,16] using an alkyl amine and benzoyl chloride or *ortho*-, *meta*- or *para*-toluoyl chloride was employed to generate the N-alkylbenzamides, following the procedure described by Sharma et al. [14]. Briefly, for preparation of N-undecyl-*o*-toluamide, N-undecylamine was dispersed in cold NaOH and an equimolar amount of *o*-toluoyl chloride was added. After vigorous shaking, the product was removed by filtration, washed, air dried and recrystallized from ethanol–water. Under our chromatographic conditions, each synthesized internal standard eluted as a single peak with a characteristic spectrum (diode array detection) insuring product purity.

To compare the chromatographic characteristics of each potential internal standard, the corrected retention time (t'_R) along with the capacity factor (k') values were calculated as:

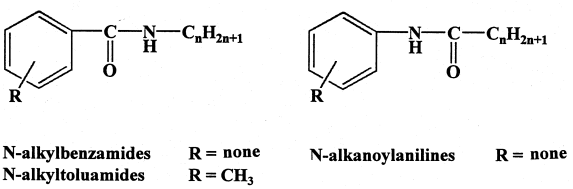

Fig. 2. Chemical structure of the N-alkylbenzamide, N-alkyltoluamide and N-alkanoylaniline internal standards. There is no R substituent in the benzamide and aniline series.

$$t'_R = t_R - t_H \text{ and } k' = \frac{t_R - t_H}{t_H}$$

where t_R is the retention time and t_H is the hold-up time.

The calculated log *P* value for each internal standard was obtained with the software CLogP for Windows (version 1.0, Biobyte, Claremont, CA, USA) based on the method of Leo [17].

2.4. Standard curves and quality controls

Standard solutions of sirolimus in methanol were added daily to samples of pooled blank whole blood or hepatic microsomes to obtain standard samples containing 0, 2.5, 5, 10, 25, 50, 100 and 200 ng of sirolimus. Duplicate quality controls were prepared in the same way to obtain the low (9 ng), medium (40 ng) and high (90 ng) standards.

2.5. Sample preparation

The applicability of the method was tested by analyzing different matrices: rat, rabbit and human whole blood along with rat hepatic microsomes. Standards, quality controls and samples (50 μl to 1 ml) were treated in exactly the same fashion, in 150×16 mm disposable borosilicate glass screw cap culture tubes wrapped in foil. Following addition of 1 μg of internal standard, the aliquots were vortex-mixed with 1 ml of 0.1 *M* Na_2CO_3 for 10 s. Ten ml of *tert*.-butyl methyl ether were added to each tube. They were tightly capped and shaken horizontally at high speed for 30 min. After a 10 min centrifugation at 1500 *g* and 4°C, the organic layer was carefully removed without disturbing the aqueous layer and transferred into a 20-ml amber glass scintillation vial and evaporated to dryness under a stream of nitrogen or purified air in a water-bath maintained at 20°C.

2.6. Chromatographic procedure

The residue was reconstituted with 200 μl of mobile phase, transferred to a glass Wisp insert vial contained in an amber injection vial. As the residue contains particles, an additional centrifugation step at 2500 *g* and 4°C was necessary to obtain a clear

extract. This organic layer was transferred to a new insert for the chromatographic analysis.

A 150-μl aliquot was injected onto the HPLC system. The mobile phase consisted of methanol–water (70:30, v/v) pumped at a flow-rate of 1.0 ml/min. The mobile phase was passed through a 0.22 μm GV Millipore filter to remove particles and degassed for 5 min with purified helium. The pre-column and column were preheated and maintained at 45°C. The total run time for each sample was 32 min. Samples can be stored overnight at 4°C without loss of sirolimus [4].

2.7. Method validation

The method validation was performed with the N-undecyl-*o*-toluamide internal standard using sirolimus–internal standard peak-area ratios for sirolimus quantitation. Sirolimus eluted as two peaks as it is present in solution as a mixture of two interconvertible isomers [5]. Only the first peak of sirolimus was used for calculations. The area of the sirolimus second peak was less than 2.5% of the first peak area and was therefore considered negligible. A variance-stabilizing transformation method without forcing the intercept to be zero was applied for the standard curve regression analysis [18,19]. All the calculations were performed using Excel (version 5.0a, Microsoft, Redmond, WA, USA).

The linearity of the method was studied over the range of 2.5 to 200 ng by determining if the peak heights and areas increased in proportion to the amount. The precision of the method was evaluated for the low, medium and high standards in terms of the variability (% C.V.) within (intra-day) and between (inter-day) runs by analyzing six (intra) or twelve (inter) replicates. The accuracy (% error) was determined by the percent difference of the mean replicate quantities from the known spiked sirolimus amounts. The limit of detection (LOD) was defined as a signal-to-noise ratio of 3:1. The lower (LOQ) limit of quantitation was determined as the lowest quantity consistently achieving adequate accuracy. According to US Food and Drug Administration (FDA) regulations [20], less than 15% error of the aforementioned statistical parameters is considered acceptable.

Recoveries of sirolimus (2.5, 9, 40 and 90 ng) and N-undecyl-*o*-toluamide (1 μg) were determined by comparing the peak areas obtained after injection of extracted standards with the values obtained following injection of known amounts of compound in mobile phase. Recovery of sirolimus relative to the internal standard was also calculated.

The assay specificity was assessed by direct injection of various drugs that might be taken as concomitant therapy (e.g., cyclosporin A, tacrolimus, prednisolone). Pre-dose samples provided specificity with respect to endogenous compounds. Rat hepatic microsome extracts provided specificity with respect to sirolimus metabolites and degradation products [6–8].

3. Results and discussion

3.1. Internal standards

Satisfactory separation between sirolimus isomers and matrix interferences was first sought by optimizing the column temperature (45°C), the mobile phase composition (methanol–water, 70:30) and flow-rate (1 ml/min) based on the characteristics of sirolimus reversible isomerization [5]. A suitable internal standard could not be found among commercially available compounds.

Based on the findings of Sharma et al. [14], seven N-alkylbenzamides containing 6 to 12 carbons in the side-chain were synthesized and evaluated in our reversed-phase HPLC system. Their corrected retention times, capacity factors and calculated log *P* values are displayed in Fig. 3 and reported in Table 1. These parameter values increased in an exponential manner with the carbon side-chain length since their log-transformed values were linearly related to the carbon side-chain length. The slope of the plot of the log-capacity factor versus carbon side-chain length was 0.207 with an intercept of -1.29 ($r^2 = 0.999$). Therefore, the capacity factor value for N-alkylbenzamides with shorter or longer carbon side-chain can be predicted with confidence and, when needed, alternative internal standards can be synthesized.

Knowing the capacity factor values for sirolimus isomers (7.72 and 11.1), and noticing the presence of numerous impurities before the first peak of

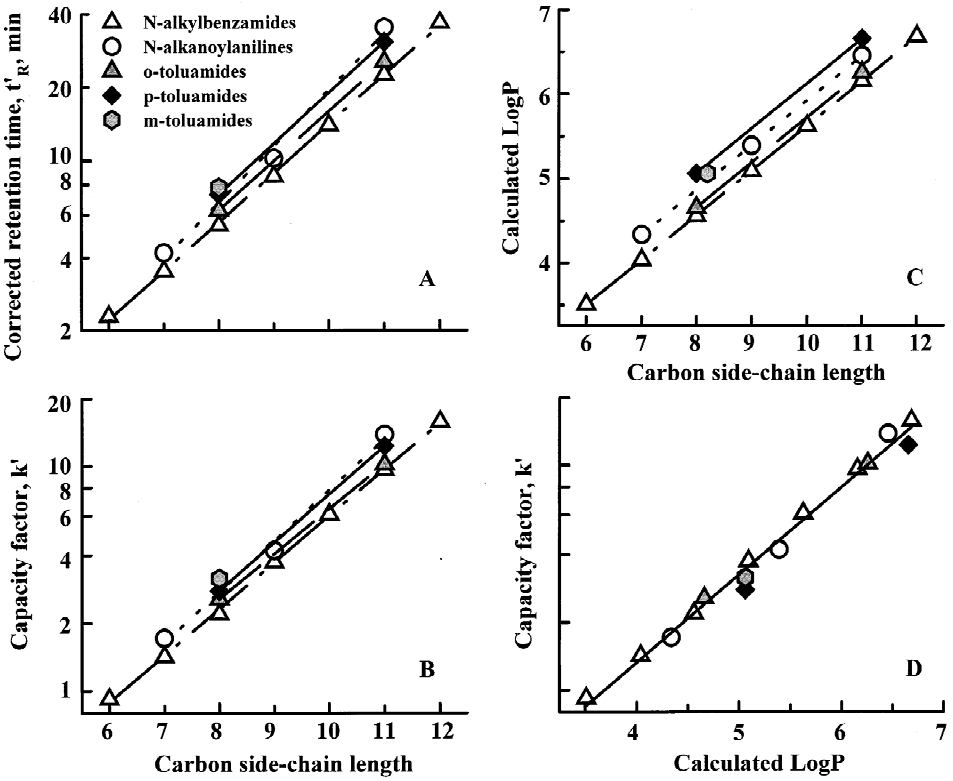

Fig. 3. Corrected retention time (A), capacity factor (B) and calculated log *P* (C) values as a function of the carbon side-chain length and capacity factor values as a function of calculated log *P* (D) for the fifteen N-alkylbenzamides, N-alkyltoluamides and N-alkanoylanilines. The lines were obtained by linear regression for each family of standards (A, B, C) or for all standards (D). The chromatographic conditions are described in Section 2.6.

sirolimus and the absence of interference between sirolimus isomers (8 min apart in our system), we decided to place our internal standard in the latter area. The most suitable of the previous standards was the N-undecylbenzamide with a corrected retention time of 22.6 min compared to 19.3 and 27.7 min for the sirolimus isomers. As the resolution between N-undecylbenzamide and the sirolimus first peak was poor and not improved by changes in the mobile phase composition (65–75% methanol–water) or column temperature (40–50°C), a new family of internal standards sharing similar chemical, physical and spectral characteristics with the N-alkylbenzamides was sought. This led to the N-alkanoylanilines.

Three N-alkanoylanilines were synthesized with the carbon side-chain lengths (*n*) of 7, 9 and 11. Their corrected retention times and capacity factors are displayed in Fig. 3 and Table 1. The slope of the relation between the log-capacity factor and the carbon side-chain length was 0.227 with an intercept of -1.37 ($r^2 = 0.999$). These values are higher than those obtained for the same molecular mass in the N-alkylbenzamide family. The capacity factor (4.21) and log *P* (5.39) values for the N-decanoylaniline are situated between the values for the N-nonyl (3.74 and 5.09) and decylbenzamides (6.09 and 5.62). The projected corrected retention time for the N-undecanoylaniline ($n = 10$) was 19.6 min. Therefore, it will interfere with sirolimus determination, rendering the N-alkanoylaniline derivatives as possible internal standards unsuitable in our system.

The introduction of a methyl group on the benzene ring of the initial N-alkylbenzamide internal standards, starting with the N-undecyltoluamides, led to the creation of three series of N-alkyltoluamide

Table 1
Capacity factor and calculated log *P* values of lipophilic benzamide and anilide derivatives

Compound	*n*[a]	Capacity factor *k'*[b]	Calculated log *P*[c]
N-alkylbenzamides and toluamides			
N-Hexylbenzamide	6	0.92	3.51
N-Heptylbenzamide	7	1.42	4.04
N-Octylbenzamide	8	2.19	4.56
N-Octyl-*o*-toluamide	8	2.56	4.66
N-Octyl-*p*-toluamide	8	2.79	5.06
N-Octyl-*m*-toluamide	8	3.15	5.06
N-Nonylbenzamide	9	3.74	5.09
N-Decylbenzamide	10	6.09	5.62
N-Undecylbenzamide	11	9.61	6.15
N-Undecyl-*o*-toluamide	11	10.19	6.25
N-Undecyl-*p*-toluamide	11	12.28	6.65
N-Dodecylbenzamide	12	15.77	6.68
N-alkanoylanilines			
N-Octanoylaniline	7	1.71	4.34
N-Decanoylaniline	9	4.21	5.39
N-Dodecanoylaniline	11	13.83	6.45

[a] *n*, Carbon side chain length.
[b] C_{18} column; 45°C; mobile phase, methanol–water (7:3, v/v).
[c] Value obtained with the CLogP for Windows software.

derivatives, the *ortho*-, *meta*- and *para*-toluamides. Their corrected retention times, capacity factors and calculated octanol–water partition coefficients were in between the N-undecyl and dodecylbenzamides (Fig. 3, Table 1). Similar findings were obtained with the N-octyltoluamides. The lower capacity factor values were obtained with the ortho methyl substituent, and the highest with *meta*, in agreement with the greater spatial influence by the methyl group in the *meta* compared to the *ortho* position on the benzene ring. The N-undecyl-*o*-toluamide was an appropriate internal standard and was evaluated most extensively.

Another way to study these internal standards was to compute their octanol–water partition coefficients. For each family of internal standards, the calculated log *P* values increased with the carbon side-chain length as seen in Fig. 3. Moreover, the log-transformed capacity factor values for all standards were linearly related to the calculated log *P* values (slope: 0.39, $r^2 = 0.988$). These relations were expected as the log *P* values were calculated from parameters derived from the chemical structures [17]. The only discordance was seen with the N-alkyltoluamide derivatives where different capacity factors were obtained for the N-octyl-*p*- and -*m*-toluamides whose calculated log *P* values were identical.

In searching for an internal standard, large flexibility in creating a proper one was demonstrated. By using the N-alkylbenzamide family for initially selecting the carbon side-chain length (11 here), adding simple substituents (e.g., methyl, ethyl) on the benzene ring at different positions (*ortho*, *meta* or *para*), and changing the atom order in the amide function (anilide derivatives) led to the synthesis of the appropriate internal standard.

3.2. Chromatographic method

Chromatograms of rabbit whole blood blank and standards (LOQ, medium QC and higher limit of quantitation) obtained from 1-ml samples are displayed in Fig. 4. The characteristics of this reversed-phase HPLC method for sirolimus quantitation are summarized in Table 2 for rabbit whole blood.

This method was found to be linear from 2.5 to 200 ng of sirolimus with a limit of quantitation of 2.5 ng using a 1-ml rabbit blood sample. The limit of detection was 1 ng and the highest standard (200 ng) was determined to be the high limit of quantitation.

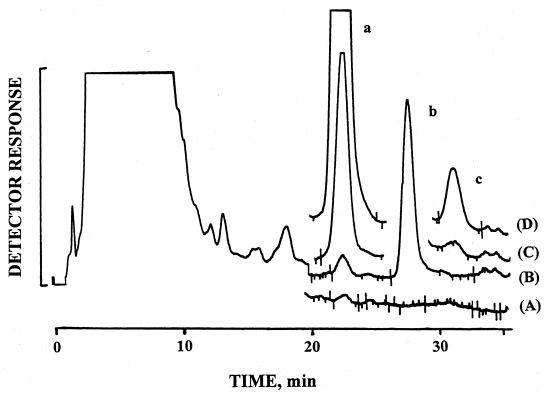

Fig. 4. Representative chromatograms of extracted 1-ml rabbit whole blood: blank (A), whole blood spiked with 2.5 ng of sirolimus (B), 40 ng (C) and 200 ng (D). Peaks: a = first sirolimus isomer; b = internal standard; c = second sirolimus isomer.

Table 3
Drugs which do not interfere with sirolimus assay

Cyclosporine A and G
Tacrolimus (FK506)
Methylprednisolone
Prednisolone and prednisone
Cortisol and corticosterone
Beclomethasone
Norethindrone
Ethinyl estradiol
Propranolol
Ketoconazole
Lorazepam
Rifampicin
Erythromycin

These values were similar to those previously reported by Yatscoff et al. [9] and Napoli and Kahan [4] and were adequate for sirolimus pharmacokinetic and drug metabolism studies where low drug concentrations are expected (10 to 60 ng/ml).

The sample clean-up method involved a one-step liquid–liquid organic extraction with a final centrifugation step prior to sample injection onto the column, making this reasonable procedure routinely applicable. The long retention time appears unavoidable [4,9]. Sirolimus was stable during extraction procedures and absolute recovery from a 1-ml rabbit whole blood sample was above 72.1% and independent of sirolimus concentrations (Table 2). These values were higher than the 35% value reported by Yatscoff et al. [9]. Napoli and Kahan [4] recovered 97% of sirolimus from human blood with a tedious multi-step sample clean-up procedure. The recovery of the N-undecyl-*o*-toluamide internal standard was 82.4% leading to a relative recovery of sirolimus between 87.5 and 95.1%.

The intra-day and inter-day precisions and accuracies were determined for samples at the limit of quantitation, and for the low, medium and high quality controls. As reported in Table 2, the accuracies (% error) were between −3.66 and 7.34% with precisions ranging from 2.96 to 11.70%, and there was no concentration dependency.

The assay specificity was demonstrated for several compounds (Table 3) which were injected onto the chromatographic system for potential interferences with sirolimus or the internal standard. Moreover,

Table 2
Characteristics of sirolimus reversed-phase HPLC method using the N-undecyl-*o*-toluamide internal standard and 1-ml rabbit whole blood samples

	LOQ (2.5 ng)	Low QC (9 ng)	Medium QC (40 ng)	High QC (90 ng)
Intra-day statistics ($n=6$)				
Accuracy (% error)	1.04	−1.53	−3.66	7.34
Precision (% C.V.)	7.64	9.56	5.54	2.96
Inter-day statistics ($n=12$)				
Accuracy (% error)	0.18	1.06	−0.85	5.72
Precision (% C.V.)	9.44	11.70	7.42	7.25
Recovery				
Absolute (%)	78.4	75.5	72.1	75.1
Relative (%)	95.1	91.5	87.5	91.1

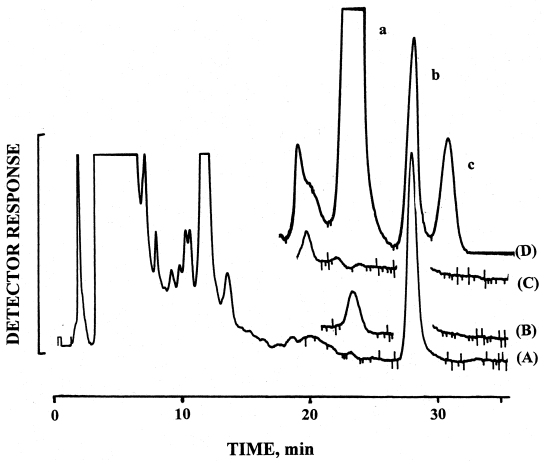

Fig. 5. Representative chromatograms of extracted rat whole blood (100 μl) before (A) and 30 min after a 1 mg/kg i.v. bolus dose (B) and of rat hepatic microsomal preparation (50 μl) after a 60-min incubation without (C) and with sirolimus at a concentration of 50 μg/ml (D). Peaks: a = first sirolimus isomer; b = internal standard; c = second sirolimus isomer.

due to their higher hydrophilicity, seco-sirolimus and the hydroxylated and desmethylated metabolites of sirolimus eluted before the first peak of sirolimus, without interfering with sirolimus quantitation. This was confirmed by the analysis of sirolimus in whole blood samples from human, rabbit and rat and in rat hepatic microsomal preparations; the chromatograms showed no apparent interfering peak from endogenous substances or sirolimus metabolites (Figs. 5 and 6). The results of these experiments will be presented in detail elsewhere.

4. Conclusions

We demonstrated that lipophilic benzamide and anilide derivatives provide a rational widely applicable series of stable and easily synthesized compounds suitable for use as internal standards for HPLC. The flexibility in synthesizing the proper internal standard allowed the successful development of a reversed-phase HPLC method for sirolimus quantitation in several biological matrices. This method provided satisfactory recovery after a simple one-step liquid–liquid extraction and complete separation of the two sirolimus isomeric forms and the

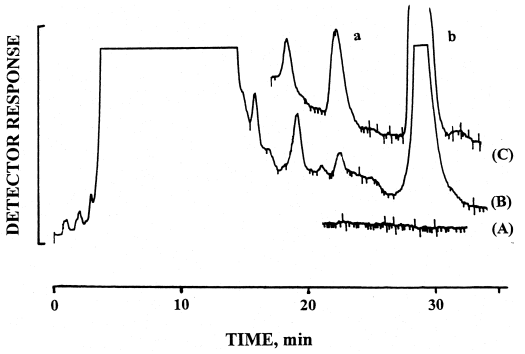

Fig. 6. Representative chromatograms of extracted human whole blood (1.0 ml) before (A) and 0.67 h (B) and 48 h (C) after a 10 mg oral dose. Peaks: a = first sirolimus isomer; b = internal standard.

N-undecyl-*o*-toluamide internal standard from endogenous components. This new HPLC method is simple, faster than previous methods, reproducible, accurate, precise, sensitive and specific. It is applicable to sirolimus quantitation in rat, rabbit and human whole blood as well as in rat hepatic microsomes.

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