Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 163–168
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Simple and rapid HPLC method for determination of amlodipine in human serum with fluorescence detection and its use in pharmacokinetic studies

Gh Bahrami*, Sh Mirzaeei

Department of Pharmacology, Biomedical Research Center, Medical School Kermanshah, University of Medical Sciences, Kermanshah 67184 69914, Iran

Received 25 August 2003; received in revised form 9 May 2004; accepted 11 May 2004

Abstract

A fast, sensitive and specific high performance liquid chromatographic method using fluorescence detection is described for analysis of amlodipine in human serum. Amlodipine is extracted from serum by ethyl acetate and involves precolumn derivatization with 4-chloro-7-nitrobenzofurazan (NBD-Cl) and reverse-phase chromatography on C18 column. The mobile phase was sodium phosphate buffer (pH 2.5) containing 1 ml/l triethylamine and methanol at flow rate of 2.8 ml/min. Propranolol was used as internal standard. The standard curve was linear over the range 0.25–16 ng/ml of amlodipine in human serum. The within-day and between-day precision studies showed good reproducibility with coefficients of variation less than 12% for all the analytes. The limit of quantification was 0.25 ng/ml of serum. The method has been applied to a bioequivalence study after administration of 10 mg amlodipine in 12 normal subjects.

Keywords: Reverse phase chromatography; HPLC; Amlodipine; Serum; Pharmacokinetic study

1. Introduction

Amlodipine is a calcium channel blocker which is used as anti-hypertensive and anti-anginal agent. It has long elimination half life and large volume of distribution. Low plasma concentration was achieved after oral administration of the drug, thus, sensitive and specific analytical methods are needed for determination of amlodipine in human serum. Several analytical methods for analysis of amlodipine in biological samples have been reported. The sensitivity of published HPLC–UV methods due to low absorbance of the drug is inadequate for pharmacokinetic studies and therapeutic drug monitoring [1,2]. In described gas chromatographic (GC) methods involving capillary column and electron capture detection although the sensitivity is improved, however, thermal decomposition of the drug under GC condition is the major problem [3–5]. Although determination of amlodipine in nanogram level was achieved by published HPLC–EC methods [6–8], however, they limited by low recovery [6], long retention time of the drug and internal standard (I.S.) [7] and/or time consuming cleaning method by use of column switching [8]. Amlodipine is quantified by HPTLC procedure which requires 2 ml of plasma at sensitivity of 2 ng/ml [9]. Also, very low limit of detection and good recoveries were achieved by use of LC–mass tandem system [10–12], however, this procedure is expensive and the analytical method requires highly trained personnel. Precolumn derivatization of the drug with 4-chloro-7-nitrobenzofurazan (NBD-Cl) and fluorescence detection has been reported by Tatar and Atmaca [13]. Although the sensitivity of their method is enough to analysis of amlodipine in pharmacokinetic studies, however, sample preparation is long, and extensive sample clean up in their method is time consuming. Because of long retention time of the I.S. their analysis had a run time of more than 20 min. In the present study, we have developed a rapid and highly sensitive HPLC–fluorimetric assay of amlodipine in human serum using NBD-Cl as fluorescence labeling agent and simple extraction procedure. The method was applied to detect amlodipine in serum samples

* Corresponding author. Tel.: +98 831 835 0197; fax: +98 831 836 8410.
E-mail address: gbahrami@kums.ac.ir (G. Bahrami).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.05.016

obtained from a bioequivalence study carried out in twelve healthy volunteers after administration of 10 mg of the drug.

2. Experimental

2.1. Chemicals

Amlodipine besylate and propranolol hydrochloride (I.S.) were kindly provided by Arya Pharmaceutical Company (Tehran, Iran). Methanol (HPLC grade), ethyl acetate, boric acid, potassium chloride, hydrochloric acid, potassium hydroxide, sodium carbonate, sodium bicarbonate, phosphoric acid and 4-chloro-7-nitrobenzofurazan (NBD-Cl) were purchased from Merck (Darmstadt, Germany). All reagents were analytical grade (methanol was HPLC grade) and were used without further purification. HPLC-grade water was obtained from a Maxima purification system (USF ELGA, England).

2.2. Preparation of standard solutions

Stock solutions of amlodipine and the I.S. were prepared by dissolving the compounds in methanol at a concentration of 100 and 200 μg/ml, respectively. Amlodipine stock solution was further diluted with methanol to give the standard solution of 1 μg/ml. Plasma calibration samples were prepared using drug-free human serum and different amounts of this solution provided concentrations of 0.25, 0.5, 1, 2, 4, 8 and 16 ng/ml. Borate buffer were prepared by dissolving 0.625 g of boric acid and 0.750 g of potassium chloride in 100 ml water and adjusting the pH to 7.8 with 0.2 M potassium hydroxide solution. A 500 μl/ml solution of NBD-Cl was prepared in ethanol. All solutions were stored at 4 °C and were stable at least for 3 weeks.

2.3. Chromatography

The chromatographic system consisted of a model LC-10A pump (Shimadzu, Kyoto, Japan), a rheodyne injection value with a 20 μl filling loop and a model RF-551 spectroflurometric detector operating at an excitations wavelength of 470 and emission wavelength of 537 nm. The HPLC column (150 mm × 6 mm i.d.) was packed with 5 μm particles of C18 packing material (Shimpack–CLC-ODS) and was maintained at 62 °C by a model Shimadzu CTO-10A column oven (Shimadzu). For quantitative calculations, a Shimadzu C-R4A data module was employed. The solvents used as the mobile phase was 0.05 M sodium phosphate buffer (pH 2.5) containing 1 ml/l triethylamine (solvent A) and methanol (solvent B). The elution gradient used was as follow (proportion of solvent B, v/v) 0–2.5 min, 69%; 2.5–4.5 min, 76%; 4.5–6.3 min, 100% and 6.3–6.5 min 69%. The eluent was filtered through a 50 μm filter (Milipore, Bedford, MA, USA), degassed (DGU- 10A Shimadzu) and delivered at a flow rate of 2.8 ml/min.

2.4. Sample preparation and derivatization

To 1 ml serum samples, 100 μl of I.S., 500 μl saturated carbonate buffer (pH 9.5) and 5 ml ethyl acetate were added. After mixing for 30 s on a vortex mixer and centrifugation (5 min at 6000 × *g*), the organic phase was removed and evaporated to dryness under stream of nitrogen at 45 °C. The residue was dissolved in 250 μl methanol, followed by addition of 100 μl the borate buffer (pH 7.8) and 100 μl of NBD-Cl solutions. The samples were kept at 80 °C for 15 min and then the mixture were cooled and diluted with 250 μl methanol. The reaction was completed with 200 μl of 2 N HCl and incubation of the samples at room temperature for 10 min. To the samples 5 ml ethyl acetate were added, followed by brief mixing on a vortex mixer. The organic phase was removed and evaporated to dryness under a stream of nitrogen at 45 °C. The residue was dissolved in 100 μl methanol and aliquot of 20 μl was injected into the HPLC system.

2.5. Optimization of the derivatization conditions

To optimize the derivatization of amlodipine the reaction time, the temperature, the pH of borate buffer, the normality of HCl and time of incubation with HCl were tested around the expected optimal values. Buffer solutions ranging from 6 to10 were used to adjust the pH to the desired value. The reaction time was optimized by following the reaction for a total 60 min. The mixture was allowed to react in a warmed water bath at temperature ranging from 40 to 90 °C. Different concentration of NBD-Cl ranging from 50 to 5000 μg/ml and HCl ranging from 0.1 to 4N were examined and to optimize the time of incubation with HCl the solutions were chromatographed on the HPLC system up to 45 min.

2.6. Calibration curves and validation of the methods

The calibration curves were obtained by the analysis of 1 ml human blank serum samples supplemented with 100 μl each of working standard amlodipine solutions. The concentration ranges of the drug were 0.25–16 ng/ml. The samples were then submitted to the procedures of extraction, derivatization and chromatographic analysis described earlier. Calibration curves were obtained by linear least-squares regression analysis plotting of peak-area ratios (amlodipine/I.S.) versus the amlodipine concentrations.

The limit of detection was defined as the concentration of drug giving a signal-to-noise ratio of 3:1. The lower limit of quantification was defined as the lowest serum concentration of amlodipine quantified with a coefficient of variation of less than 20% (range recommended by the

Conference Report on Bioanalytical Methods Validation [14]). The recovery of amlodipine from serum was studied at the concentration ranges of 0.25, 2, 8, and 16 ng/ml. The recovery was calculated by comparing peak areas obtained after derivatization of amlodipine extracted from serum with peak areas obtained after derivatization of the same amounts of unextracted amlodipine. The presence of disturbing endogenous peaks was examined on 12 human serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure except from the addition of the I.S. Inter-day variation was determined by repeated ($N = 6$) analysis of different controls in a single analytical run. Intra-day variation was based on repeated analysis of the same concentration controls in ten analytical run performed on different days.

3. Application of the method

The method was applied to the determination of amlodipine in serum, following oral administration of 10 mg of both the market leader and a generic equivalent in 12 healthy volunteers in randomized single-dose cross-over study. They received single dose of 10 mg amlodipine after an overnight fasting. Blood samples were taken from each of the subjects prior to administration of the drug and at suitable intervals up to 48 h.

4. Results and discussion

Chromatograms obtained with human blank serum and human blank serum spiked with amlodipine (16 ng/ml) and the I.S. are shown in Fig. 1A and B, respectively. Amlodipine and the I.S. were well resolved with good symmetry with respective retention times of 3.7 and 5.4 min. No endogenous interfering peaks from serum were observed. Fig. 1C and D show the chromatograms of serum samples obtained at 9 and 48 h after a single oral dose of 10 mg amlodipine from a normal subject. Fluorescence response can be increased by increasing the amount of derivatization reagent. However, significant band-broadening was seen for a large excess of NBD-Cl. Suitable responses for different concentration of amlodipine were observed for 100 μl NBD-Cl (500 μg/ml). The derivatization reaction appeared to be highly dependent on reaction temperature (Fig. 2A), the pH of the borate buffer (Fig. 2B), normality of HCl (Fig. 2C), time of incubation with HCl (Fig. 2D). The optimal condition were found to be: a reaction temperature of 80 °C for 10 min, the pH of 7.6–8.5 of borate buffer and 10 min incubation with 2N HCl. The time course of the reaction of amlodipine with NBD-Cl in abovementioned conditions is presented in Fig. 3.

Detection of amlodipine by this method is sensitive enough for therapeutic drug monitoring of patients and pharmacokinetic studies dosed as low as 10 mg. The detection limit defined as a *S*/*N* of 3 was 0.1 ng/ml serum. The limit of

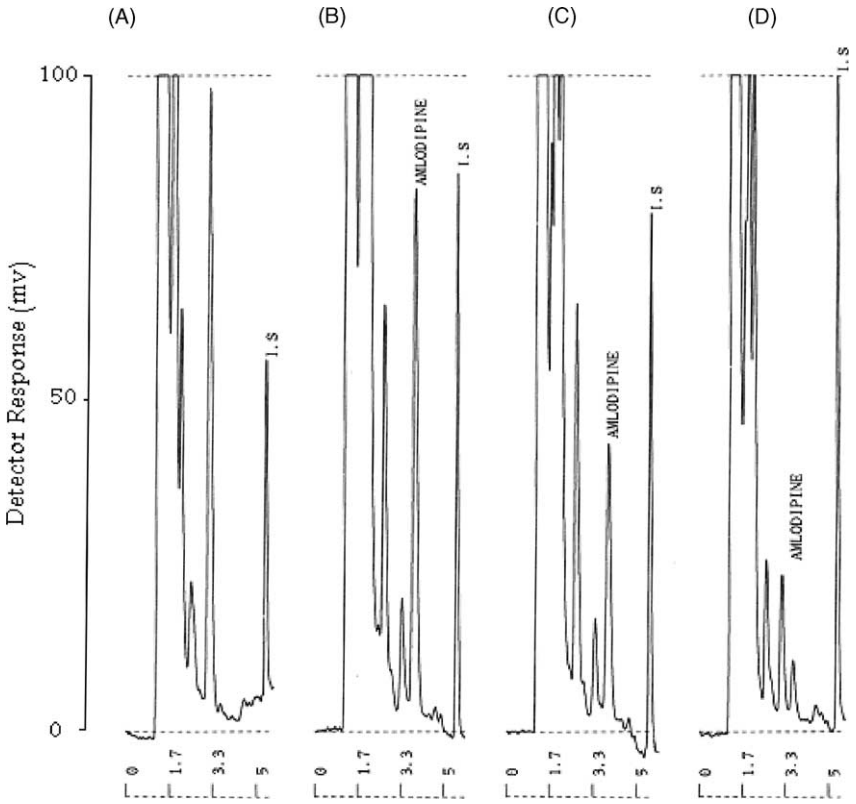

Fig. 1. Typical chromatograms obtained from an extract of (A) human blank serum (B) human blank serum spiked with 16 ng/ml amlodipine and the I.S. (C) and (D) serum samples from a volunteer, 9 and 48 h after a single oral dose of 10 mg drug containing 8 and 1.3 ng/ml of amlodipine, respectively.

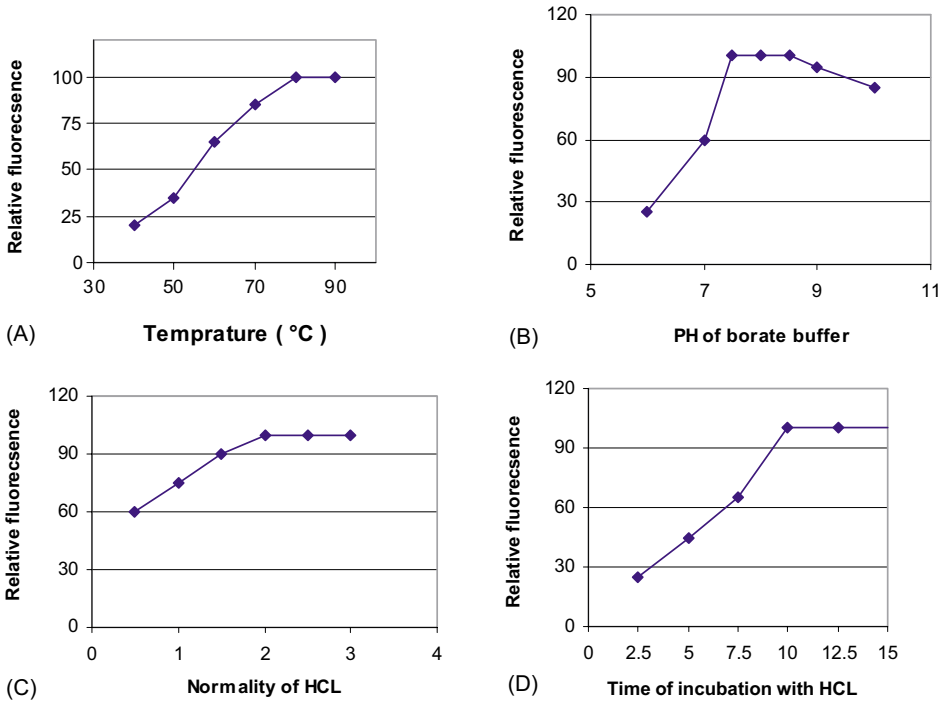

Fig. 2. Effects of (A) temperature (B) different pH values of borate buffer (C) HCl normality and (D) incubation time with HCl at room temperature on the reaction of amlodipine with NBD-Cl. Condition as in Section 2.

quantification corresponding with a coefficient of variation of less than 20% was 0.25 ng/ml. The calibration curves were linear over the concentration ranges of 0.25–16 ng/ml with a coefficient of 0.9979. The accuracy and precision of the method were investigated by replicate analysis of pooled serum samples containing added concentrations of amlodipine. The intra- and inter-day accuracy and precision data are presented in Table 1. The intra-assay accuracy ranged from 108 to 98.7% and the inter-assay accuracy ranged from 108 to 101.5%. The repeatability coefficients of variation were relatively low and do not exceed the 12% limit, however this value for lower concentrations (less than 0.25 ng/ml) was not acceptable. Thus, lowest concentration measured with acceptable precision and accuracy in our method was 0.25 ng/ml. The extraction efficiency was determined in human serum for different concentrations of amlodipine as well as for the I.S. The mean recovery of amlodipine was 90 ± 6% at four different concentrations and that of the I.S. was 85% at the concentration used.

The amlodipine and the I.S. kept at 4 °C and no decrease in their concentrations was observed over a period of 30 days. The derivatized solutions were found to be stable (>95%) for 4 h. After 30 days storing of the serum in −40 °C the concentration of amlodipine were found to be 101% of the initial value.

Various extraction procedures including protein precipitation methods and liquid–liquid extraction were investigated. Direct protein precipitation with either acetonitrile or trichlroacetic acid solution gave low recovery with high background noise. Prior to selection of extracting solvent of amlodipine from serum, the factors affecting the separation were studied in order to obtain the optimum conditions. The pH adjustment is a main factor affecting the recovery and

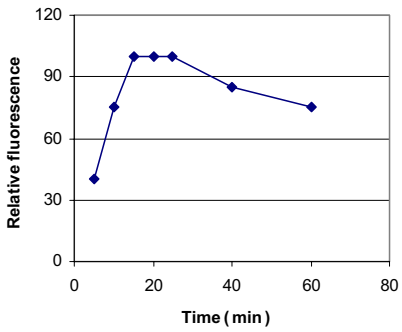

Fig. 3. Time course for the reaction of amlodipine with NBD-Cl. Amlodipine (8 ng/ml) was derivatized with 100 μl of NBD-Cl (500 μg/ml) and 100 μl the borate buffer (pH 7.8) in 80 °C.

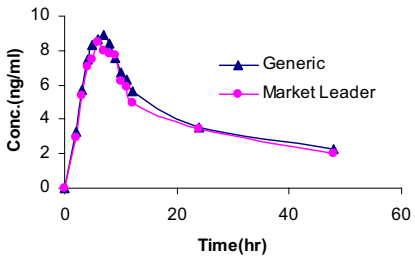

Fig. 4. Mean serum concentration–curve of amlodipine in 12 human volunteers after administration of a single 2 mg × 5 mg oral dose.

Table 1
Precision and accuracy for determination of amlodipine in human serum by the HPLC method

Known concentration (ng/ml)	Concentration found (mean ± S.D.)	Coefficient of variation (%)	Accuracy (percentage difference)
Within-day ($n = 6$)			
0.25	0.27 ± 0.026	9.7	+8
0.5	0.53 ± 0.045	8.7	+6
1	1.05 ± 0.065	6.2	+5
2	2.05 ± .083	4.09	+2.5
4	4.07 ± 0.163	4.01	+1.75
8	8.12 ± 0.24	2.2	+1.5
16	16.3 ± 0.44	2	+1.9
Between-day ($n = 10$)			
0.25	0.27 ± 0.06	11.1	+8
0.5	0.52 ± 0.6	11	+4
1	1.02 ± 0.11	10.9	+2
2	2.06 ± 0.16	7.9	+3
4	4.1 ± 0.231	5.3	+1
8	8.14 ± 0.34	4.2	+2.5
16	15.8 ± 0.33	2.4	−1.3

Percentage difference = [(mean concentration found − known concentration)/known concentration]100.

better recovery is resulted in alkalinized serum. Addition of a salt to the serum reduces the degree of hydration of the analyte and tend to promote its transfer into the organic phase. Extraction efficiency of different solvents including ethyl acetate, hexane, diethyl ether, dichloromethane and chloroform each alone and in combination with different percents of 2-propanol or isoamyl alcohol were compared and with regard to both recovery and interfering with endogenous peaks ethyl acetate gave the best recovery for amlodipine and the I.S. without interfering peaks. Thus, salting out approach using saturated carbonate/bicarbonate buffer and ethyl acetate as the extracting solvent was selected. However, in blank serum unknown peak with long retention time which interferes with the next analysis was eluted. Additional washing steps, application of different analytical columns (C8, CN, and TMS) or change in composition and the pH of the mobile phase did not eliminate this peak. Thus gradient elution was selected and in order to earlier elution of the unknown peak and its elimination in the next analysis, after elution of amlodipine and the I.S. the organic phase in the eluent was increased for a short time.

A number of drugs with secondary or primary aliphatic amines (e.g. fluoxetine, norfluoxetine, atenolol, metoprolol, desipramine, nortriptyline and metocarbamol) were tested but rendered unsuitable because of low recovery or late elution. Propranolol was selected as internal standard because of high recovery and suitable retention time.

The derivatization of amlodipine with NBD-Cl has been reported by Tatar and Atmaca [13]. However, their method involves several steps of extraction and cleaning of the samples and is time consuming. In their method, nortriptyline with long retention time was used as the I.S. and the total run time was more than 20 min.

In our method however, the resolution has been improved and less time is needed for preparing, derivitization and analysis of amlodipine in the human serum.

Table 2
Pharmacokinetic parameters of amlodipine in human volunteers after administration of a single 2 mg × 5 mg oral dose

Parameter	Market leader (n =12)	Generic (n=12)	P-value
T_{max} (h)	6.7	6.8	NS
C_{max} (ng/ml)	9.7	10.9	NS
$AUC_{0–48}$ (ngh/ml)	170.0	175.2	NS
$AUC_{0–00}$ (ngh/ml)	263.3	254.6	NS
$T_{1/2}$ (h)	30.4	27.3	NS

T_{max}: time of maximum concentration, C_{max}: maximum concentration, AUC: area under the concentration time curve, $T_{1/2}$: elimination half life, n: no. of volunteers, NS: not significant.

4.1. Application of the method

The method was applied to the determination of amlodipine in serum following single oral administration of 10 mg of both the market leader and a generic equivalent in 12 healthy volunteers. Fig. 4 shows a typical serum concentration–time profile for both formulation and resulted pharmacokinetic parameters are summarized in Table 2.

In conclusion, this paper describes a sensitive, specific and rapid reverse-phase HPLC method with fluorescence detection for the determination of amlodipine in serum. This method has been demonstrated to be suitable for use in pharmacokinetic studies of amlodipine. The advantages of our method are short time of analysis, improved resolution, a simple sample extraction and clean-up compared to multiple extraction and washing steps, and a longer analysis time of the previously published method [13].

Acknowledgements

This work was supported by Arya Pharmaceutical Company and in part by Kermanshah University of Medical

Sciences. We would like to thanks from Mr. Amir Kiani for his valuable assistance in analysis of the samples.

References

[1] J. Lluksa, D.J. Josic, M. Kremser, Z. Kopitar, S. Milutinovic, J. Chromatogr. B 703 (1997) 185–193.
[2] R. Klinkenberg, B. Streel, A. Ceccato, J. Pharm. Biomed. Anal. 32 (2003) 345–352.
[3] A.P. Beresford, P.V. Macrae, D.A. Stopher, B.A. Wood, J. Chromatogr. 420 (1987) 178–183.
[4] F. Scharpf, K.D. Riedel, H. Laufen, M. Leitold, J. Chromatogr. B 655 (1994) 225–233.
[5] S.C. Monkman, J.S. Ellis, S. Cholerton, J.M. Thomason, R.A. Seymour, J.R. Idle, J. Chromatogr. B 678 (1996) 360–364.
[6] K. Shimooka, Y. Sawada, H. Tatematsu, J. Pharm. Biomed. Anal. 7 (1989) 1267–1272.
[7] M. Josefsson, A.L. Zackrisson, B. Norlander, J. Chromatogr. B 672 (1995) 310–313.
[8] M. Josefsson, B. Norlander, J. Pharm. Biomed. Anal. 15 (1996) 267–277.
[9] K.K. Pandya, M. Satia, T.P. Gandhi, I.A. Modi, R.I. Modi, B.K. Chakravarthy, J. Chromatogr. B 667 (1995) 315–320.
[10] T. Yasuda, M. Tanaka, K. Iba, J. Mass Spectrom. 31 (1996) 879–884.
[11] A. Marzo, L.D. Bo, P. Mazzucchelli, N.C. Monti, F. Crivelli, S. Ismaili, M.R. Uhr, P.L. Commare, Arzneim-Forsch. Drug Res. 50 (2000) 688–695.
[12] B. Streel, C. Laine, C. Zimmer, R. Sibenaler, A. Ceccato, J. Biochem. Biophys. Methods 54 (2002) 357–368.
[13] S. Tatar, S. Atmaca, J. Chromatogr. B 758 (2001) 305–310.
[14] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Eur. J. Drug Matab. Pharmacokin. 16 (1991) 249–255.