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# Isocratic high-performance liquid chromatographic method for the separation of isradipine and its main metabolites

## Application to in vitro metabolization by h3A4/OR cells

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### Abstract

A reversed-phase HPLC method was developed for the study of isradipine oxidation in vitro. The drug and its main metabolites were determined after extraction from culture media and from h3A4/OR cells having the cytochrome P450 isoenzyme involved in the xenobiotic metabolization. The HPLC assay was fully validated and was used to follow the biotransformation kinetics of isradipine.

*Keywords:* h3A4/OR cells; Isradipine

### 1. Introduction

Isradipine (PN200-110), isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate (ISR), is a dihydropyridine derivative and a nifedipine analogue well known as calcium antagonist. This drug, like other compounds of this class has a low bioavailability in humans due to an extensive first pass metabolism [1]. Interestingly, the cytochrome P450 3A4 isoenzyme (CYP 3A4) was described [2] to have a preponderant place in their transformation.

The aim of this work was to develop a model useful to study, in vitro, the working of the cytochrome P450 3A4 as well as the interactions of drugs or nutriment on its function. The metabolization of

isradipine as well as the interactions when incubated in more complex systems (microsomes) will be further envisaged and the analytical method was developed to allow a wide utilization.

The choice of isradipine was made not because it is a calcium antagonist widely used in therapeutics but because it is an excellent substrate for isoenzyme CYP 3A4. Moreover, contrarily to nifedipine, ISR has the advantage of being stable in daylight.

Assay procedures for ISR in biological matrix have precedently used gas chromatography with mass spectrometric detection [3]; <sup>14</sup>C labelled methods [1], radioimmunoassay [4] and high performance liquid chromatography (HPLC) with UV detection [5]. These methods were developed to determine either ISR or ISR and its first metabolite.

In this paper, we developed for the first time to our knowledge, an isocratic HPLC assay for the

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determination of ISR and four metabolites in cells and in culture medium.

The choice of the column and the mobile phase as well as the extraction conditions of these molecules, which have a wide polarity range, were particularly studied.

This method was used to follow ISR oxidation *in vitro* by the human lymphoblastoid cell line h3A4/OR.

## 2. Experimental

### 2.1. Chemicals

Isradipine (PN200-110), PN204-144, PN204-145, PN204-755 and PN203-831 were gratefully donated by Sandoz (Basle, Switzerland) (Fig. 1).

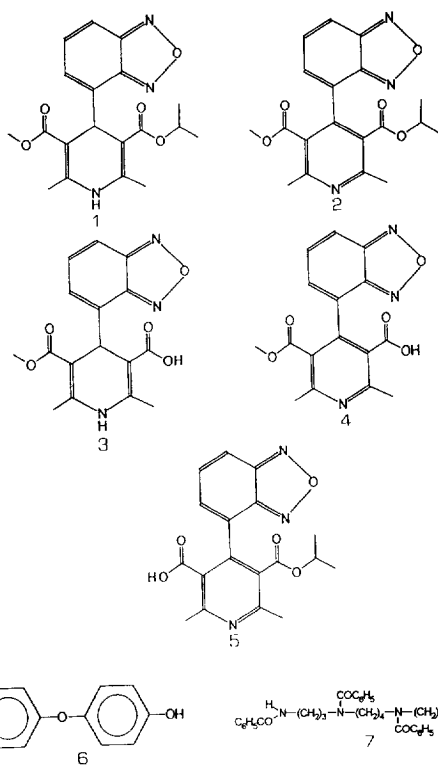


Fig. 1. Chemical structures of isradipine (ISR), its main metabolites and internal standards. 1=ISR, 2=PN 203-831, 3=PN 204-144, 4=PN 204-145, 5=PN 204-755, 6=*p*-phenoxyphenol and 7=benzoylated spermine.

Sodium dihydroxide pellets, sodium dihydrogen-phosphate monohydrate GR, chloroform GR, hydrochloric acid 1 *M* and citric acid of analytical grade were purchased from E. Merck (Darmstadt, Germany). Tetrabutylammonium hydrogen sulfate (TBA), 98%, was supplied by Acros organics (Geel, Belgium). Tetrahydrofuran RS was of analytical grade and provided by Farmitalia Carlo Erba (Milan, Italy). *N,N',N'',N'''*-tetrabenzoyl-4,9-diazododecane-1,12-diamine was prepared in our laboratories by benzylation of spermine [6] and identified by LC-MS; *p*-phenoxyphenol was purchased from Janssen Chimica (Geel, Belgium) (Fig. 1).

Methanol was purified by home redistillation and deionised water was prepared on site and further purified using a Milli-Q system (Millipore, Brussels, Belgium).

Penicillin-streptomycin (5000 IU/ml–5000  $\mu$ g/ml), horse serum, L-glutamine 200 mM and Nunclon (80 cm<sup>2</sup>, 260 ml) Nunclon Delta were supplied by Gibco Life Technologies (Gaithersburg, MD, USA).

RPMI 1640 containing 2 mM histidinol without L-histidine and h3A4/OR cell line were purchased from Gentest (Woburn, MA, USA).

### 2.2. Preparation of solutions

Stock solutions were prepared in methanol and were further diluted either in mobile phase to be used as chromatographic standards or in water to spike biological samples.

The concentration range was between 0.7 and 39  $\mu$ M (Table 1).

### 2.3. Chromatographic analysis

Separation was carried out by an isocratic reversed-phase HPLC technique using a Gilson Model 305 pump (Middletown, WI, USA), a Rheodyne 7125 injector (Cotati, CA, USA) and a Chromspher C<sub>8</sub> column (100×4.6 mm I.D., 5  $\mu$ m particle size) from Chrompack (Middelburg, Netherlands).

Isradipine and its metabolites were detected spectrophotometrically at 240 nm with a diode array detector, Module 168 from Beckman (Berkeley, CA, USA).

Table 1

Concentration range used to spike solutions and to prepare aqueous standards for linearity studies

Sample	Concentration ( $\mu\text{M}$ )				
	ISR	PN 204-144	PN 204-145	PN 204-755	PN 203-831
Aqueous standards	3.62–18.12	1.30–6.50	1.36–6.81	1.91–9.54	3.9–19.49
h3A4/OR cells	1.81–18.12	0.65–6.50	0.68–6.81	0.95–9.54	1.95–19.49
Culture medium	3.624–36.24	1.30–12.99	1.36–13.63	1.91–19.08	3.90–38.98

The detector output was integrated and quantified on the Gold system (Beckman). The mobile phase was 55.4% water with 0.16 M  $\text{NaH}_2\text{PO}_4$  and 0.017 M tetrabutylammonium hydrogensulfate, 43.6% of methanol and 1% of tetrahydrofuran; the pH of the aqueous phase was 3.0 and the flow-rate 1 ml/min.

Separations on other columns namely Chromspher 5 poly  $\text{C}_{18}$  (100 $\times$ 3 mm I.D., 5  $\mu\text{m}$  particle size) from Chrompack and Ultrasphere ODS (150 $\times$ 4.6 mm I.D., 5  $\mu\text{m}$  particle size) from Beckman were also investigated.

#### 2.4. Standard curves

The following ranges of concentrations were used for spiking solutions and to prepare aqueous standards for linearity studies (Table 1).

The concentrations of the internal standards (I.S.) in spiking solutions were, respectively, 10.63  $\mu\text{M}$  for the *p*-phenoxyphenol and 2.815  $\mu\text{M}$  for the benzoylated spermine.

#### 2.5. Cell line and culture medium

h3A4/OR cells were maintained at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  in air and in an 80  $\text{cm}^2$  flask. Cells were grown in RPMI 1640 (containing 2 mM 1-histidinol without 1-histidine) supplemented with 9% of horse serum (HS), 2 mM L-glutamine, 100 UI/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin solutions.

The timing of cell manipulation was calculated from the day of passage (=day 0). Cells were subcultured on day 3 ( $1 \times 10^5$  cells per ml). Stocks of cells were routinely frozen and stored in liquid  $\text{N}_2$ .

#### 2.6. Extraction procedures

##### 2.6.1. From culture medium

A 5-ml volume of culture medium (RPMI+9% HS) was spiked with 200  $\mu\text{l}$  of an aqueous solution of isradipine (ISR), its metabolites and the internal standards (I.S.). The pH sample was adjusted to 8.0 with 1 M NaOH, 5 ml of  $\text{CHCl}_3$  were added and the solution was mixed for 30 min at 30 rpm. After centrifugation for 5 min at 1000 g, the chloroformic phase was withdrawn. The pH of the remaining aqueous phase was adjusted to pH 3.0 with HCl (1 M) and 1 ml of a citrate buffer (pH 3; 0.1 M) and 1 ml of TBA (0.037 M) was added. The aqueous phase was reextracted with 5 ml of  $\text{CHCl}_3$  for 30 min at 30 rpm. The solution was centrifuged (5 min, 1000 g) and the chloroformic phase was withdrawn and added to the first extraction phase.  $\text{CHCl}_3$  was evaporated to dryness under a nitrogen stream and the residue was dissolved in 300  $\mu\text{l}$  of mobile phase, sonicated for 1 min and 50  $\mu\text{l}$  were injected.

##### 2.6.2. From h3A4/OR cells

A 100- $\mu\text{l}$  volume of a cell suspension ( $40 \cdot 10^6$  cells) was spiked with 100  $\mu\text{l}$  of a solution containing isradipine, its metabolites and the internal standards. Immediately the mixture was vortex-mixed for 1 min and sonicated for 3 min. A 200- $\mu\text{l}$  volume of  $\text{CH}_3\text{OH}$  was added, vortex-mixed for 1 min and precipitated proteins were separated by centrifugation for 5 min (2000 g), 50  $\mu\text{l}$  of the supernatant was then injected.

##### 2.6.3. In vitro metabolism of isradipine

Cells ( $40 \cdot 10^6$ ) were incubated in 25 ml of RPMI medium spiked with dexamethasone (enzyme inducer) at a concentration of  $4 \cdot 10^{-6}$  M.

At the end of this induction period the medium was discarded and cells were reincubated in fresh medium (25 ml) containing ISR at the concentration of 9.3  $\mu\text{M}$ . Cells or medium were sampled at different times (every 3 h, for 30 h), extracted and analyzed by HPLC to determine ISR and its metabolite PN 203-831.

#### 2.6.4. Validation procedures

**Limits of detection (LOD) and quantification (LOQ).** The LOQ was quoted as the concentration yielding a signal-to-noise ratio of 10 by analyzing six samples at this value. The accepted precision was 10%. For the LOD a ratio of 2:1 was the yield.

**Recovery.** The recoveries of ISR and its four metabolites were determined for both biological media at five concentrations in the range stated for linearity studies.

The extraction efficiency of the analytes was determined by comparing the peak area ratios (analyte/I.S.) after extraction and without processing. *p*-Phenoxyphenol and benzoylated spermine were, respectively, the I.S. for the quantification of the three first eluting peaks (PN204-145; PN204-144; PN204-755) and the two last eluting peaks (PN 203-831; ISR).

**Intra- and inter-assay precision.** Intra-assay precision of the method was determined by analyzing six independent biological samples at each concentration of the linearity range in a one day experiment. Inter-assay precision was obtained by assaying one sample at all concentrations on three different days. Each sample was injected three times.

### 3. Results and discussion

#### 3.1. Chromatographic analysis

Our goal was to develop an isocratic HPLC method which allows the total separation and the determination of ISR and its main metabolites. The challenge was thus the separation of compounds with a wide polarity range in a limited time span. By using a  $\text{C}_{18}$  column, a complete resolution of the

system was done within 47 min with a mobile phase prepared by mixing 55% water with  $\text{NaH}_2\text{PO}_4$  0.20 M and 45% of methanol, the pH of the aqueous phase was 3.5. On a polymerized Chromspher 5 poly  $\text{C}_{18}$ , which had a lower selectivity, the separation of polar metabolites was not possible even with mobile phases with a very low percentage of organic solvent. However, it was demonstrated that ISR and its metabolite PN 203-831 were separated and could be determined by using this type of column within a short analysis time. The best solution was found by analyzing these compounds by ion pair chromatography on a  $\text{C}_8$  column. Tetrabutylammonium hydrogensulfate was used as cationic counter ion. The chromatogram (Fig. 2) indicates a good resolution for all isradipine derivatives and both internal standards within 37 min. The influence of several mobile phase variables was tested on this column: the pH of the aqueous phase, the concentration of TBA and the percentage of  $\text{CH}_3\text{OH}$ . TBA was used to form ion pairs with the more polar metabolites of ISR, i.e. PN 204-145, PN 204-144 and PN 204-755, which are molecules with a carboxylic function. In this way, the retention time of these drugs was increased while the retention time of ISR and PN 203-831, on which the acid function is esterified by alkyl groups, was not modified.

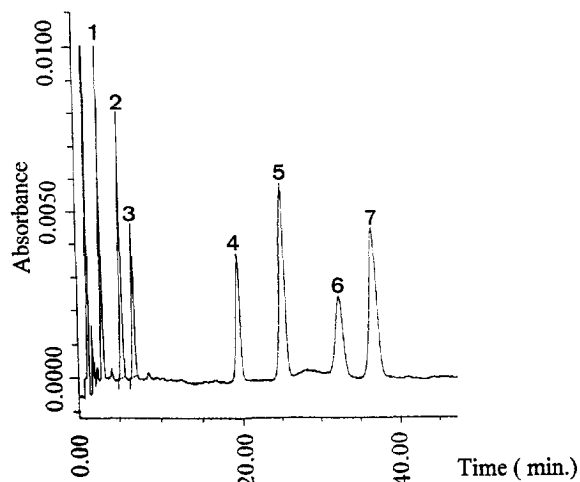


Fig. 2. Representative chromatogram of isradipine, its main metabolites and internal standards dissolved in aqueous solutions. Peaks: 1=PN 204-145, 2=PN 204-144, 3=PN 204-755, 4=*p*-phenoxyphenol, 5=PN 203-831, 6=benzoylated spermine and 7=ISR.

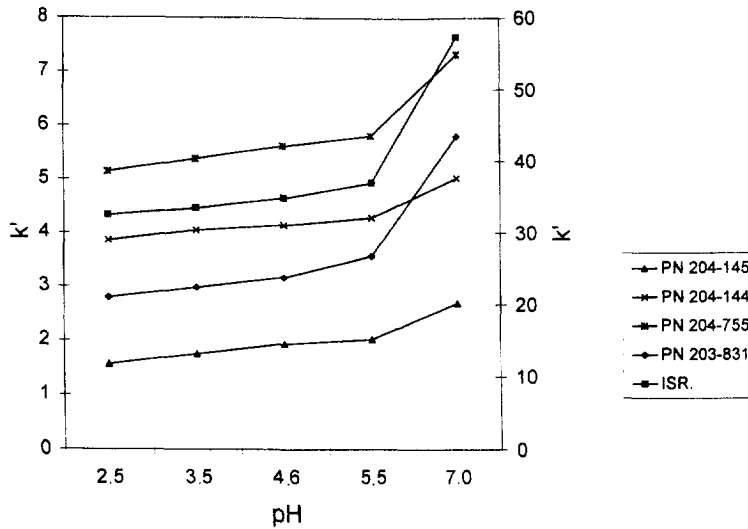


Fig. 3. Influence of the mobile phase pH on the capacity factor ( $k'$ ) of compounds.  $k'$  scale for PN 204-145, PN 204-144 and PN 204-755 is on the left side of the graphic; for PN 203-831 and ISR on the right.

Table 2  
Coefficients of correlation, intra-day, inter-day precision and recoveries for drugs extracted from h3A4/OR cell pellets

Compound	Concentration added ( $\mu M$ )	Intra-day recovery (n=6) (%)	Intra-day C.V. (%)	Inter-day recovery (n=3) (%)	Inter-day C.V. (%)	$r^2$ (mean $\pm$ S.D.) (n=3)
ISR	18.12	104.4	2.2	99.6	4.2	0.996 $\pm 0.004$
	12.08	104.4	1.8	98.8	5.3	
	9.06	103.5 mean: 102.0	2.5 mean C.V.	98.5 mean: 98.5	4.5 mean C.V.	
	6.04	97.7	2.6 2.5	98.2	0.7 3.6	
	1.81	99.8	3.3	97.4	3.4	
PN 204-144	6.50	97.5	2.0	98.6	1.8	0.993 $\pm 0.002$
	4.33	98.1	0.7	99.5	1.2	
	3.25	97.4 mean: 97.6	2.7 mean C.V.	97.6 mean: 99.8	3.2 mean C.V.	
	2.17	81.7	9.1 4.1	96.4	13.2 5.0	
	0.65	113.3	6.0	106.7	5.4	
PN 204-145	6.81	100.9	1.8	93.1	10.8	0.981 $\pm 0.009$
	4.54	100.5	1.3	100.7	4.1	
	3.41	97.3 mean: 102.3	5.3 mean C.V.	90.9 mean: 97.3	12.3 mean C.V.	
	3.27	118.8	7.7 5.1	104.5	11.9 8.5	
	0.68	93.9	9.6	97.3	3.5	
PN 204-755	9.54	108.1	1.0	105.5	2.3	0.996 $\pm 0.002$
	6.36	103.3	3.5	107.2	7.8	
	4.77	109.1 mean: 101.1	6.9 mean C.V.	106.2 mean: 102.3	4.2 mean C.V.	
	3.18	102.9	6.1 4.6	100.5	2.3 5.2	
	0.95	82.0	5.6	92.1	9.5	
PN 203-831	19.50	97.5	1.1	102.4	4.3	0.997 $\pm 0.003$
	13.00	98.2	1.5	104.7	5.8	
	9.75	97.6 mean: 99.7	2.8 mean C.V.	101.5 mean: 103.3	3.4 mean C.V.	
	6.50	102.0	3.2 2.3	102.4	2.3 3.7	
	1.95	103.7	2.7	105.3	2.9	

Despite the fact that pH 3.0 generally does not allow complete ionization of the carboxylic acid functions, the experimental results showed a complete resolution for all peaks. An increase of the pH higher than 5.5 resulted in increased retention time for all drugs (Fig. 3). This phenomenon could be easily explained for drugs with a carboxylic group by a better ionization. For ISR and PN 203-831, it could be the consequence of ion suppression on the amine group. In these conditions, it should be assumed that the chromatographic behaviour of the metabolites PN 204-144, PN 204-145 and PN 204-755 was the result of a mixed influence of ion pair formation and amine protonation suppression when pH increases.

As could be expected, an increase in TBA concentration and a decrease in methanol proportion increased the retention time of all the compounds. Compromise concentrations were chosen considering the range of polarity of the products.

### 3.2. Validation

#### 3.2.1. Standard curves

The coefficients of correlation are collected in Tables 2 and 3 and show that calibration curves exhibit a good linearity for all drugs determined in culture medium or in cells. However, the slopes and intercepts are statistically different ( $P < 0.05$ ) from day to day. Thus, the samples are quantified against standards injected the same day. The intercepts were never different from zero ( $P < 0.05$ ).

#### 3.2.2. Recovery and precision

The relative recoveries were close to 100% for all drugs in both media and at all concentrations of the linearity range. The precisions of intra-day and inter-day assays appeared to be excellent in all cases, with a mean value which remained under 10% (Tables 2 and 3). During the extraction of the analytes from the

Table 3  
Coefficients of correlation, intra-day, inter-day precision and recoveries for drugs extracted from RPMI medium

Compound	Concentration added ( $\mu M$ )	Intra-day recovery ( $n=6$ ) (%)	Intra-day C.V. (%)	Inter-day recovery ( $n=3$ ) (%)	Inter-day C.V. (%)	$r^2$ (mean $\pm$ S.D.) ( $n=3$ )
ISR	36.24	101.9	2.9	100.3	2.1	0.996 $\pm 0.003$
	27.18	100.2 mean: 102.4	3.2	99.0	4.6	
	18.12	102.7	1.3 mean C.V.	105.8 mean: 101.6	2.6 mean C.V.	
	9.06	107.4	4.6 3.3	102.9	4.7 3.3	
	3.62	99.8	4.5	99.9	2.7	
PN 204-144	13.00	–	–	–	–	–
	9.74	–	–	–	–	
	6.50	73.5 mean: 82.5	1.8 mean C.V.	73.9 mean: 81.5	8.7 mean C.V.	
	3.25	69.4	3.7 4.5	74.7	64. 10.4	
	1.30	104.6	8.1	90.6	16.1	
PN 204-145	13.63	–	–	–	–	0.997 $\pm 0.002$
	10.22	101.4	2.7	102.3	3.4	
	6.82	101.6 mean: 102.7	2.3 mean C.V.	105.2 mean: 104.3	3.1 mean C.V.	
	3.41	102.2	4.3 4.4	105.2	2.9 2.7	
	1.36	105.6	8.3	104.5	1.4	
PN 204-755	19.08	101.3	1.3	99.1	3.1	0.983 $\pm 0.009$
	14.31	116.3	3.3	103.9	10.7	
	9.54	80.5 mean: 93.0	1.7 mean C.V.	90.9 mean: 94.6	4.1 mean C.V.	
	4.77	74.9	2.3 3.3	83.8	15.3 9.6	
	1.91	91.8	7.9	95.1	14.7	
PN 203-831	38.98	100.2	1.7	101.1	1.2	0.996 $\pm 0.004$
	29.24	102.8	3.4	102.7 mean: 97.6	2.6	
	19.49	100.8 mean: 100.9	1.5 mean C.V.	94.8	6.5 mean C.V.	
	9.75	97.1	2.2 2.2	98.3	3.9 5.3	
	3.90	103.5	2.3	90.9	12.4	

medium, it should be pointed out that TBA was only added for the second extraction run to prevent too many impurities in the organic phase which give interfering peaks in the chromatogram. The choice of pH 3.0 for this extraction step, was also done for specificity purposes. It is important to note that the quantitative extraction of ISR and PN 203-831 from culture medium was achieved in a one-step extraction by  $\text{CHCl}_3$  at pH 8.0 without any addition of TBA, as it was described in the first part of Section 2.6. However TBA was an absolute requisite to extract the more polar metabolites (PN 204-145, PN 204-144, PN 204-755) with a high efficiency.

### 3.2.3. Limits of detection and quantification

The LOD and LOQ in cells and culture medium were obtained by two different methods and expressed in two different ways according to the material used. They are in all cases at the low nmol level as indicated in Table 4.

### 3.2.4. Specificity

A typical chromatogram of blank medium is represented in Fig. 4 which does not reveal any interfering peaks. The analysis of biological samples spiked with all derivatives was performed by the Gold system according to the evolving factor analysis which did not detect any coeluting impurity. These results were confirmed by the spectral overlay technique. The stability of I.S. throughout the analysis procedure was investigated. No degradation product was found in the chromatogram.

### 3.3. Metabolization study

Fig. 5 shows the concentration profile of PN 203-831 in the culture medium and in cells after

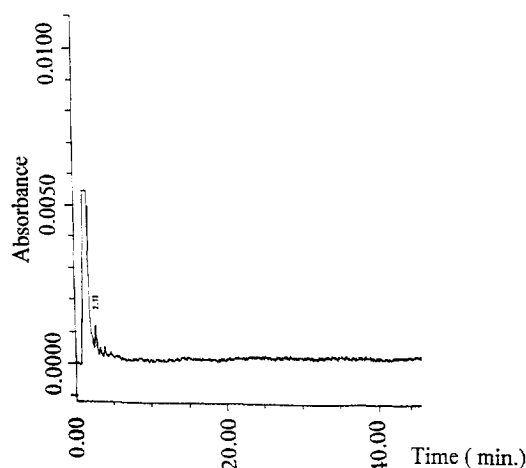


Fig. 4. HPLC chromatogram of blank sample from culture medium (5 ml).

different incubation times of cells with isradipine. Each point was the mean of three independent assays. It was found that the concentration of the metabolite was linearly increasing with time while its concentration was nearly constant in cells. Fig. 6 illustrates the metabolization of ISR by h3A4/OR cells after 27 h of incubation.

## 4. Conclusion

An isocratic reversed-phase HPLC method has been developed and validated for the resolution of ISR and its main metabolites. This method was used to measure the cytochrome P450 3A4 isoenzyme activity in h3A4/OR cells.

The analytical technique described coupled with the use of this transformed cell line is an interesting tool to study changes in the CYP 3A4 activity after

Table 4  
Limits of detection and quantification

Compound	LOD		LOQ	
	Cells nmol/10 <sup>6</sup> cells	Culture medium nmol/ml	Cells nmol/10 <sup>6</sup> cells	Culture medium nmol/ml
ISR	0.11	0.21	0.35	0.70
PN 204-144	0.0375	0.075	0.125	0.25
PN 204-145	0.0390	0.078	0.13	0.26
PN 204-755	0.0375	0.11	0.125	0.37
PN 203-831	0.11	0.228	0.38	0.76

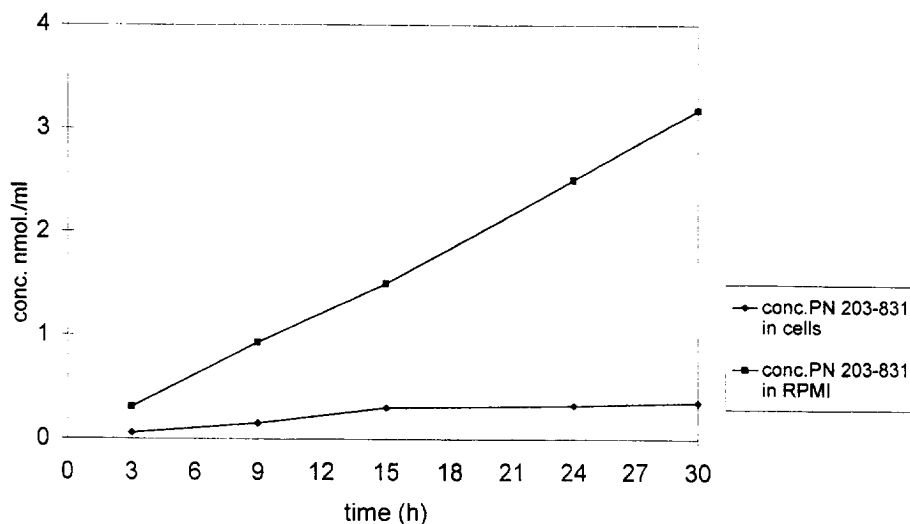


Fig. 5. Concentration–time data for PN 203-831 extracted from culture medium and from cells after different incubation times.

treatment with inhibitors or inducing agents and represents an alternative method to the use of testosterone [7] or nifedipine [8] as substrates to monitor biotransformations by CYP 3A4.

Moreover, the HPLC determination of ISR metab-

olites should be suitable for pharmacokinetic studies with little modification.

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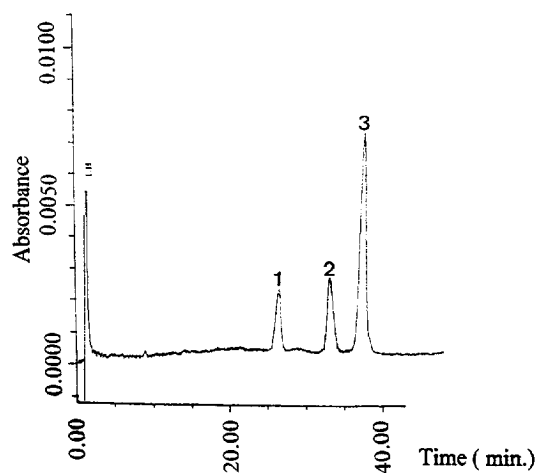


Fig. 6. HPLC chromatogram of ISR (3), its main metabolite PN 203-831 (1) and the internal standard, benzoylated spermine (2).