ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
26 (2001) 7–14

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

A sensitive method for the determination of gemfibrozil in human plasma samples by RP-LC

E. González-Peñas [a,*], S. Agarraberes [a], A. López-Ocariz [b], E. García-Quetglas [b], M.A. Campanero [b], J.J. Carballal [c], J. Honorato [b]

[a] *Departamento de Química Orgánica y Farmacéutica, Facultad de Farmacia, Universidad de Navarra, 31008 Pamplona, Spain*
[b] *Servicio de Farmacología Clínica, Clínica Universitaria de Navarra, Universidad de Navarra, 31008 Pamplona, Spain*
[c] *Laboratorios CEPA SL, Madrid, Spain*

Received 8 October 2000; received in revised form 23 November 2000; accepted 10 December 2000

Abstract

A sensitive high-performance liquid chromatographic assay for the quantitative determination of gemfibrozil is described in this work. Ibuprofen was used as internal standard. The assay involved a single cyclohexane extraction and LC analysis with fluorescence detection. Chromatography was performed at 40°C on a Hypersil ODS column. The mobile phase was a mixture of a solution of phosphoric acid 0.4% and acetonitrile (45:55). The method was validated. The detection limit of this method was 0.025 μg ml^{-1}; only 0.5 ml of the plasma sample was required for the determination. The calibration graph was linear from 0.05 to 0.5 μg ml^{-1} and required a cubic equation from 0.5 to 30 μg ml^{-1}. Intra and inter-day precision (C.V.) did no exceed 15%. Mean recoveries were of $90.15 \pm 6.9\%$ (C.V.'s $< 8\%$) for gemfibrozil and 93.10% for ibuprofen Applicability of the method was demonstrated by a pharmacokinetic study in normal volunteers who received gemfibrozil by oral route.

Keywords: Reverse-phase chromatography; Gemfibrozil in human plasma; Pharmacokinetics

1. Introduction

Gemfibrozil (CAS No. 25812-30-0) is a fibrate hypolipidaemic agent demonstrated to lower the incidence of coronary heart disease in humans [2]. Gemfibrozil is well absorbed in the intestinal tract with a plasmatic half-life of ~ 1.5 h. [3]. Gemfibrozil is extensively metabolized and excreted in urine as a glucuronide together with four oxidized metabolites [4]. In pharmacokinetic studies it is very important to have very sensitive methods that permit measuring low concentrations of analyte such as those likely to be present in vivo in

* Corresponding author. Tel.: +34-425653 Ext 6371; fax: +34-455652.
E-mail address: mgpenas@unav.es (E. González-Peñas).

0731-7085/01/$ - see front matter
PII: S0731-7085(01)00369-7

plasma in order to obtain an adequate characterization of the levels-time curves. Some methods are currently available for the quantitation of gemfibrozil from plasma and urine. A gas chromatographic method was published in 1984 to determine gemfibrozil in biological fluids; it included a derivatisation procedure prior to analysis with a sensitivity of 500 ng ml^{-1} of plasma [1]. More recently, some HPLC methods have been reported in order to achieve higher sensitivity with less tedious sample handling. In 1985, Hengy and Kölle reported an HPLC-UV detection method for gemfibrozil in plasma samples with a quantification and detection limits of 200 and 50 ng ml^{-1}, respectively [5]; it is, to our knowledge, the lowest limit of detection described in literature. In 1986, Randinitis et al. using HPLC-UV, reported a quantification limit of 0.5 μg ml^{-1} [6]. Later, in 1991, fluorescence detection was first introduced by Nakagawa et al., reporting a detection limit of 0.10 μg ml^{-1} [4].This paper describes a simple and validated high performance liquid chromatography method to study the pharmacokinetics of unchanged gemfibrozil based on that of Hengy and Kölle [5] but with some modifications, such as fluorescence detection, in order to achieve higher sensitivity.

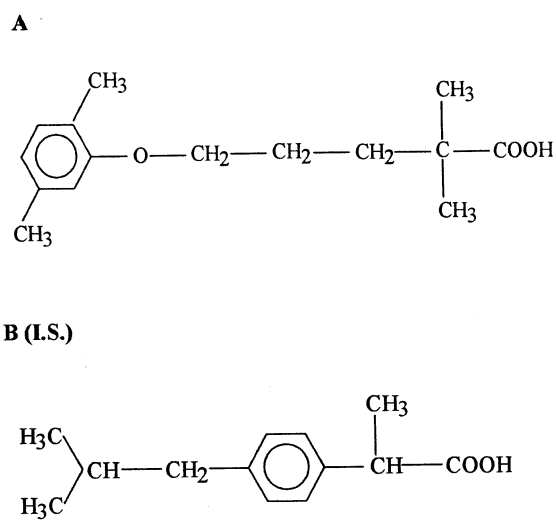

Fig. 1. Chemical structure of gemfibrozil (A) and I.S. (B).

2. Experimental

2.1. Chemicals and reagents

Gemfibrozil (Fig. 1) were supplied by Lab. CEPA S.L. Ibuprofen was provided by SIGMA Chemicals (Madrid, Spain). Acetonitrile HPLC-grade was obtained from Scharlau (Barcelona, Spain); cyclohexane HPLC-grade and phosphoric acid analytical grade were obtained from Merck (Barcelona, Spain). Hydrochloric acid 1 N SV was from Panreac (Barcelona, Spain).

2.2. Apparatus and chromatographic conditions

A Hewlett Packard high performance liquid chromatograph (Madrid, Spain), Mod. 1090 equipped with a HP 1046A fluorescence detector were used. The excitation and emission wavelength were 242 and 300 nm, respectively. The cut-off filter was 295 nm and PMTgain was 6. The computer programs used were Chemstation 3D for chromatographic analysis, and Microsoft Excel 5.0 for statistic analysis.

In setting up the analytical method, the following factors have been studied: detector conditions (emission and excitation wavelengths, PMTgain or sensitivity of the fluorescence detector, quantity of the PI to be added to the samples) and plasma extraction conditions for gemfibrozil and internal standard. Selection of the most adequate wavelengths for fluorescence detection was based on the scan of excitation and emission of the gemfibrozil peak; the sensibility of the detector was fixed in an attempt to obtain the better signal-noise ratio for gemfibrozil peak in the concentration range studied. The quantity of PI in the sample was determined in order to obtain a peak of intermediate size from among those of gemfibrozil in the concentration range expected.

With respect to the extraction process, pentane and cyclohexane have been studied. Pentane was discarded because the sample extract presented chromatographic peaks with the same retention time as that of the Internal standard.

The stationary phase used for the separation was an Hypersil ODS column (250 × 4.0 mm I.D.) packed with a 5 μm particle Teknokroma

(Barcelona, Spain). A guard column C_{18} (Teknokroma) was also used. Separations were conducted using a mobile phase of acetonitrile and phosphoric acid solution 0.4% (55:45). Mobile phase was filtered though a 0.45 μm pore-size membrane filter. The flow-rate was 2 ml min^{-1}. The analytical and guard columns were operated at 40°C.

2.3. Standard solutions and samples

A stock solution of gemfibrozil (1 μg ml^{-1}) was prepared by dissolving 0.1 mg of gemfibrozil in 100 ml of acetonitrile. Two standard solutions (0.1 and 0.01 μg ml^{-1}) were made by further dilution of the stock solution with acetonitrile.The internal standard stock solution of ibuprofen (10 mg ml^{-1}) was prepared in acetonitrile.

Plasma standard solutions for calibration curve were prepared by adding appropriate volumes from the corresponding standard solution of gemfibrozil to each one of 10 tubes. After the solvent was evaporated, 0.5 ml of drug-free plasma and 10 μl of the internal standard solution were added to each tube. Plasma solutions had the following gemfibrozil concentrations: 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 25 and 30 μg ml^{-1}.

2.4. Extraction and isolation procedures

Human plasma (0.5 ml) was pipetted into a 15-ml glass stoppered tube and was spiked with 10 μl of internal standard solution (10 mg ml^{-1}) and 0.2 ml of HCl 1 N. The samples were extracted with 5 ml of cyclohexane using a rotatory shaker for 20 min. The organic layer was separated after centrifugation at 3500 rpm for 5 min. The separated organic layer was evaporated to dryness in a waterbath at 40°C under a stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase and vortex-mixed. A 25 μl aliquot was then injected onto an HPLC column.

2.5. Application of the method

The present method was applied for the determination of the plasma concentration of gemfibrozil after a single oral administration of the drug to healthy adult male volunteers (600 mg, Trialmin®, Lab. Menarini (Badalona, Spain)) as a part of a Phase I study. Venous blood samples were withdrawn and plasma fractions were separated immediately prior to dosing and at 0.5, 1, 1,5, 2, 2,5, 3, 4, 5, 6, 8, 10, 12 and 24 h after dosing. Plasma samples were stored frozen (-40°C until analyses).

3. Results

3.1. Chromatography

Gemfibrozil and ibuprofen (I.S.) had a retention time of 5.9 and 4.0 min, respectively, in the chromatographic conditions described. Fig. 2 shows a typical subject's plasma chromatograms 2.5 and 24 h post-dose with 8.51 and 0.09 μg ml^{-1} of gemfibrozil, respectively.

3.2. Extraction efficiency

Recoveries of gemfibrozil and ibuprofen from samples spiked with each analyte were determined as follows: from the standard solutions of gemfibrozil, the following quantities of gemfibrozil were placed in two series of tubes (A and B):0.025, 0.25, 2.5 and 12.5 μg. A total of 10 μl of the internal standard stock solution were added to each tube. Once the solvent had evaporated completely with the aid of a stream of nitrogen, 0.5 ml of plasma was added to each tube of the A series. Each tube was stirred and then subjected to the previously described sample treatment process. 100 μl of the mobile phase were added to each one of the tubes belonging to the B series and then the tubes were stirred. All of these samples were chromatographied and the peak areas obtained for both products in all of the tubes were recorded. The recuperation for each concentration was calculated by dividing the area obtained for each peak in the series A tubes by the area obtained in the series B tubes and then multiplying the result by 100. The mean value of recovery of the three concentrations was considered to be the recovery value of gemfibrozil. The extraction recovery of gemfibrozil was 90.15% (C.V. =

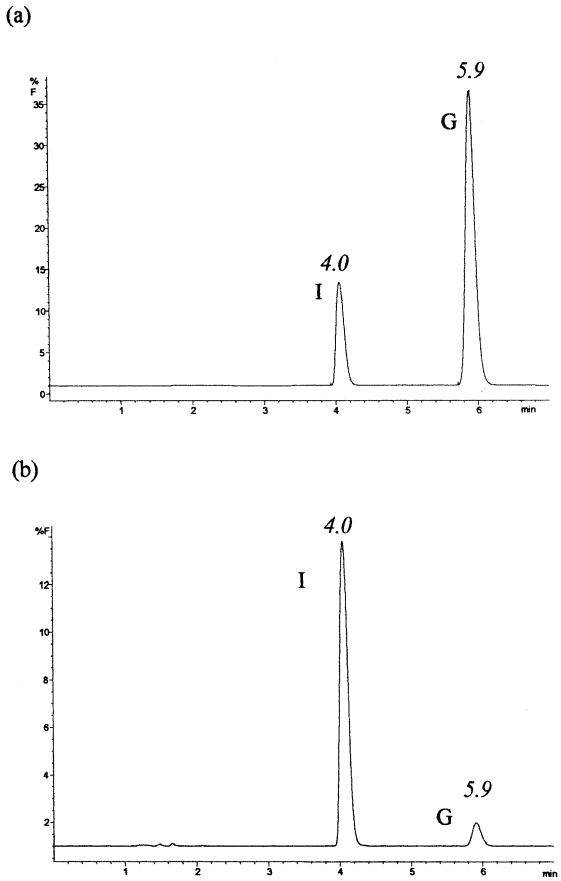

Fig. 2. Chromatograms resulting from the analysis of human plasma samples obtained at 2.5 (a) and 24 h (b) from a subject who received a single oral dose of gemfibrozil (600 mg). Ibuprofen: 4.0 min (I); Gemfibrozil: 5.9 min (G).

7.7%). Ibuprofen has very similar extraction (93.10%) and chromatographic behaviour when compared to that of gemfibrozil.

3.3. Selectivity of the assay

The resolution between gemfibrozil and I.S. was satisfactory as can be observed in Fig. 2.

The selectivity of the assay was examined in relation to interferences from endogenous substances in drug-free plasma from six different subjects without internal standard addition. Under the chromatographic conditions described, no endogenous sources of interference were observed in plasma. Fig. 3 shows these six superimposed chromatograms obtained from drug free-plasma.

A possible interference due to gemfibrozil metabolites was unlikely. Because of their increased polarity they were not readily extracted from plasma into cyclohexane (4). In order to exclude this, plasma samples obtained at 10 h after oral administration of gemfibrozil have been treated and chromatographied in a greater proportion of aqueous phase in the mobile phase (60%). Moreover, real plasma samples have been treated and chromatographied in the original conditions, without addition of internal standard, 1 month after being frozen. The appearance of interfering peaks with gemfibrozil or internal standard has not been observed in any of the cases.

3.4. Sensitivity of the assay

Detection limit (LOD) of the assay method was determined by the signal-to-noise ratio of decreasing and known concentrations of gemfibrozil. The LOD was considered to be the concentration of gemfibrozil in plasma that would give a peak of ~ 3 times the signal-to-noise ratio. The estimated LOD was 0.025 μg ml^{-1}. The quantification limit (LOQ) was calculated as 10 times the signal-to-noise ratio. The estimated LOQ was 0.05 μg ml^{-1}. This LOQ was confirmed for plasma, using calibrators with nominal concentration of 0.05 μg ml^{-1}. The mean assay result was 0.04 μg ml^{-1} ($n = 9$), with C.V. $< 16\%$.

3.5. Linearity of the assay

The assays exhibited linearity between the response (y) (peak-area ratio of gemfibrozil and the internal standard) and the corresponding concentration of gemfibrozil (x), over the 0.05–0.5 and 0.5–5 μg ml^{-1} range in plasma (mean equations: $y = 0.214x - 0.004$ for 0.05–0.5 μg ml^{-1} range, $y = 0.200x + 0.012$ for 0.5–5 μg ml^{-1} range), and required a cubic adjustment over the 5–30 μg ml^{-1} range ($y = 4.896x^3 - 4.555x^2 + 0.264x - 0.205$). The relationship between peak area ratio and gemfibrozil concentration is demonstrated by the correlation coefficients obtained for the calibrations curves that were 0.9999, 0.9999 and 1.000, respectively.

Moreover, a test comparing calculated standard points to the nominal ones was carried out on calibration curves in order to test the quality of the fitting. The assays exhibited linearity ($r > 0.999$), with a slope near to unit (0.9999) and an intercept not statistically different from zero.

3.6. Accuracy of the assay

Accuracy values in intra-day variation studies at low, medium and high concentrations of gemfibrozil in plasma were within acceptable limits ($< 15\%$ except for 0.05 μg ml^{-1} $< 20\%$) [7] at all concentrations (Table 1).

3.7. Precision of the method

In this work, precision of the method was tested both within-day and between-day variability in plasma.

3.7.1. Within-day variability of the assay

Within-day variability of the assay method was determined by repeated analysis of three quality control samples at 0.05, 0.5, 5 and 25 μg ml^{-1} concentrations on the same day. The results are shown in Table 1. This data indicates that the assay method is precise within the same day [7].

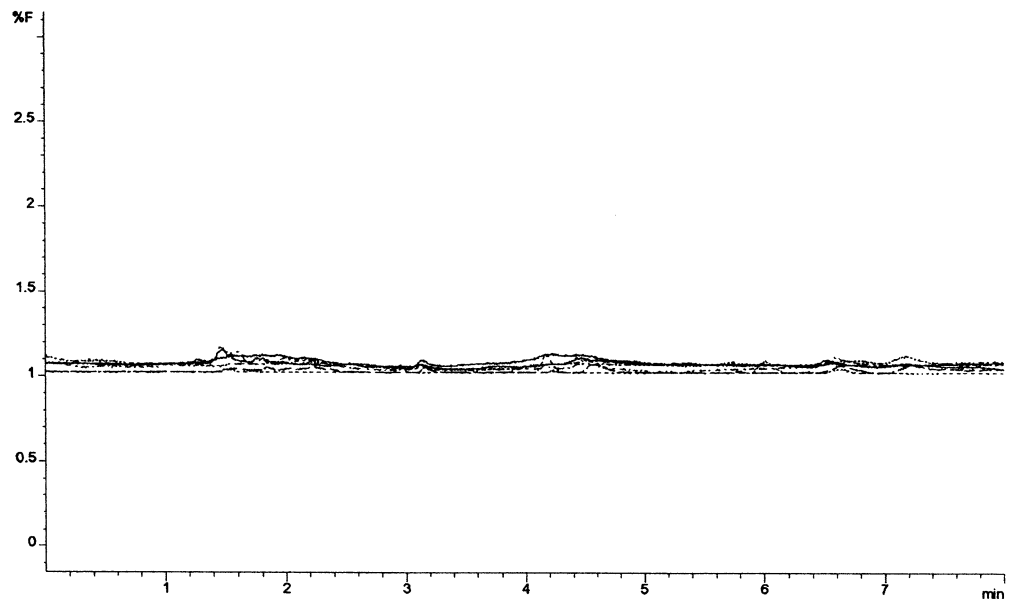

Fig. 3. Selectivity: six superimposed chromatograms obtained form drug-free plasma.

Table 1
Within and between-day precision of the HPLC method for determining gemfibrozil concentrations in plasma samples (A: Accuracy)

Concentration added (μg ml^{-1})	Within-day precision and accuracy ($n = 3$)			Between-day precision ($n = 9$)	
	Concentration found (mean ± S.D.) (μg ml^{-1})	C.V. (%)	A (%)	Concentration found (mean ± S.D.) (μg ml^{-1})	C.V. (%)
0.05	0.0403 ± 0.0006	1.5	19.4	0.0410 ± 0.006	15.9
0.5	0.52 ± 0.02	3.8	4.0	0.49 ± 0.07	13.7
5	4.86 ± 0.18	3.7	2.8	4.69 ± 0.19	4.0
25	22.99 ± 2.51	10.9	8.0	23.60 ± 0.63	11.1

Table 2
Stability of gemfibrozil during the analytical procedure

Concentration prepared (μg ml^{-1})	Concentration found $t = 0$ h (μg ml^{-1})	Concentration found $t = 24$ h (μg ml^{-1})	Accuracy (%)
0.5	0.46	0.44	4.34
5	5.07	4.80	5.32
25	25.05	25.48	1.72
In plasma samples at −20°C			
Plasma sample	Concentration found $t = 0$ days (μg ml^{-1})	Concentration found $t = 30$ days (μg ml^{-1})	
1	17.47	18.64	6.7
2	16.13	16.31	1.1
3	25.71	21.92	14.7

3.7.2. Between-day variability of the assay

Between-day variability of the assay method was determined in method validation by repeated analysis of three quality control samples at 0.05, 0.5, 5 and 25 μg ml^{-1} concentrations on three different days. The results are shown in Table 1. These data indicate that the assay method is precise within different days [7].

3.8. Stability of gemfibrozil

3.8.1. Stability of the analyte during the analytical procedure

Six samples of 0.5, 5 and 25 μg ml^{-1} of gemfibrozil in plasma were extracted. Of each concentration three samples were chromatographied immediately after being prepared and the remaining three, after having been stored for 24 h at room temperature in the injector of the chromatograph. The samples remained stable for at least this time, which was superior to the time that the real samples remained in the injector before being analyzed (Table 2).

3.9. Stability of the analyte in plasma

The bibliography [5] cites that plasma samples containing gemfibrozil can be stored at −18°C for at least 6 months without decomposition. In this study it has been proven that samples which have been analyzed and later frozen, maintained the same levels of gemfibrozil during at least 1 month, a time which was superior in all of the cases in which samples were maintained before being analyzed (Table 2).

4. Ruggedness of the method

For ruggedness studies, the influence of mobile phase (percentage of acetonitrile ranged from 60 to 45%) on the analytical procedure was evaluated. Modifications in the percentage of acetonitrile in the mobile phase significantly alter the gemfibrozil (4.7–14.6 min) and IS retention times (3.4–8.7 min).

4.1. Control of analytical method

During the analysis of gemfibrozil in real plasma samples, two plasma standard samples of the following concentrations: 0.5, 5 and 25 μg ml^{-1} were intercalated each day between real samples. The results for each concentration are presented in Table 3 and demonstrate that the method has been maintained precise and accurate during the time of the study.

5. Discussion

We have developed an specific HPLC method that is sensitive, with adequate precision and accuracy and suitable for the quantification of

Table 3
Control of analytical method (A: Accuracy)

Concentration added (μg ml^{-1})	Concentration found ($n = 13$) (mean ± S.D.) (μg ml^{-1})	C.V.(%)	A(%)
0.5	0.50 ± 0.04	8.8	0.5
5	4.62 ± 0.47	10.2	7.5
25	24.11 ± 2.39	9.9	3.5

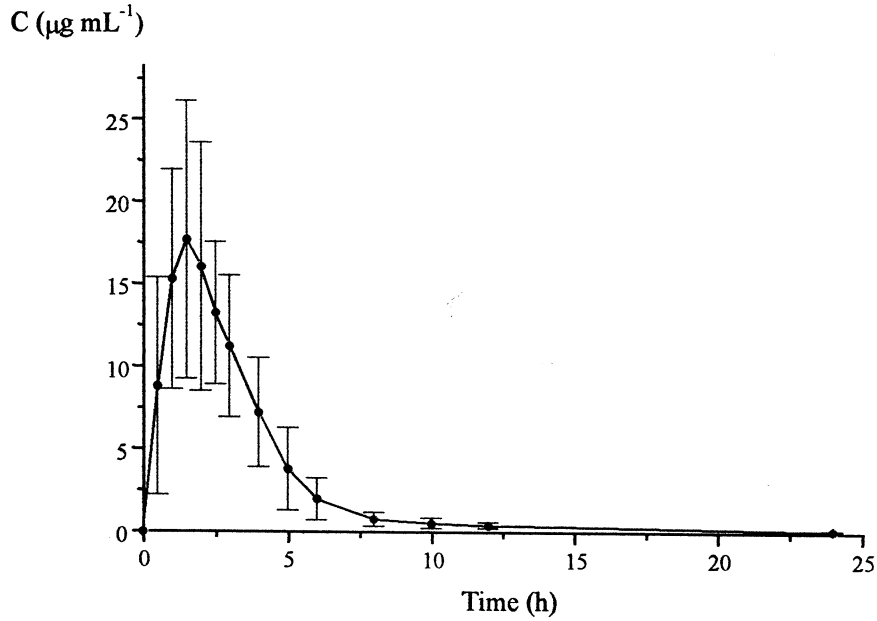

Fig. 4. Mean concentration–time curve of gemfibrozil.

gemfibrozil in human plasma. Sensitivity of this method was found to be better than previous published methods, thus permitting the determination of small concentrations of gemfibrozil as low as 25 ng ml^{-1}.

For the determination of gemfibrozil by HPLC, fluorescence detection was used because this compound was found to have fluorescence derived from the phenoxy moiety. This detection was more advantageous than UV detection in its stability of chromatographic baseline and selectivity for the compounds of interest in body fluids.

This method has been used extensively to study gemfibrozil pharmacokinetics in human volunteers. Fig. 4 shows the corresponding mean concentration–time curve of gemfibrozil and the mean pharmacokinetic parameters measured for gemfibrozil are indicated in Table 4.

Table 4
Mean pharmacokinetic parameters measured for gemfibrozil (Trialmin®)

AUC (mg h l^{-1})	63.98 ± 11.84
C_{max} (mg l^{-1})	24.55 ± 5.86
T_{max} (h)	1.67 ± 0.62

References

[1] E.J. Randinitis, A.W. Kinkel, C. Nelson, T.D. Parker III, J. Chromatogr. B 307 (1984) 210–215.
[2] B.C. Sallustio, B.A. Fairchild, J. Chromatogr. B 665 (1995) 345–353.
[3] C.M. Smith, A.M. Reynard, Textbook of Pharmacology. W.B Saunders, Philadelphia, PA, 1992, p. 608.
[4] A. Nakagawa, A. Shigeta, H. Iwabuchi, M. Horiguchi, K. Nakamura, H. Takahagi, Biomed. Chromatogr. 5 (2) (1991) 68–73.
[5] H. Hengy, E.U. Kölle, Arzneim.-Forsch./Drug Res. 35 (1985) 1637–1639.
[6] E.J. Randinitis, T.D. Parker III, A.W. Kinkel, J. Chromatogr. 383 (1986) 444–448.
[7] F. Bressolle, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3–10.