Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 827 (2005) 165–172
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Verapamil quantification in human plasma by liquid chromatography coupled to tandem mass spectrometry
An application for bioequivalence study

Ney Carter do C. Borges [a], Gustavo D. Mendes [b,c], Rafael E. Barrientos-Astigarraga [b], Paulo Galvinas [b], Celso H. Oliveira [b], Gilberto De Nucci [a,b,c,*]

[a] *Department of Pharmacology, State University of Campinas, P.O. Box 6111, Campinas, SP, Brazil*
[b] *Cartesius Development of Clinical Research, Jesuino Marcondes Machado Ave. 415, 13092-320 Campinas/SP, Brazil*
[c] *Department of Internal Medicine, State University of Campinas, Campinas/SP, Brazil*

Received 25 November 2003; accepted 6 July 2005
Available online 3 October 2005

Abstract

An analytical method based on liquid chromatography with positive ion electrospray ionization (ESI) coupled to tandem mass spectrometry detection (LC–MS/MS) was developed for the determination of Verapamil in human plasma using Metoprolol as the internal standard. The analyte and internal standard were extracted from the plasma samples by liquid–liquid extraction and chromatographed on a C_8 analytical column. The mobile phase consisted of methanol–water (70:30; v/v) + 12 mM formic acid. The method had a chromatographic total run time of 3.5 min and was linear within the range 1.00–500 ng/mL. Detection was carried out on a Micromass Quattro Ultima tandem mass spectrometer by multiple reaction monitoring (MRM). The intra-run imprecision was less than 5.1% calculated from the quality control (QC) samples, and 16.3% from the limit of quantification (LOQ). The accuracy determined from QC samples were between 92.9 and 103.1%, and 95.2 and 115.3% from LOQ. Concerning the inter-batch analysis, the imprecision was less than 5.8% and 17.3% from QC samples and LOQ, respectively. The accuracy varied between 98.2 and 100.8% from QC and it was 103.1% from LOQ. The protocol herein described was employed in a bioequivalence study of two tablet formulations of Verapamil.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Verapamil; Bioequivalence; LC–MS/MS; Metoprolol

1. Introduction

Verapamil, benzenacetonitrile, (±)-(alpha)[3-[[2-(3,4-dimethoxyphenyl)ethyl]methylamino]propyl]-3,4-dimethoxy-(alpha)-(1-methylethyl) monohydrochloride is a calcium ion influx inhibitor (slow channel blocker or calcium ion antagonist) [1–2], and it has demonstrated to be effective in the treatment of angina, arrhythmia, essential hypertension, certain cardiomyopathies and recently modifying agent in tumors which express P-glycoprotein [3–5]. Verapamil is administered as a racemic mixture of the *R* and *S* enantiomers and is not chemically related to other cardioactive drugs. The empirical formula is $C_{27}H_{38}N_2O_4$ HCl with a molecular mass is 491.08 g/mol [1–2]. Verapamil has been determined in plasma and other biological fluids using several types of analytical techniques such as gas chromatography–mass spectrometry (MS) [6], mass fragmentography [7], high-pressure liquid chromatography (HPLC) coupled to fluorimetric detection [8–12], high-pressure liquid chromatography coupled to ultra-violet detection [14–17], liquid chromatography coupled to mass spectrometry with electrospray ionization (LC–ESI-MS) [13,18], chiral high-pressure liquid chromatography coupled mass spectrometry–mass spectrometry with API ionisa-

* Corresponding author. Tel.: +55 19 3251 6923; fax: +55 19 3252 1516.
E-mail address: denucci@dglnet.com.br (G. De Nucci).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.07.012

tion (HPLC–API-MS-MS) [19], In tube SPME–liquid chromatography coupled mass spectrometry with electrospray ionization (SPME–LC–ESI-MS) [20]), liquid chromatography coupled mass spectrometry-mass spectrometry with RAM and MSPD [21], liquid chromatography coupled mass spectrometry-mass spectrometry (LC–MS-MS) [22,27], molecularly imprinted polymer coupled liquid chromatography–mass spectrometry (MIP–LC–MS) [23].

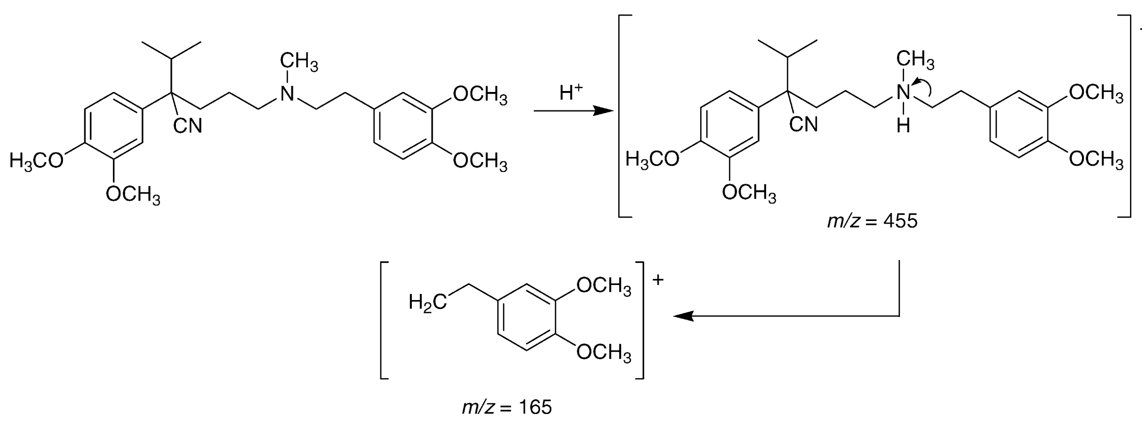
Walles et al. [22], using solid phase extraction in HPLC–MS-MS, observed a LOD 0.5–2.5 ng/mL in cell cultures, however the RT was between (15.3–49.8 min).

In this work, we describe a rapid, sensitive and selective high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS), method for the quantitation of Verapamil using Metoprolol as the internal standard (Fig. 1). The method was developed for a study of bioequivalence of two formulations of Verapamil (80 mg tablet formulation tablet from Apotex do Brasil Ltda. as test formulation and Dilacoron® from Knoll Produtos Químicos e Farmacêuticos Ltda. as reference formulation).

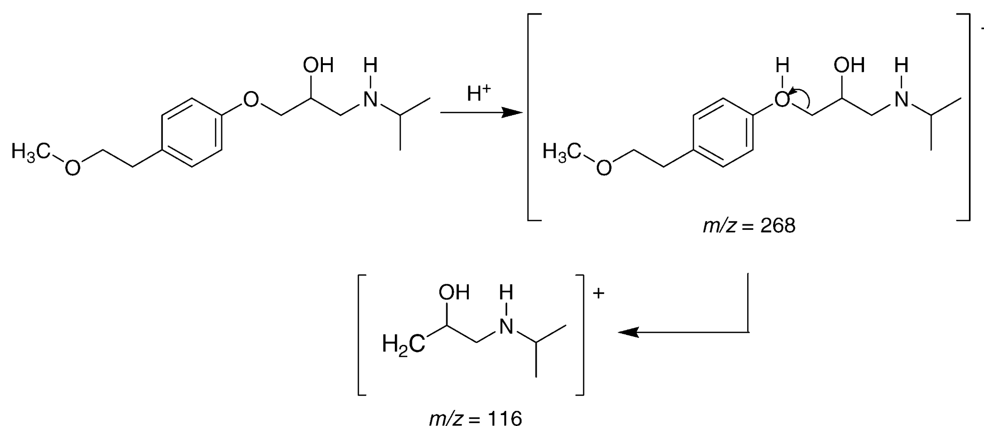
2. Experimental

2.1. Chemicals and reagents

Verapamil hydrochloride (lot CG0960, 99.9%) was provided by Apotex Ltda., Brazil, and metoprolol tartarate (lot 139777, 100%) was a generous gift from Novartis, Brazil. Methanol (HPLC-grade) and diethyl-ether (analytical-grade) were purchased from Mallinckrodt (Paris, KY, USA). Formic acid (88%, analytical-grade) was purchased from Merck (Rio de Janeiro, RJ, Brazil). Ultra-pure water was obtained from an Elga UHQ system (Elga, UK). Blank human blood was collected from healthy, drug-free volunteers. Human plasma was obtained by centrifugation of blood treated with



(a) Proposed fragmentation pathway for Verapamil ($m/z = 455 > 165$).



(b) Proposed fragmentation pathway for Metoprolol ($m/z = 268 > 116$).

Fig. 1. Chemical structures and fragmentation pathways proposed for Verapamil (a) and Metoprolol, the internal standard (b).

the anticoagulant sodium heparin. Pooled human plasma was prepared and stored at approximately −20 °C until needed.

2.2. Calibration standards and quality control

The master solutions prepared at 1000 μg/mL for the Verapamil and Metoprolol were prepared in methanol–water (50:50 v/v). Calibration curves of Verapamil were prepared by spiking the pooled blank human plasma at concentrations of 1.00, 2.00, 5.00, 10.0, 20.0, 50.0, 100, 500 ng/mL and the analysis was carried out in duplicate for each concentration. The quality control samples were prepared in pooled blank human plasma at concentrations of 3.0, 40.0 and 400 ng/mL (QCA, QCB and QCC, respectively). The spiked human plasma samples (standards and quality controls) were extracted on each analytical batch along with the unknown samples.

2.3. Sample preparation

All frozen human plasma samples were previously thawed at ambient temperature and centrifuged at 2550 × *g* for 5 min at 8 °C to precipitate solids. 50 μL of the internal standard solution (1 μg/mL Metoprolol in 50/50; v/v methanol–water solution) were added to a 200 μL aliquot of plasma sample. The tubes were briefly vortex-mixed and the compounds of interest were extracted with 4 mL of diethyl-ether. The mixture was vortex-mixed for approximately 40 s, and the organic phase was removed and evaporated under N_2 at 40 °C. The dry residues were reconstituted to 200 μL with a solution of mobile phase and vortex-mixed for 10 s. The solutions were then transferred to the appropriate 96-well plates and placed them into the auto-injector racks.

2.4. Chromatographic conditions

We used a liquid chromatograph Shimadzu Corporation (Model LC-10AD) and auto injector CTC Analytics (MXY01-01B). An aliquot (20 μL) of each plasma extract was injected into a guard column Alltech Prevail C_8 analytical column, 5 μm (7.5 mm × 4.6 mm I.D.) and Alltech Prevail C_8 analytical column, 5 μm (150 mm × 4.6 mm I.D.) operating at room temperature. The compounds were eluted by pumping the mobile phase (methanol/water; 70/30; v/v + 12 mM formic acid) at a flow-rate of 1.25 mL/min.

Under these conditions, typical standard retention times were 2.3 min for Verapamil and 2.0 min for Metoprolol, and back-pressure values of approximately 105 bar were observed. The split ratio selection was performed so as to make detection response ideal. The evaluated ratios were 1:2, 1:5, 1:10, 1:15 and 1:20. Based on the results, it was observed that the split of the column eluant of approximately 1:15, which allows an entrance volume of 83 μL/min into the mass spectrometer, was the most suitable one. The temperature of the autosampler was kept at 8 °C, the injection volume was 10 μL and the total run-time was 3.5 min.

2.5. Mass-spectrometric conditions

The mass spectrometer (Micromass model Quattro Ultima) equipped with an electrospray ionization source running in positive mode (ES^+), was set up in multiple reaction monitoring (MRM), monitoring the transitions 268.5 > 116.4 455.4 > 165.2, for Metoprolol and Verapamil, respectively. Fig. 2 shows the full scan spectra (upper trace) and the product ion spectra (lower trace) obtained for Metoprolol (panel A) and Verapamil (panel B). In order to optimize all the MS parameters, a standard solution of the analyte and I.S. was infused into the mass spectrometer. For both Verapamil and Metoprolol, the following optimized parameters were obtained: the dwell time and the collision gas 23 pressure (argon) were 0.2 s and 0.20 s × 1.0×10^{-3} mbar, respectively. The cone voltage and the collision energy were 60 V and 25 eV for Verapamil and 17 V and 45 eV for Metoprolol, respectively. Data acquisition and analysis were carried out using the software MassLynx (v 3.5) running under Windows NT (v 4.0) on a Pentium III PC.

2.6. Linearity

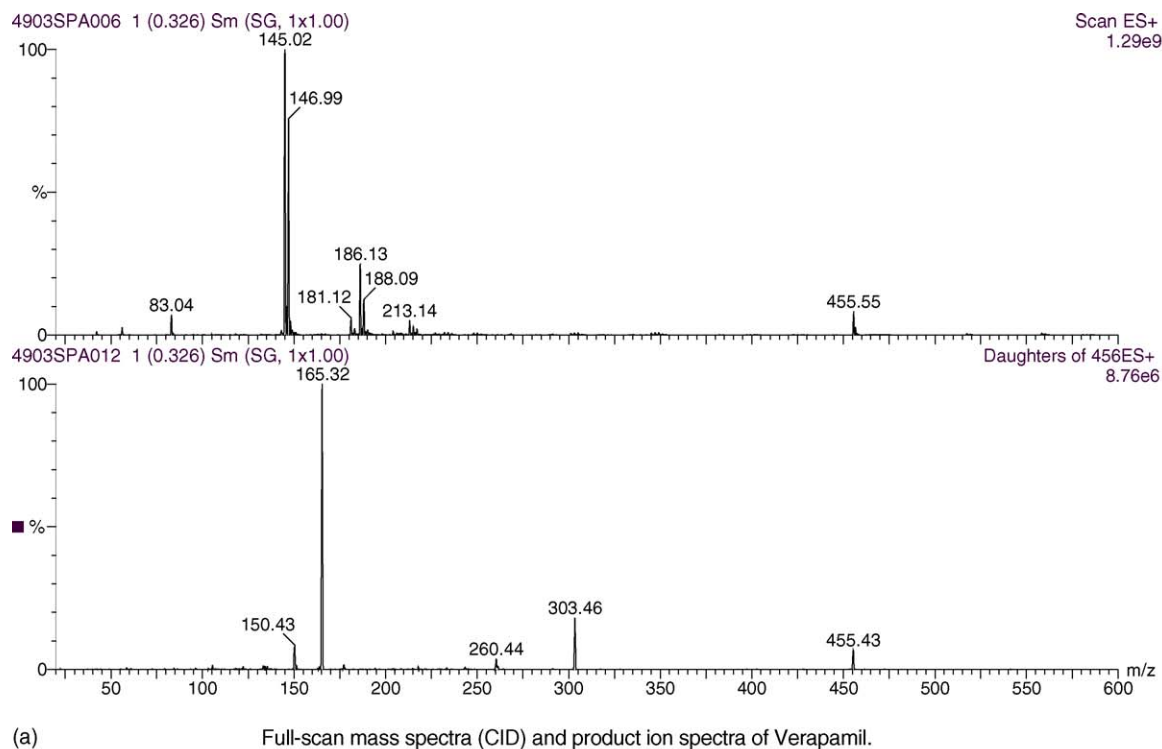
Linearity was determined to assess the performance of the method. A linear least-squares regression with a weighting index of $1/x^2$ was performed on the peak area ratios of Verapamil/Metoprolol versus Verapamil nominal concentrations of the eight plasma standards (1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0 and 500.0 ng/mL) in duplicate to generate a calibration curve.

2.7. Stability

Stability quality control plasma samples (3.00, 40.0 and 400 ng/mL) were subjected to short-term (6 h) room temperature, three freeze-and-thaw (−20 to 25 °C) cycles and to 62.5 h autosampler (8 °C) stability tests. Subsequently, the Verapamil concentrations were measured compared to freshly prepared samples.

2.8. Recovery

The recovery was evaluated by calculating the mean of the response of each concentration and dividing the extracted sample mean by the unextracted (spiked blank plasma extract) sample mean of the corresponding concentration. The comparison between the unextracted samples, spiked on plasma residues and the extracted sample was done in order to eliminate matrix effects, giving a true recovery. The matrix effect experiments were carried out using the ratio between spiked mobile phase solutions and unextracted samples, spiked on plasma residues.



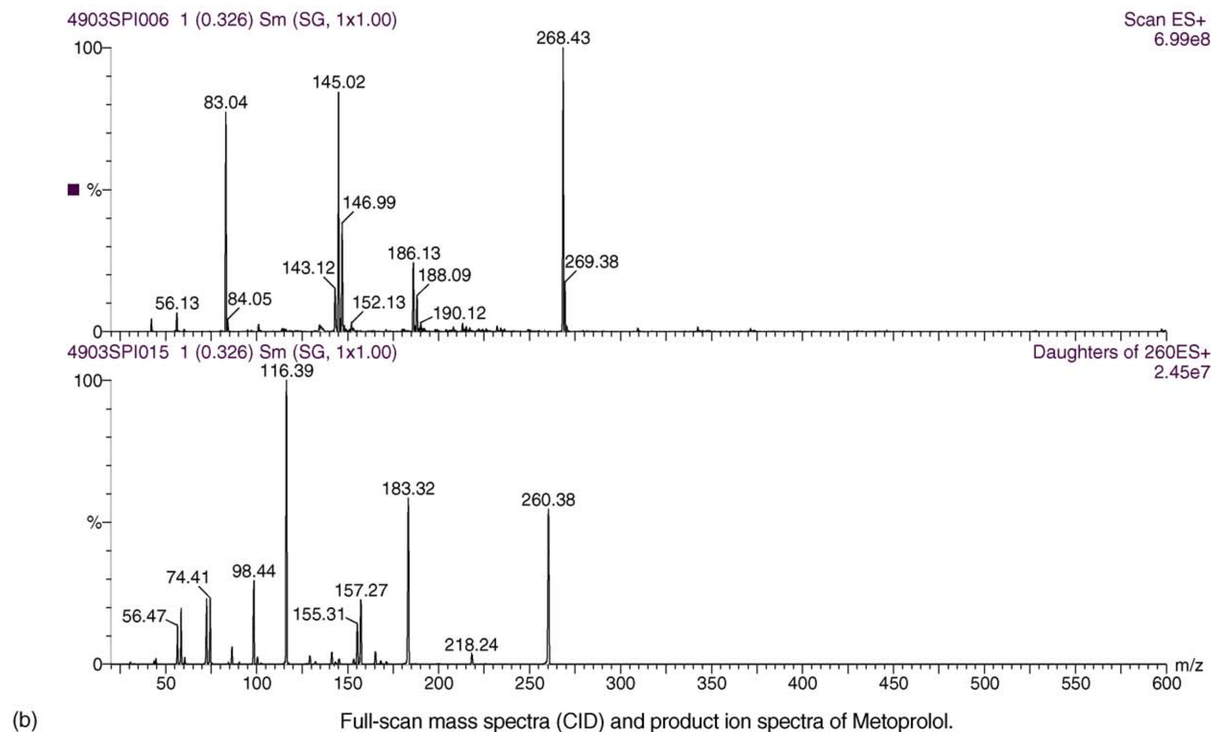


Fig. 2. Full scan mass spectra in upper trace and product ion spectra in lower trace of (a) Verapamil and (b) Metoprolol.

2.9. Ion supression

Supression of the MS signal ("ion supression") can be caused by contaminants (e.g. salts) in the LC eluant entering the MS. Thus, a non-specific extraction procedure may produce ion suppression that could interfere with the analysis of the samples. The effect of the sample preparation method (for the matrix that is being analyzed) on the vari-

ability of the electrospray ionization response should be determined.

To assess the effect of ion suppression on the MS/MS signal of the analyte, Verapamil, and the internal standard, Metoprolol, was evaluated. The experimental consisted of an infusion pump connected to the system by a "zero volume tee" before the split and the HPLC system pumping the mobile phase, which was the same as that used in the routine analysis of Verapamil, i.e. methanol/water (70/30; v/v) + 12 mM formic acid at 1.25 mL/min. The infusion pump was set to transfer (50 μL/min) of a mixture of analyte and internal standard in mobile phase (both 50 ng/mL). A sample of human pooled blank plasma sample was extracted. The reconstituted extract was injected into the HPLC system while the standard mixture was being infused. In this system any ion suppression would be observed as a depression of the MS signal.

2.10. Bioequivalence study

The method was applied to evaluate the bioequivalence of two tablets formulations of Verapamil in healthy volunteers: Verapamil (test formulation from Apotex do Brasil Ltda.; lot no. GF 5927 expiry date September 2004) and Dilacoron®—80 mg tablet (standard reference formulation from Knoll Produtos Químicos e Farmacêuticos Ltda.; lot no. 910098 F 02 expiry date January 2006). The study consisted of an open study of 35 healthy volunteers. After screening and wash-out period (of at least 2 weeks), the individuals who qualified were confined for two periods of approximately 30 h. Each confinement was intervaled by a period of 1 week. Study schedule pre-study period: medical history, general physical examination, electrocardiogram, clinical laboratory examination, confined period: a 4 mL blood

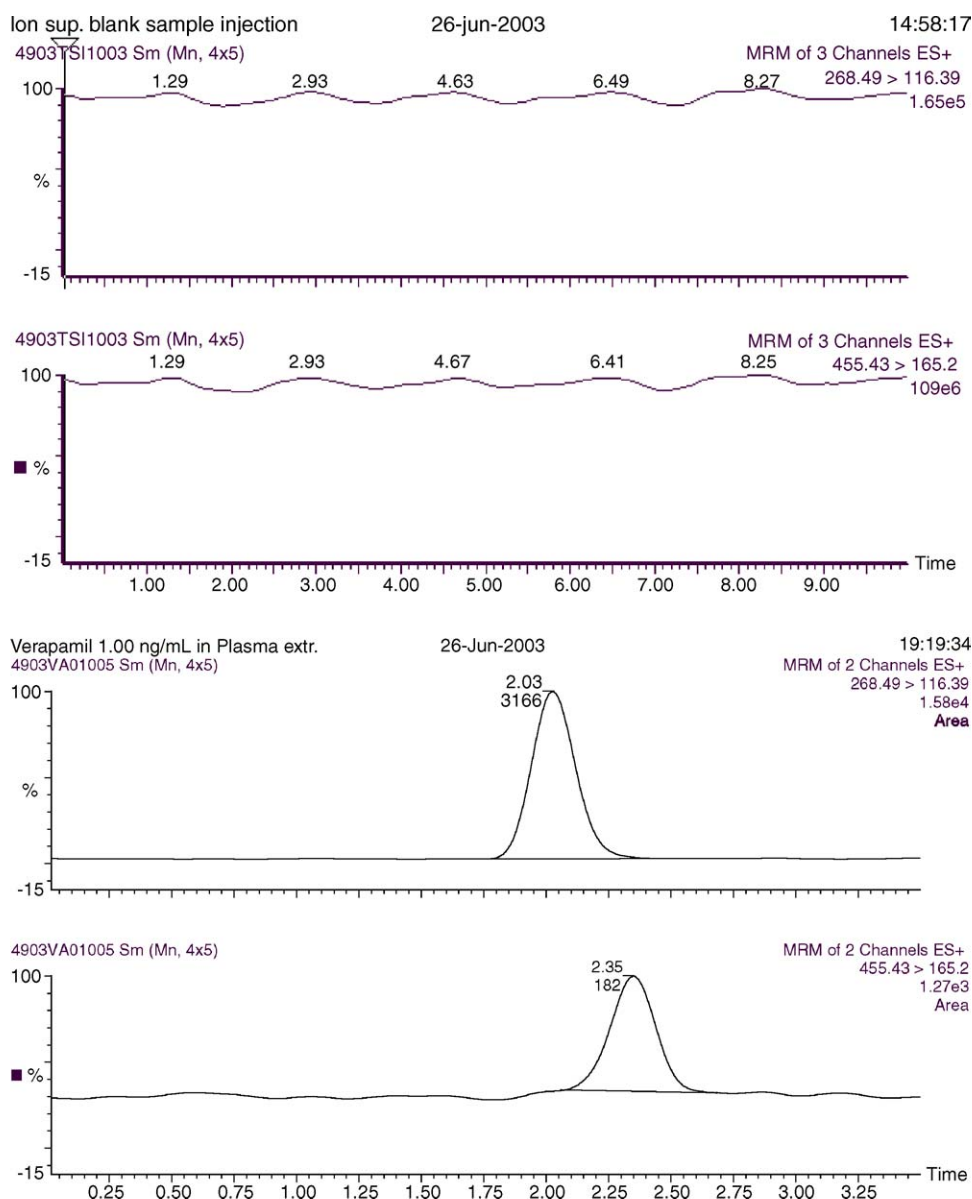


Fig. 3. Ion suppression experiment (baseline profile after blank plasma extract injection and standard peak at the LOQ level).

sample was collected before dosing and 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 10 and 12 h post-dosing. Post-study period: general physical examination, electrocardiogram, clinical laboratory examination.

3. Results

As shown in Fig. 1 the proposed fragmentation route for Verapamil. The method was linear for Verapamil into the range from 1.00 to 500 ng/mL (calibration curve $0.0565320x + 0.00281062$, $r^2 > 0.998151$). A linear least-squares regression with a weighting index of $1/x^2$ was carried out on the peak area ratios of Verapamil/Metoprolol versus Verapamil concentrations of the eight plasma standards (1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0 and 500.0 ng/mL) in duplicate to generate a calibration curve. In the case of Verapamil and their internal standard, Metoprolol, there was no significant ion suppression in the region where the analyte and internal standard eluted (Fig. 3). As we can see in Fig. 4, no endogenous peak was observed in the mass chromatogram of blank plasma. The chromatogram for the standard limit of quantification (LOQ) sample is shown in Fig. 5, in which the retention times for both the internal standard and Verapamil were 2.0 and 2.3 min, respectively can be observed.

The recoveries observed (value ± C.V.%, $n = 5$) were 94 ± 3, 91 ± 13 and 96 ± 2.7% (3.00, 40.0 and 400 ng/mL, respectively) for Verapamil, and 93 ± 5% for the I.S. (180 ng/mL). No significant (i.e. less than 10%) matrix effect was observed. The lower limit of quantification, defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was 1.00 ng/mL. Table 1 shows the between-run calibration quality data, LOQ, accuracy and precision.

Table 1
Data for quantified concentration (ng/mL) of individual QC samples and LOQ for intra-batch and inter-batch validation

Nominal concentration (ng mL^{-1})	LOQ	3.00	40.0	400
Intra-batch				
Mean	0.950	3.05	41.2	407
Accuracy (%)	95.2	101.5	103.1	101.8
Precision (C.V.%)	15.0	5.1	4.4	2.7
Inter-batch				
Mean	1.03	2.98	40.3	393
Accuracy (%)	103.1	99.2	100.8	98.2
Precision (C.V.%)	17.3	5.8	4.4	4.8

Table 2
Mean pharmacokinetic parameters obtained from 35 volunteers after administration of each 80 mg Verapamil tablet formulation

	Dilacoron®		Verapamil	
AUC_{last} ((ng h)/mL)	207.9	88.0	232.1	99.9
AUC_{inf} ((ng h)/mL)	231.3	99.2	260.4	112.0
AUC_{all} (0-12h) ((ng h)/mL)	208.9	88.4	233.2	100.3
C_{max} (ng/mL)	66.3	35.4	73.8	40.9
$T_{1/2}$(h)	3.83	0.80	3.91	1.02
T_{max} (h)—median	0.75[a]	0.50–2.50[b]	0.75[a]	0.50–2.00[b]

[a] Median.
[b] Range.

Stability analysis was carried out with plasma quality control samples (3.0, 40 and 400 ng/mL). All samples showed no significant degradation under the conditions previously described in Section 2.

The mean Verapamil plasma concentrations versus time profiles after a single oral dose of each 80 mg tablet formulation of Verapamil is shown in Fig. 6. Table 2 shows the mean pharmacokinetics parameters obtained from 35 volunteers after the administration of 80 mg Verapamil tablet.

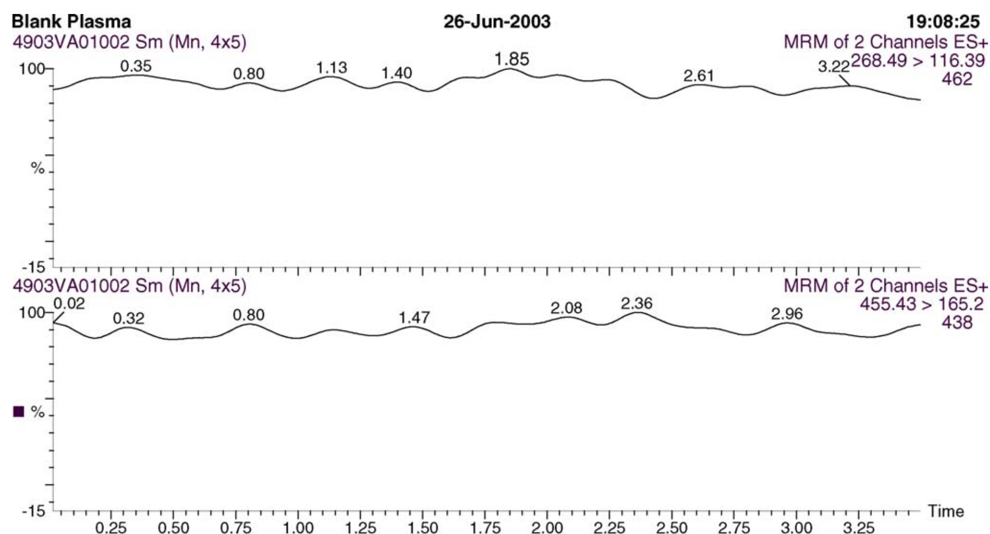


Fig. 4. MRM chromatograms of blank normal pooled human plasma: Metoprololol and Verapamil.

Fig. 5. (A) MRM chromatogram of the LOQ sample (1.00 ng/mL) and (B) a representative volunteer samples.

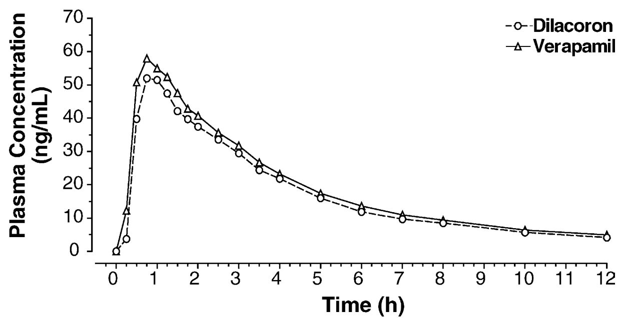


Fig. 6. Mean plasma concentrations versus time curve for two Verapamil tablet formulations ($n = 35$).

4. Discussion

Although it is well known that Verapamil and Metoprolol are not stable at low pH, no perceivable degradation of the analyte and I.S. was observed under the described liquid chromatographic conditions. The fact that the mobile phase contained a low amount of formic acid did not interfere with the analysis, since the total run time (3.5 min), under these condition of acidity, was not long enough to cause significant degradation. Kolbah and Zavitsanos [19], described in plasma using chiral HPLC–API-MS-MS employed simple liquid–liquid extraction the LOQ 1 ng/mL, the retention

time of Verapamil was longer than our method (6.1 min) as expected for chiral separation.

The method (LC–MS/MS), herein presented is less sensitive than many other available assays. On the other hand one should say that it is simple and straightforward. However, this level of sensitivity was sufficient to perform the bioequivalence study. The LC–ESI-MS method described by Richter et al. [13] has showed a LOQ of 0.500 ng/mL, however it has a longer retention time 12.9 min and that method employs a solid phase extraction, which one require SPE cartridges becoming more expensive, laborious than liquid–liquid extraction, as employed in our method.

Recently Hedeland et al. [27], using LC–MS/MS method with liquid–liquid extraction in human plasma has demonstrated a sensitivity (LOQ of 50 pg/mL), but the retention time of Verapamil (RT—15 min) was extremely higher than our method (RT—2.3 min).

It is worth say that in current pharmacokinetics studies, it is very desirable to increase the throughtput having shorter analytical run-times [27]. As demonstrated in this assay, this method is perfectly suitable for a high-throughput routine, such as in a bioequivalence study.

After the oral administration of the Verapamil tablets to the volunteers, the observed Verapamil peak plasma concentration (C_{max}) values and the time values taken to be achieved (T_{max}) were similar to those reported in the literature [9] and equivalent between the formulations (Fig. 6; Table 2). In addition, the calculated 90% CI for mean C_{max}, AUC_{last} and $AUC_{0\text{-}inf}$ Verapamil/Dilacoron individual ratios were within the 80–125% interval defined by the US Food and Drug Administration [25].

Verapamil is commonly regarded as drug with highly variable pharmacokinetics [26] primarily due to its high first pass metabolism [24]. For this reason, it was required to employ a higher number of volunteers ($n = 35$) in order to assure proper statistical power.

5. Conclusion

The HPLC/MS/MS method described here for Verapamil quantification in human plasma agrees with the concepts of high sensitivity, specificity, high samples throughput and straightforward required for pharmacokinetic assays such as bioequivalence studies.

References

[1] A. Fleckenstein, Circ. Res. 52 (1983) I3.
[2] J.E.F. Reynolds, The Extra Pharmcopeia, 29th ed., 1989, p. 89.
[3] D. Kang, D. Verotta, M.E. Krecic-Shepard, N.B. Modi, S.K. Grupta, J.B. Schwartaz, Clin. Pharmacol. Ther. 73 (2003) 31.
[4] H. Thomas, H.M. Coley, Cancer Control 10 (2003) 159.
[5] J.P. Tassin, J. Dubois, G. Atassi, M. Hanocq, J. Chromatogr. B. 691 (1997) 449.
[6] P.A. Hynning, P. Anderson, U. Bonodesson, L.O. Boréus, Clin. Chem. 34 (1988) 2502.
[7] B. Spiegelhalder, M. Eichelbaum, Arzneimitettel-forschung 27 (1977) 94.
[8] M.N. Muscara, G. De Nucci, Braz. J. Med. Biol. Res. 26 (1993) 753.
[9] A. Ceccato, P. Chiap, P. Hubert, B. Toussaint, J. Crommen, J. Chormatogr. A 750 (1996) 351.
[10] Y.C. Tsang, R. Pop, P. Gordon, J. Hems, M. Spino, Pharm. Res. 13 (1996) 846.
[11] C. Horne, G. Stenzhorn, H. Blume, H. Knauf, E. Mustscheler, Arch. Pharm. 325 (1992) 531.
[12] Z.B. Salama, C. Dilger, W. Czogalla, R. Otto, H. Jaeger, J. Chormatogr. B Arzneimitettel-forschung 39 (1989) 210.
[13] O.V. Richter, M. Eichelbaum, F. Schönberg, U. Hofmann, J. Chromatogr. B 738 (2000) 137.
[14] Y. Long, J. Feng, S. Tong, Zhongguo Yiyao Gongye Zazhi. 24 (1993) 267.
[15] O.H. Drummer, S. Horomidis, S. Kourtis, M.L. Syrjanen, P. Tippet, J. Anal. Toxicol. 18 (1994) 134.
[16] H. Soini, M.L. Riekkala, M.V. Novotny, J. Chormatogr. 608 (1992) 265.
[17] M.A. Garcia, J.J. Aramayana, M.A. Bregante, L.J. Fraile, C. Solans, J. Chormatogr. B 693 (1997) 337.
[18] J.M. Dethy, B.L. Ackermann, C. Delatour, J.D. Henion, G.A. Schultz, Anal. Chem. 75 (2003) 805.
[19] T.A. Kolbah, A.P. Zavitsanos, J. Chromatogr. A 759 (1997) 65.
[20] M. Walles, W.M. Mullett, K. Levsen, J. Borlak, G. Wunsch, J. Pawliszyn, J. Pharm. Biomed. Anal. 30 (2002) 307.
[21] M. Walles, J. Borlak, K. Levsen, Anal. Bioanal. Chem. 347 (2002) 1179.
[22] M. Walles, T. Thum, K. Levsen, J. Borlak, J. Chromatogr. B 798 (2003) 265.
[23] W.M. Mullett, M. Walles, K. Levsen, J. Borlak, J. Pawliszyn, J. Chromatogr. B 801 (2004) 297.
[24] M. Eichelbaum, M. Ende, G. Remberg, M. Schomerus, H.J. Dengler, Drug Metab. Dispos. 7 (1979) 145.
[25] Food and Drug Administration, Fed. Reg. 63 (1998) 64222.
[26] H.H. Blume, B. Scheidel, M. Siewart, Proc. Bio-Int. 92 (1993) 44.
[27] M. Hedeland, E. Fredriksson, H. Lennernas, U. Bondesson, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 804 (2004) 303.