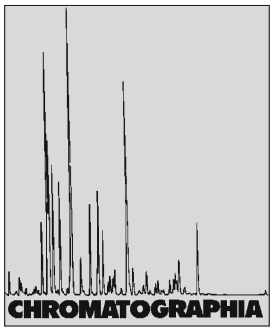
A Validated RP–LC Method for Simultaneous Determination of Losartan Potassium and Amlodipine Besilate in Pharmaceutical Preparations



2009, *69*, S201–S206

Carlos F. Pedroso✉, Julcemara G. de Oliveira, Francinete R. Campos, Alan G. Gonçalves, Angela C. L. B. Trindade, Roberto Pontarolo

Departamento de Farmácia, Universidade Federal do Paraná, Curitiba, Paraná, Brazil; E-Mail: cpedroso@ufpr.br; pontarolo@ufpr.br

Received: 30 September 2008 / Revised: 1 December 2008 / Accepted: 7 January 2009
Online publication: 5 February 2009

Abstract

A simple RP–LC method for simultaneous quantification of losartan and amlodipine and separation of their degradation products has been developed. For this purpose we tested appropriated mobile phase pH range, flow rate, temperature and different columns. The method was validated with an ODS column. A gradient of acetonitrile and phosphate pH 3.0 buffer was utilized as mobile phase. The linearity was determined at 50–150% level. Individual recoveries at 70–130% level ranged from 98.8 to 100.5% for losartan and 96.4–101.2% for amlodipine. The robustness was also evaluated. Although losartan has much higher quantities than amlodipine in commercial tablets, this method allowed simultaneous quantification for both drugs.

Keywords

Column liquid chromatography
Losartan potassium
Amlodipine besilate
Method validation

Introduction

Amlodipine besilate, 3-ethyl 5-methyl (4*RS*)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzenesulphonate [1] is a potent calcium channel antagonist used for the treatment of hypertension [2]. LC–UV have been performed for raw material [1] and for combination with a second drug in commercial tablets [3–5]. Pharmacokinetic studies employing liquid chromatography coupled to tandem mass spectrometry [6, 7], UV detection [8] or fluorescence detection [9] has also been described.

Losartan, monopotassium salt of 2-butyl-4-chloro-1-[*p*-(*o*-1*H*-tetrazol-5-yl-phenyl)benzyl]imidazole-5-methanol is a non-peptide AT_1 receptor antagonist utilized for the treatment of hypertension [10]. A number of analytical methods have been related in literature for losartan determination. For raw material [11] and commercial tablets [12, 13] LC–UV has been commonly used. In the simultaneous determination of losartan and its degradation products techniques such as LC–MS–MS [14], RP–TLC [15] have been reported. Williams and coworkers [16] demonstrated comparative analyses using LC, CE and SFC. LC quantification of the combined losartan and hydrochlorothiazide have also been related [12, 17, 18]. Determination of losartan in plasma has been conduct by use of LC–UV [19, 20] and with fluorescence detection [21–23].

Monotherapy for control of blood pressure has been shown to be inefficient; in this way, combination drug therapy has become a clinical routine practice [24, 25]. Furthermore, the development of drug combinations at the same pharmaceutical preparation is widespread in pharmaceutical industry [26].

Separation Analysis Applied to Pharmaceutical Sciences in Brazil.

Combination of losartan and amlodipine at the same pharmaceutical preparation (100/5.0 and 50/2.5 mg, respectively) have been used for better therapy compliance. However, there is no available LC method in the literature for simultaneous quantification of these two drugs in the same chromatographic separation. This kind of separation is not readily performed because of the distinct losartan and amlodipine acid-base behavior and the high difference in the amount between them in the available commercial tablets.

Here we described the development and validation of RP–LC with DAD detection for the simultaneous quantification of losartan and amlodipine, and separation of their major degradation products in commercial preparations.

Experimental

Chemicals and Reagents

Losartan potassium salt and amlodipine besilate were purchased from United States Pharmacopoeia Reference Standards. Pharmaceutical preparation containing 50/2.5 and 100/5.0 mg of losartan and amlodipine were manufactured by Biosintética Farmacêutica. Placebos were prepared in the laboratory using United Stated Pharmacopoeia grade excipients. Acetonitrile and methanol (LC grade), sodium dihydrogen phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$), phosphoric acid 85%, DMSO-d_6 were purchased from Merck (Darmstadt, Germany); ammonium acetate crystal, ammonium formate 97%, formic acid 88% and acetic acid glacial from J.T. Baker (Mexico); TMS from Aldrich (Germany). For sample dilution a mixture of acetonitrile and 10 mM phosphate buffer solution at pH 3.0 (50:50 *v*/*v*) was prepared.

Working Standard Solution, Sample Preparation

The content of 20 capsules were crushed and mixed to homogeneous powder and the mass equivalent of one capsule was weighed and transferred to a 50 or 100 mL volumetric flask, dissolved in diluent and sonicated for 10 min. The resulting solution was filtered through a Millipore Millex PVDF 0.45 μm filter and diluted with diluent to the final concentration of 40 and 2.84 μg mL^{-1} for losartan and amlodipine, respectively.

Chromatography

Chromatographic separations were performed on an Agilent 1100 LC system, consisting of G1311A quaternary pump, a G1379A degasser, G1329A automatic injector, G1315B DAD (Germany), and Chemistation Rev. A. 10.02[1757] software. The following columns were adapted to the equipment for this study: XBridge C18 (150 × 4.6 mm I.D., 5 μm particle size), Spherisorb ODS2 C18 (150 × 4.6 mm, 5 μm), Symmetry C18 (150 × 3.9 mm, 5 μm) purchased from Waters Corporation (Ireland). Zorbax Eclipse C18 XDB column (150 × 4.6 mm, 5 μm) was purchased from Agilent Technologies (USA) and Microsorb-MV C18 110-5 (150 × 4.6 mm, 5 μm) from Varian (USA). The SPE OASIS HLB 1 cc used was purchased from Waters (USA).

For method development, mixtures of phosphate, fomate and acetate buffers in different concentrations (5–30 mM) and pH ranges (3.0–7.0) were tested. Gradients were performed with acetonitrile or methanol with different gradient ramps. Varying of column temperatures (20–50 °C), and types and manufactures of ODS packing were also evaluated. Chromatograms were then analyzed and compared for a better selection of chromatographic and time efficiency.

The selected conditions for method validation were as follows: mobile phase with phosphate buffer solution 10 mM pH 3.0 (channel A) and acetonitrile (channel B) in a linear gradient from A to B (65:35, *v*/*v*) in 10 min A–B (35:65, *v*/*v*) to A–B in 15 min (65:35, *v*/*v*) and 5 min for column re-equilibration. The flow rate was 1 mL min^{-1} with 20 μL being injected. The UV detection was performed at 237 nm on an XBridge column.

Analytes and Degradation Products Identification

The solution containing losartan, amlodipine and their degradation products was injected repeated times and each analyte was collected and purified by SPE for subsequent characterisation by tandem mass spectrometry and NMR analysis.

Low-resolution ESI–MS and ESI–MS–MS data were acquired, using an API 3200 of Applied Biosystems instrument, equipped with electrospray ionization (ESI) and atmospheric pressure ionization (APCI) sources. A Turbo-IonSpray (ESI) probe in the positive-ion mode was utilized for these experiments. Full scan and product ions mass spectra were obtained by direct infusion into the mass spectrometer with acetonitrile/water (50:50, *v*/*v*) containing 5 mM ammonium formate as diluents. The typical ion source parameters were: declustering potential (DP): 50 V (losartan, amlodipine and Impurity D), 58 V (Compound E and Compound F), and 5,500 V (IS); collision energy (CE): 20 eV (losartan), 50 eV (amlodipine), 30 eV (Impurity D), 40 eV (Compound E and Compound F); entrance potential (EP) 10 V; collision cell exit potential (CXP) 3.8 V; spray voltage, 5,500 V and ion temperature, 300 °C. Nebulizer gas (NEB), curtain gas (CUR) and collision gas (CAD) were set to 40, 10, 5 psi, respectively. Nitrogen gas was used for CUR, CAD, NEB.

All NMR data were recorded at 293 K in DMSO-d_6 as the solvent and TMS as the internal standard on a Bruker AVANCE DRX 400 spectrometer operating at 9.4 T, observing ^{1}H at 400.13 and ^{13}C at 100.61 MHz.

Method Validation

The validation approach was executed under ICH guideline for validation of analytical procedures [27].

Selectivity

Individual injections of diluent solution, placebo, losartan and amlodipine

stressed solution were performed. The chromatograms were compared with the response of the working solutions the contribution for losartan and amlodipine quantification was then evaluated.

Linearity

The linearity of the detector response was determined at level ranges of 50–150% for losartan (20–60 μg mL^{-1}) and amlodipine (1.4–4.2 μg mL^{-1}). Evaluations were conducted by analysis of three working standard solutions of losartan and amlodipine in nine concentration levels. Peak areas and analyte concentrations were plotted and subjected to regression analysis to calculate correlation coefficient, *y*-intercept and slope of the regression line.

Limits of Detection and Quantification

The limits of detection (LOD) and limits of quantification (LOQ) were established by decreasing analyte amounts to S/N of 3:1 and 10:1, respectively. Injection of six different solutions with the supposed LOD and LOQ concentrations for amlodipine were then performed.

Precision

The precision of the method was evaluated in terms of repeatability (intra-day) and Intermediate precision (inter-day). Determinations were executed with three independent replicates in five concentration levels (70, 80, 100, 120, 130%).

Accuracy

The study of accuracy was conducted analyzing the recovery of known amounts of the analytes in spiked blank matrices through three independent replicates in five concentration levels (70, 80, 100, 120, 130%).

Robustness

The evaluation of robustness was made by analyses of the influence of variations in pH (2.8–3.2), buffer concentration (5–20 mM) and acetonitrile (±1%) in the mobile phase. Column temperature

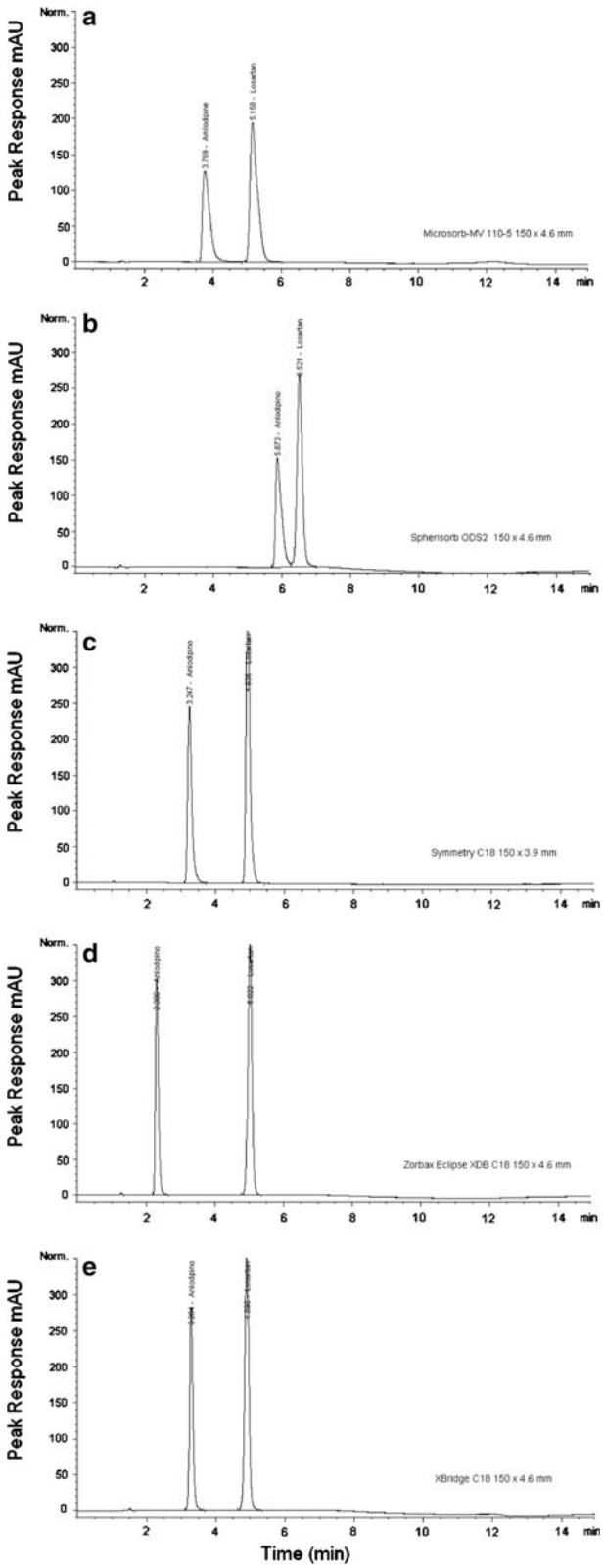


Fig. 1. LC chromatogram of losartan and amlodipine with different ODS columns (**a** Microsorb, **b** Spherisorb, **c** Symmetry, **d** Zorbax, **e** XBridge). Separation conditions: gradient of 10 mM phosphate pH 3.0 buffer and acetonitrile, 1.0 mL min^{-1} flow rate, room temperature, 20 μL injection volume and UV detection at 237 nm

Table 1. System suitability test

	Amlodipine's Impurity	Amlodipine	Losartan	Losartan's Compound E	Losartan's Compound F
k'	1.89	2.76	3.35	7.57	9.83
T	1.35	1.62	0.94	0.92	0.97
N	11,881	16,739	24,383	91,800	14,038
R_s	–	7.86	5.16	5.66	7.1
RSD	0.54%	0.10%	0.20%	0.87%	0.22%

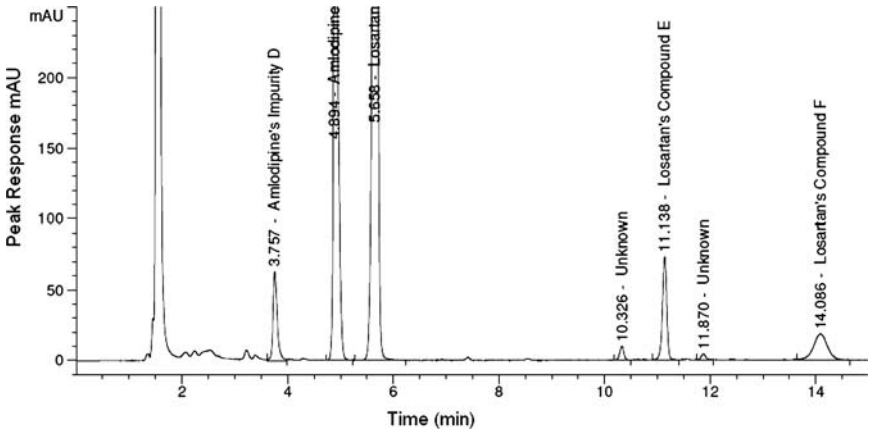


Fig. 2. LC chromatogram of losartan and amlodipine with their major degradation products

(20–30 °C) and mobile phase flow rate (0.9–1.1 mL min^{-1}) were also evaluated.

System Suitability

System suitability testing is an integral part of many analytical procedures. These tests are used to verify if the resolution and repeatability of the system are adequate for the analysis to be executed [11, 27, 28]. The tests were performed injecting six times the solution for the study of losartan and amlodipine degradation products. The method of generation of amlodipine degradation products is described in the British Pharmacopoeia [1]. For losartan, the specific methodology is described by Hertzog et al. [12].

Results and Discussion

Degradation Products Identification

Amlodipine and Impurity D have characteristic UV spectra, but losartan, Compounds E and F have similar UV spectra that prevent their differentiation by use of DAD detection in the chromatographic experiments. For unambiguous determination of the mentioned compounds we introduced NMR and ESI–MS analysis of the collected fractions from repeated stressed solution injections. Full scan ESI–MS analysis of the compounds presented protonated species $[M + H]^+$ at m/z 409, 407, 423, 827, and 827 for amlodipine, Impurity D, losartan, Compounds E and F, respectively. Tandem mass spectrometry analysis of Compounds F and E also gave similar spectra [14]. Both impurities were then submitted to 1 and 2D NMR analysis. Differently from the two isolated methylene of losartan, the ^1H NMR spectrum of Compound E revealed the presence of four methylene signals at δ 4.30, 5.21, 4.94, and 4.74 which correspond to the carbons at δ 51.5, 46.5, 46.7 and 39.5, respectively as evidenced on the one-bond ^1H-^{13}C heteronuclear correlation (HSQC) experiment. On the other hand, for Compound F, four new other ^1H NMR signals were observed at δ 4.35, 5.22, 5.24, and 5.78 that correspond to carbons at δ 51.5, 47.1, 46.9, and 45.2, respectively. Therefore, both degradation products of losartan could be identified by means of NMR experiments as the combination of two losartan monomers that result in two position dimmer isomers. This data allowed us to correlate each impurity structure to the corresponding chromatographic retention time.

Method Development

The methodology described here was developed in order to separate and quantify losartan and amlodipine in commercial preparation. We also provided the separation of their major degradation products for stability studies. For this purpose, we selected ODS columns and mobile phase gradient involving polar solvents and aqueous buffers. For the first pH and gradient condition studies, we utilized a Spherisorb ODS2 column with the gradient composed by methanol or acetonitrile with phosphate, acetate or formate buffer.

Better conditions were achieved using a gradient of acetonitrile and phosphate buffer (0 min 35 and 65%; 10 min 35 and 65%, 15 min 35 and 65%, respectively) with 5 min in the initial condition for column re-equilibration. Column temperature and mobile phase flow rate were also tested. Tests with different column temperatures (20–50 °C) did not lead to significant changes in the chromatogram, hence room temperature was preferred to provide longer column lifetime. The mobile phase flow rate was tested from 0.5 to 1.5 mL min^{-1}, with the best relationship between time and pressure at 1.0 mL min^{-1}.

The pH buffer tests were performed with values ranging from 3.0 to 7.0. All evaluated pH conditions were able to separate the main analytes, however, at pH 7.0 losartan was not retained and amlodipine had an excessive slowing in the elution time. At pH 3.0, 4.0, 4.5 and 5.0 the main analytes were well resolved ($R_s > 2.0$), however, at pH 4.0 and 4.5 the losartan major degradation products were co-eluted. Even though in pH 5.0 condition the analytes and their major degradation products presented a good

Table 2. Accuracy by recovery and precision study ($n = 3$)

Approximate level	Losartan				Amlodipine			
	Conc. ($\mu g\ mL^{-1}$)	Accuracy	Precision		Conc. ($\mu g\ mL^{-1}$)	Accuracy	Precision	
		Recovery mean (%)	Intra-day RSD (%)	Inter-day RSD (%)		Recovery mean (%)	Intra-day RSD (%)	Inter-day RSD (%)
70	28.01	98.8	0.66	1.49	1.98	100.6	2.87	3.05
80	32.01	99.8	0.89	0.75	2.27	101.2	4.23	4.48
100	40.01	100.0	0.55	0.28	2.83	96.4	1.51	4.93
120	48.02	100.3	1.03	0.66	3.40	97.1	1.34	4.94
130	52.02	100.4	0.54	0.62	3.68	97.4	3.15	3.09

resolution, the method appeared less robust for losartan retention times in different chromatographic runs and types of column. This was probably caused by the closeness of losartan pK_a value (4.9) to the buffer pH value in this condition. Analyses performed at pH 3.0 gave the best results: all main analytes and the degradation products were well resolved with the appropriate robustness and, additionally, the more retained analyte (losartan, in this pH) appeared with the lower retention time in comparison to the other conditions.

We also evaluated different types of columns and manufacturers (Fig. 1). All the columns tested (L1 packing by USP, with the same dimensions) quantified losartan and amlodipine with different chromatographic efficiencies. All N values were higher than 1,000, the tailing factor for amlodipine lower than 2.0 and resolution higher than 2.0. This data indicated that all columns presented acceptable chromatographic parameters to provide the proposed analysis. In this study XBridge column provided the best results.

Separation of related degradation products was also evaluated. In this case, Spherisorb, Symmetry and XBridge columns were able to separate the analytes in the proposed run time (15 min). The best overall results were obtained by use of the XBridge column, thus, it was employed for the method validation.

Validation

System Suitability

The test was performed by six repeated injections of losartan and amlodipine stressed solutions. The values of resolution (R_s), USP tailing (T), plate number (N), retention factor (k') and RSD are presented in Table 1 and a typical chromatogram is showed in Fig. 2.

Selectivity

Selectivity assay demonstrated that the placebo and diluents had negligible contribution in the chromatogram, and the major degradation products had a good resolution from the analytes, indicating that amlodipine and losartan can be appropriately labeled (Fig. 2).

Linearity

The linear relationship between detector response and analyte concentration was performed from 50 to 150% levels. For losartan, the concentration range was 20–60 $\mu g\ mL^{-1}$, the ration line with $y = 48.78163x - 9.08904$ and correlation coefficient of 0.99994. For amlodipine the values were 1.4–4.2 $\mu g\ mL^{-1}$; $y = 36.87527x + 0.972512$; 0.99964 as indicating for losartan.

Limits of Detection and Quantification

The LOD and LOQ were determined only for amlodipine because it is normally present in a lower amount in commercial tablets and capsules than losartan. The LOD and LOQ values were 0.0875 and 0.175 $\mu g\ mL^{-1}$, respectively.

Precision

Losartan and amlodipine intra-day RSD were from 0.54 to 1.03% and from 1.34 to 4.23%, respectively. The RSD inter-day ranged from 0.28 to 1.49% (losartan) and 3.05–4.94% (amlodipine), as shown in Table 2.

Accuracy

The accuracy was evaluated by determination of analytes recovery percentage. For losartan the value ranged from 98.8 to 100.4% and for amlodipine from 96.4 to 101.2%.The results are listed in Table 2.

Robustness

For robustness evaluation, small changes in flow rate (±10%), pH buffer (2.8 and 3.2) buffer concentration (5 and 20 mM), Acetonitrile content (±1%) and column temperature (±5 °C) did not give significant changes in resolution and recovery.

Conclusion

As far as we know, there was no available method for the simultaneous quantification of losartan and amlodipine in pharmaceutical preparations. Here we described a new and simple method that has been established for this purpose. Although commercial tablets present much higher quantities of losartan (20 times) in comparison to amlodipine, the method here described allowed a reliable simultaneous quantification of this kind of product. In addition, ESI–MS and NMR tools were employed to determine the related degradation products unambiguously.

References

1. British Pharmacopoeia Commission (2007) British Pharmacopoeia, vol 1, 5th edn. The Stationery Office, London
2. Meredith PA, Elliott HL (1992) Clin Pharmacokinet 22:22–31
3. Mohammadi A, Rezanour N, Ansari Dogaheh M, Ghorbani Bidkorbeh F, Hashem M, Walker RB (2007) J Chromatogr B Analyt Technol Biomed Life Sci 846:215–221. doi:10.1016/j.jchromb.2006.09.007
4. Naidu KR, Kale UN, Shingare MS (2005) J Pharm Biomed Anal 39:147–155. doi:10.1016/j.jpba.2005.04.001
5. Patel YP, Patil S, Bhoir IC, Sundaresan M (1998) J Chromatogr A 828:283–286. doi:10.1016/S0021-9673(98)00652-9
6. Ma Y, Qin F, Sun X, Lu X, Li F (2007) J Pharm Biomed Anal 43:1540–1545. doi:10.1016/j.jpba.2006.11.015
7. Streel B, Laine C, Zimmer C, Sibenaler R, Ceccato A (2002) J Biochem Biophys Methods 54:357–368. doi:10.1016/S0165-022X(02)00133-1
8. Zarghi A, Foroutan SM, Shafaati A, Khoddam A (2005) Farmaco 60:789–792. doi:10.1016/j.farmac.2005.06.012
9. Tatar S, Atmaca S (2001) J Chromatogr B Biomed Sci Appl 758:305–310. doi:10.1016/S0378-4347(01)00197-9
10. Conlin PR (2001) Curr Ther Res Clin Exp 62:673–673. doi:10.1016/S0011-393X(01)80019-9
11. Seburg RA, Ballard JM, Hwang TL, Sullivan CM (2006) J Pharm Biomed Anal 42:411–422. doi:10.1016/j.jpba.2006.04.030
12. Hertzog DL, McCafferty JF, Fang X, Tyrrell RJ, Reed RA (2002) J Pharm Biomed Anal 30:747–760. doi:10.1016/S0731-7085(02)00385-0
13. Lusina M, Cindric T, Tomaic J, Peko M, Pozaic L, Musulin N (2005) Int J Pharm 291:127–137. doi:10.1016/j.ijpharm.2004.07.050
14. Zhao Z, Wang Q, Tsai EW, Qin XZ, Ip D (1999) J Pharm Biomed Anal 20:129–136. doi:10.1016/S0731-7085(99)00004-7
15. McCarthy KE, Wang Q, Tsai EW, Gilbert RE, Ip DP, Brooks MA (1998) J Pharm Biomed Anal 17:671–677. doi:10.1016/S0731-7085(97)00251-3
16. Williams RC, Alasandro MS, Fasone VL, Boucher RJ, Edwards JF (1996) J Pharm Biomed Anal 14:1539–1546. doi:10.1016/0731-7085(96)01740-2
17. Carlucci G, Palumbo G, Mazzeo P, Quaglia MG (2000) J Pharm Biomed Anal 23:185–189. doi:10.1016/S0731-7085(00)00268-5
18. Erk N (2001) J Pharm Biomed Anal 24:603–611. doi:10.1016/S0731-7085(00)00434-9
19. Soldner A, Spahn-Langguth H, Mutschler E (1998) J Pharm Biomed Anal 16:863–873. doi:10.1016/S0731-7085(97)00128-3
20. Yeung PK, Jamieson A, Smith GJ, Fice D, Pollak PT (2000) Int J Pharm 204:17–22. doi:10.1016/S0378-5173(00)00453-1
21. Farthing D, Sica D, Fakhry I, Pedro A, Gehr TW (1997) J Chromatogr B Biomed Sci Appl 704:374–378. doi:10.1016/S0378-4347(97)00489-1
22. Gonzalez L, Lopez JA, Alonso RM, Jimenez RM (2002) J Chromatogr A 949:49–60. doi:10.1016/S0021-9673(01)01496-0
23. Ritter MA, Furtek CI, Lo MW (1997) J Pharm Biomed Anal 15:1021–1029. doi:10.1016/S0731-7085(96)01948-6
24. Society British Hypertension (2004) J Fam Pract 53:528–550
25. Wolf-Maier K, Cooper RS, Kramer H, Banegas JR, Giampaoli S, Joffres MR, Poulter N, Primatesta P, Stegmayr B, Thamm M (2004) Hypertension 43:10–17. doi:10.1161/01.HYP.0000103630.72812.10
26. Qin XZ, Demarco J, Ip DP (1995) J Chromatogr A 707:245–254. doi:10.1016/0021-9673(95)00349-R
27. ICH Validation of analytical procedures: text and methodology *Q*2(*R*1) (2005). International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use
28. Shabir GA (2003) J Chromatogr A 987:57–66. doi:10.1016/S0021-9673(02)01536-4