ELSEVIER

Journal of Chromatography B, 690 (1997) 195–202

JOURNAL OF CHROMATOGRAPHY B

On-line solid-phase extraction and high-performance liquid chromatographic determination of chlorthalidone in urine

Salvador Salado C.[a], Luz E. Vera-Avila[b,*]

[a]*Centro A.F. de Estudios Tecnológicos S.A., Nicolás San Juan 1024, 03100 Mexico D.F., Mexico*

[b]*Departamento de Química Analítica, Facultad de Química, Universidad Nacional Autónoma de México, 04510 Mexico D.F., Mexico*

Received 24 April 1996; revised 21 August 1996; accepted 22 August 1996

Abstract

A simple and rapid on-line method for the determination of chlorthalidone in urine is proposed. The sample containing the internal standard is injected in a CN precolumn. After a 2-ml water rinsing, the precolumn is coupled for 30 s to the HPLC column via a switching valve, allowing the on-line elution of the compounds of interest. Analysis is carried out by reversed-phase chromatography with an acetonitrile–0.01 *M* phosphate buffer pH 7 (20:80, v/v) eluent, using UV detection at 214 nm. While the LC separation is performed, the precolumn is regenerated and conditioned, and is ready to receive the next sample at the end of the run. Accurate (>95%) and precise (<10%) analyses, in the range of 0.1–20 μg/ml of chlorthalidone in urine, have been achieved using this method.

Keywords: Chlorthalidone

1. Introduction

Chlorthalidone is a diuretic of low toxicity and extended action used in the hypertension therapy. For a bioequivalence/bioavailability study on chlorthalidone drug products, it is necessary to analyze hundreds of samples in a relatively short time [1]. Thus, a reliable, rapid and sensitive method for the determination of this compound or one of its metabolites in blood or urine is required.

Current methods for the determination of chlorthalidone in biological fluids generally involve liquid–liquid extraction of the sample, evaporation of the organic solvent and reconstitution of the extract in an appropriate solvent or in the mobile phase prior to RP-HPLC analysis with UV detection [2–4]. However, for urine samples, some works report the direct injection of the diluted sample in the chromatograph [5,6]. Off-line solid-phase extraction (SPE) on C_{18} cartridges has also been used for the analysis of diuretics in urine [4,7]. All these methods present some disadvantages: liquid–liquid extraction methods are long and cumbersome and require extensive sample handling; direct injection methods are not sufficiently selective and the probability of interference from endogenous compounds of the matrix is very high; finally, off-line SPE also requires some sample handling and the cost of the disposable cartridges renders the method rather expensive for bioequivalence studies.

In the last decade, an increasing number of publications have demonstrated the potential of column-switching techniques for the determination of drugs in biological samples [8]. The use of these

*Corresponding author.

PII S0378-4347(96)00396-9

systems has greatly facilitated the sample preparation step, providing better precision and accuracy in the whole analysis, the possibility of fully automation and a considerable time reduction.

This work describes a very simple on-line solid-phase extraction method for the determination of chlorthalidone at low μg/ml concentration levels in urine. The management of samples has been reduced to a minimum and hence the assay time is considerably shortened.

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile and methanol were from Mallinckrodt (St. Louis, MO, USA). Potassium dihydrogen phosphate, potassium monohydrogen phosphate and sodium hydroxide were analytical grade reagents from Merck (Darmstadt, Germany). HPLC water from a Milli-Q deionizer (Millipore, Bedford, MA, USA) was used in all experiments. Analytical grade chlorthalidone and 2,7-dihydroxynaphthalene, used as internal standard, were from Sigma (St. Louis, MO, USA). Reference USP chlorthalidone was used for the validation study.

Stock solutions of chlorthalidone and the internal standard (I.S.), 1000 μg/ml each, were prepared separately in methanol. Standards of chlorthalidone in blank urine were prepared from the stock solution. Appropriate aliquots of these solutions were diluted with urine to give a range of spiked samples from 0.02 to 20 μg/ml. All samples were stored until analysis at −40°C. A standard containing 3 μg/ml of the I.S. in water was also prepared from the corresponding stock solution.

2.2. Apparatus

The sample preparation system consisted of a Waters (Milford, MA, USA) flow unit for four solvents, Model 600 E (pump P_1), and a Waters automatic injector, Model 717 (injector I_1). The HPLC system, also from Waters, consisted of an isocratic pump, Model 510 (pump P_2), equipped with a Model U6K injector (injector I_2), a Model 486 variable-wavelength detector set at 214 nm and a Model 745 integrator. Both systems were coupled through a six-port switching valve (Waters) with the SPE precolumn connected to ports 1 and 4. A Waters 600 controlling unity, connected to pump P_1, injector I_1, the detector, the integrator and the switching valve, was used to automatize the analysis. Fig. 1 shows the diagram of the experimental setup.

2.3. Columns and mobile phase

A reversed-phase analytical column, 250×4.6 mm I.D., packed with 5 μm, C_{18} Ultrasphere (Beckman-Altex, San Ramon, CA, USA) was used throughout. The SPE precolumn was a small, 12.5×4 mm I.D., stainless steel column prepacked with 5 μm, Stable Bond-CN from Zorbax (Dupont, Wilmington, DE, USA). Preliminary off-line experiments were carried out on 100 mg Spe-ed C_{18} and CN cartridges from Applied Separations (Allentown, PA, USA).

The isocratic HPLC separation was carried out with acetonitrile–0.01 *M* phosphate buffer, pH 7 (20:80, v/v) at a flow-rate of 2 ml/min. Temperature was ambient.

2.4. Procedures

The following procedure for the on-line coupling of sample preparation and HPLC analysis was established:

With the setup in position 1, pumps P_1 and P_2 are activated. The first pump delivers water to the CN precolumn and the second delivers the mobile phase to the analytical column. A 400-μl volume of the aqueous I.S. standard is added to 600 μl of the urine sample and 50 μl of this mixture are injected in the precolumn via the automatic injector I_1. After 2 min, the precolumn is switched to position 2 for 30 s, allowing the backflush transfer of the compounds of interest to the analytical column for their separation and analysis. Meanwhile, the precolumn, switched back to position 1, is first regenerated with an acetonitrile–water (95:5, v/v) mixture and then it is conditioned with water. When the HPLC run is finished, the precolumn is ready to receive the next sample. Table 1 presents the time-table corresponding to this procedure.

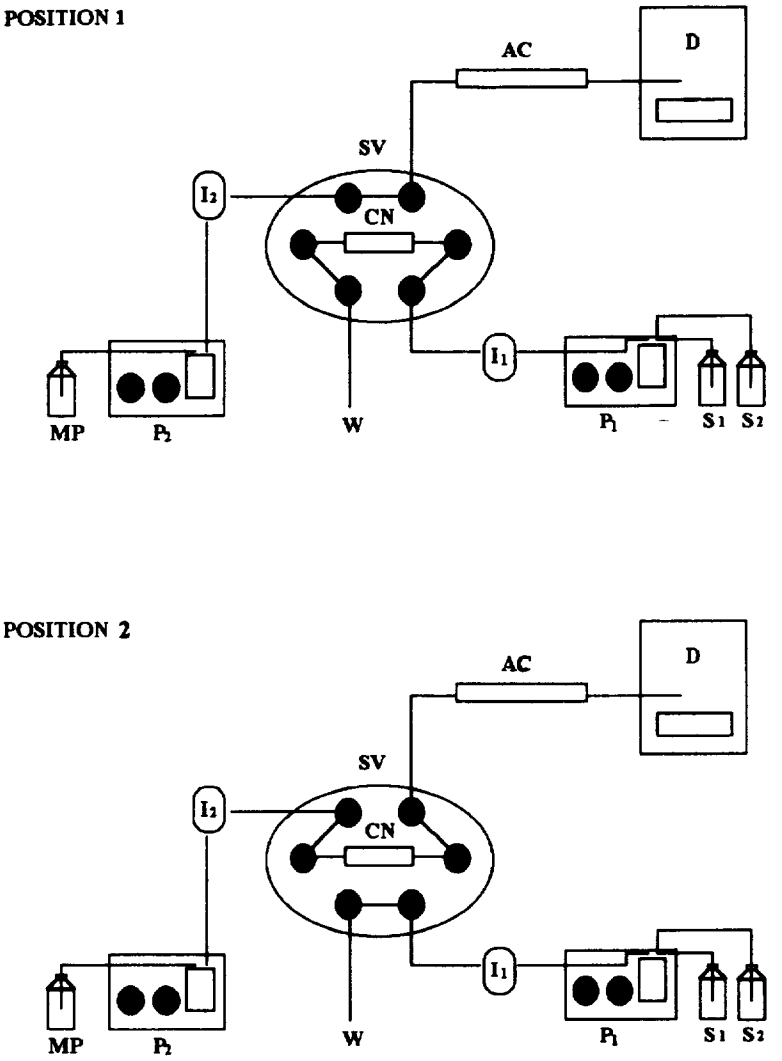

Fig. 1. Diagram of the experimental setup for the on-line extraction, sample cleanup and HPLC analysis of chlorthalidone in urine. P_1, sample preparation gradient pump; P_2, HPLC isocratic pump; I_1, automatic injector; I_2, manual injector; SV, switching valve; D, UV detector; W, waste; S_1, water; S_2, acetonitrile; MP, mobile phase. Columns: AC, 250×4.6 mm I.D. reversed-phase analytical column packed with 5 μm C_{18} Ultrasphere; CN, 12.5×4 mm I.D. precolumn packed with 5 μm Stable Bond-CN.

3. Results and discussion

Preliminary experiments to test the solid-phase extraction of chlorthalidone from urine samples were performed in the off-line mode using C_{18} and CN cartridges. After loading the sample, the cartridges were rinsed with water or with different methanol–water mixtures and the elution of the analyte was carried out with 1 ml of acetone methanol (50:50, v/v). The extract was evaporated to dryness, reconstituted in the mobile phase and injected in the chromatograph. These experiments showed that the retention of chlorthalidone on both adsorbents was adequate; however, the adjustment of conditions for a good cleanup of the sample was easier with the CN phase. Indeed, with only a 3-ml water rinsing of the

Table 1
Time-table for the analysis of chlorthalidone in urine

Time[a] (min)	Event (valve position)	Solvent (P_1) CH_3CN-H_2O (v/v)	Flow-rate (P_1) (ml/min)
0.0	Inject sample and rinse precolumn (1)	0–100	1.0
2.0[b]	Switch valve (2)	0–100	1.0
2.5	Switch valve (1)	0–100	1.0
3.5	Regenerate precolumn (1)	95–5	2.0
5.0	Regenerate precolumn (1)	95–5	2.0
6.0	Condition precolumn (1)	0–100	2.0
14.0[c]	Condition precolumn (1)	0–100	2.0
15.0	Begin new cycle	0–100	1.0

[a] During the 15-min cycle, pump P_2 delivers the mobile phase at 2 ml/min in the HPLC system.
[b] Beginning of the chromatographic run.
[c] End of the chromatographic run.

CN cartridge most of the endogenous interferences of the sample were eliminated and a good recovery of chlorthalidone was achieved, ca. 90%.

For the choice of the internal standard, the urine samples were spiked with different compounds and treated in the CN cartridge using the conditions mentioned above. The best results concerning absolute recovery and chromatographic retention were obtained with 2,7-dihydroxynaphthalene, even though some of the compounds tested, like chlorothiazide, hydrochlorothiazide and amiloride, have a closer structural resemblance to chlorthalidone. The compound finally chosen fulfils the requirements for a good I.S.: readily available with a high degree of purity, never present in the samples, good recovery (80%) and retention time close to the chlorthalidone peak but completely resolved from it and from its main metabolite, 2-(3-aminosulfonyl-4-chlorobenzoyl) benzoic acid [9].

Considering the advantages of using column-switching techniques for sample preparation and the results of the preliminary experiments, the procedure described in Section 2.4 was finally adopted. Typical chromatograms are shown in Fig. 2. The excellent cleanup of the sample achieved by the method can be appreciated by comparing Fig. 2A and D; both of them correspond to a diluted blank urine, but the former was analyzed using the on-line system and the latter was directly injected in the analytical column by means of the injector I_2. Fig. 2B and C illustrates the application of the method to urine samples spiked with chlorthalidone at the lowest and the highest concentration of the range studied in this work (0.10 and 20.0 μg/ml respectively). Finally, Fig. 2E shows the chromatogram of the urine sample from a volunteer obtained 72 h after a single dose administration of 50 mg of chlorthalidone.

In fact, the procedure followed in this method is equivalent to a heart-cutting of the sample. The most polar compounds of it are discarded during the 2-min rinsing of the precolumn with water. Then, the portion containing the compounds of interest is transferred to the analytical column for their separation and analysis. Finally, the least polar part of the sample, remaining in the CN precolumn, is washed out by the acetonitrile rich solvent used in the regeneration step.

3.1. Selectivity

Urine samples of seven healthy volunteers were analyzed by this method. No interfering peaks were found for any of the samples examined and all the chromatograms were as clean as that of Fig. 2A. Besides, the chromatogram of Fig. 2E also demonstrates that there are no interferences from chlorthalidone metabolites in the analysis of samples from excretion studies.

To confirm the adequacy of the method for bioequivalence/bioavailability studies, other compounds added to the urine samples were tested for possible interference: 2-(3-aminosulfonyl-4-chloro-

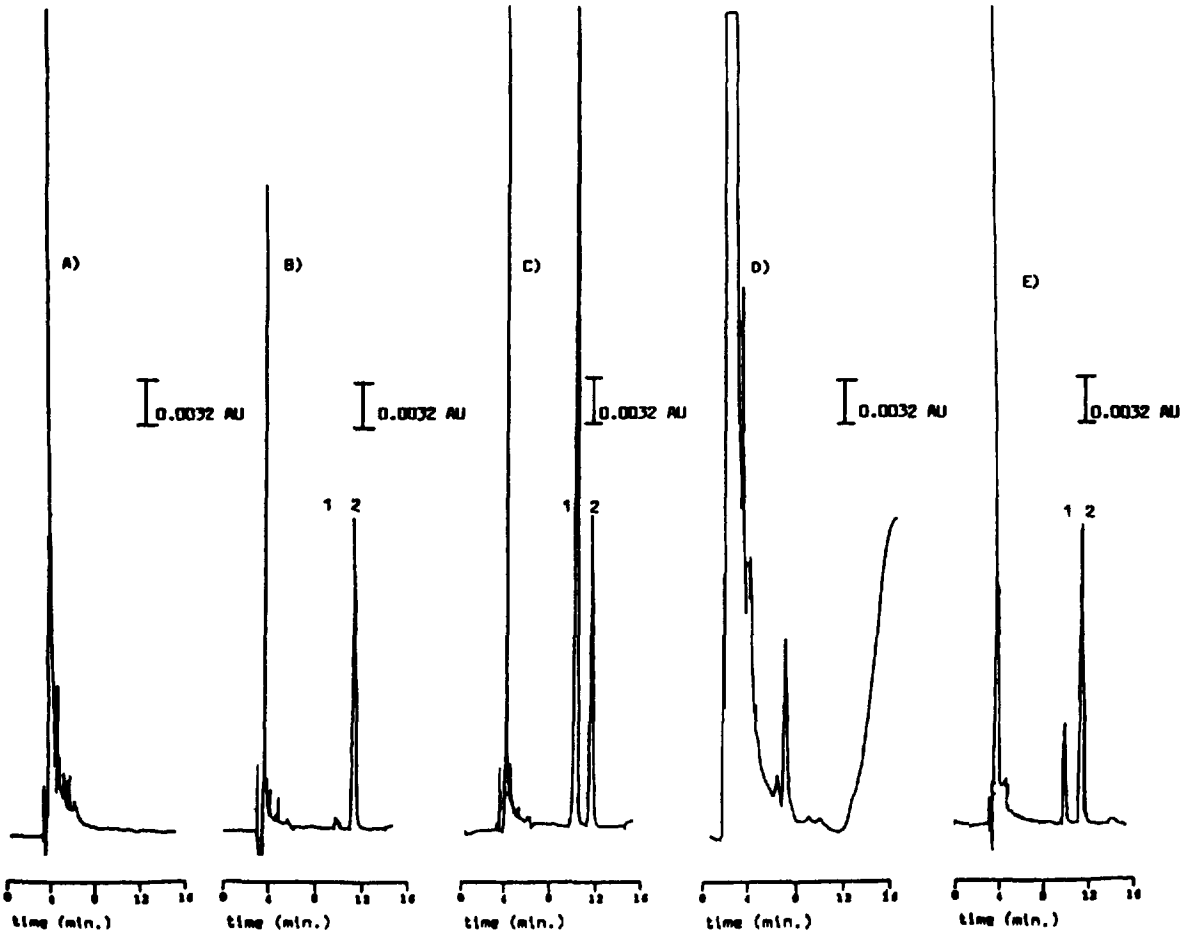

Fig. 2. Chromatograms corresponding to the analysis of urine samples using the method described (A–C and E) and by direct injection (D). (A, D) Blank urine, (B) urine spiked at 0.1 μg/ml of chlorthalidone, (C) urine spiked at 20.0 μg/ml of chlorthalidone, (E) urine collected 72 h after a single dose administration of 50 mg of chlorthalidone. Peaks: (1) chlorthalidone, (2) 2,7-dihydroxynaphthalene (internal standard). Sample preparation and chromatographic conditions described in Section 2.

benzoyl) benzoic acid, the main degradation product of chlorthalidone, and also some products that can be eventually present in the samples of volunteers, such as salicylic acid (from acetyl-salicylic acid), naproxen, caffeine and methyldopa.

Table 2 shows the retention times of these compounds in the conditions of the method. Methyldopa and salicylic acid were not detected, either because they were not retained in the CN precolumn or because they were not transferred to the analytical column. From these results, for the compounds tested, the only interference in the analysis of chlorthalidone is naproxen.

3.2. Linearity

Urine samples fortified at six different concentrations of chlorthalidone, in the range 0.1–20 μg/ml, were analyzed using the method. The calibration curves, peak-height ratio (R) vs. concentration (C), generated in three different days were linear, with a correlation coefficient $r=1.000$. Eqs. (1a), (1b), (1c), represent the three calibration lines.

$$R = 0.002 + 0.412C \quad (1a)$$

$$R = 0.010 + 0.415C \quad (1b)$$

Table 2
Retention times of chlorthalidone, the I.S. and some potential interference compounds

Compound	Retention time (min)
Chlorthalidone	9.8
2,7-Dihydroxynaphthalene (I.S.)	11.2
2-(3-Aminosulfonyl-4-chlorobenzoyl)benzoic acid	5.3
Caffeine	4.1
Naproxen	9.5
Methyldopa	Not detected
Salicylic acid	Not detected

$$R = -0.002 + 0.421C \quad (1c)$$

The quality of fit was evaluated from the statistical analysis of the back calculated concentrations, using the corresponding linear regression equation for each experimental point. Table 3 shows the results of this work, which corroborate the linear behavior in the range of concentrations studied.

3.3. Accuracy, precision, limit of quantitation and limit of detection

Intra-day accuracy and precision were evaluated from the results of six replicate analysis of urine samples spiked at four different concentrations (Table 4). For the evaluation of inter-day variations, single analysis of samples at four concentrations were carried out on six different days (Table 5).

Generally accepted criteria [10] to consider an analytical method sufficiently precise and accurate for bioequivalence/bioavailability studies, specify that the coefficient of variation should be lower than 15% and the error must be in the range of ±15% of the real value. Tables 4 and 5 show that the proposed method fulfils these requirements in the range of chlorthalidone concentrations from 0.100 to 20.0 μg/ml. Therefore, the former value may be considered as the limit of quantitation. On the other hand, the limit of detection of the method, determined for a signal-to-noise ratio of 3, is 0.02 μg/ml.

Table 3
Quality of fit in the calibration curves of three different days

Concentration added (μg/ml)	Concentration back calculated (μg/ml)	C.V. (%)	Error (%)
0.100	0.102	13.33	2.0
0.200	0.198	5.35	−1.0
0.500	0.501	4.09	0.2
1.00	0.991	0.47	−0.9
5.00	5.01	1.03	0.2
20.0	20.0	0.06	0.0

Table 4
Intra-day accuracy and precision ($n = 6$)

Concentration added (μg/ml)	Concentration found (μg/ml)	C.V. (%)	Error (%)
0.100	0.0952	6.55	−4.8
0.300	0.299	2.83	−0.3
7.00	6.86	1.59	−2.0
16.0	15.6	4.97	−2.5

3.4. Absolute recoveries

Absolute recoveries were calculated by comparing the mean peak heights obtained from five replicate analysis of urine samples and aqueous samples fortified at the same concentration of chlorthalidone. The urine samples were analyzed using the method; the aqueous samples were mixed with the I.S. standard in the ratio 6:4 (v/v) and 50 μl were directly injected in the analytical column.

Four different concentrations of chlorthalidone

Table 5
Inter-day accuracy and precision[a]

Concentration added (μg/ml)	Concentration found (μg/ml)	C.V. (%)	Error (%)
0.100	0.103	9.46	3.3
0.300	0.312	6.21	4.0
7.00	7.07	3.19	1.0
16.0	16.11	3.71	0.7

[a] Single analysis on six different days.

Table 6
Absolute recovery of chlorthalidone and the I.S.

Compound	Concentration (μg/ml)	Recovery (%)	C.V. (%)
Chlorthalidone ($n=5$)			
	0.100	95.6	13.2
	0.300	96.0	4.4
	7.00	89.7	3.3
	16.0	88.2	2.4
2,7-Dihydroxynaphthalene ($n=20$)		82.8	5.3

were studied, 0.1, 0.3, 7 and 16 μg/ml. The coefficient of variation of recovery (C.V.) per individual chlorthalidone concentration was calculated as follows:

$$C.V. = [(C.V._h)^2_{urine} + (C.V._h)^2_{water}]^{0.5} \quad (2)$$

where $(C.V._h)_{urine}$ is the coefficient of variation of the peak height for the five urine samples, and $(C.V._h)_{water}$ is the same parameter for the five aqueous samples.

The same procedure was used to calculate the recovery and the coefficient of variation of recovery for the internal standard in the samples examined.

The results of these experiments, presented in Table 6, show that the absolute recovery of chlothalidone is sensibly constant in the range of concentrations studied.

3.5. Stability of the samples

A test to check the stability of the samples was carried out as follows: first, urine samples spiked at three different concentrations of chlorthalidone were analyzed, then, the fortified samples were subjected to three 24-h freeze–thaw cycles and re-analyzed. Table 7 shows that there are no clear signs of sample degradation during this experiment, considering the criteria of accuracy and precision mentioned before.

3.6. Excretion study

Urine samples from a healthy male volunteer (age 23 years) were collected at scheduled time intervals after a single dose administration of 50 mg of chlorthalidone. The samples were analyzed using the method for the determination of unchanged drug. Table 8 shows excretion data of chlorthalidone. It was excreted in urine until at least five days after dosage and about 35% of the dose was recovered in the urine.

4. Conclusions

A simple, economical and rapid analytical method for the determination of chlorthalidone in urine, using 2,7-dihydroxynaphthalene as internal standard was developed.

The method, based on the on-line coupling of the

Table 7
Stability of the sample after freeze–thaw cycles

Concentration added (μg/ml)	Concentration found (μg/ml)		C.V. (%)		Error (%)	
	1	2	1	2	1	2
0.300	0.299	0.320	2.83	0.95	−0.33	6.56
7.00	6.86	6.53	1.59	0.80	−2.00	−3.69
16.0	15.6	14.9	4.97	0.58	−2.55	−7.13

(1) Initial analysis.
(2) Analysis after three 24-h freeze–thaw cycles.

Table 8
Excretion of chlorthalidone after a single oral dose (50 mg)

Time (h) interval	Urine volume (ml)	Concentration (μg/ml)	Amount (μg)	(%) Excreted
0–2	325	0.11	0.036	0.07
2–4	275	5.02	1.38	2.76
4–6	415	5.59	2.32	4.64
6–8	290	3.52	1.02	2.04
8–12	440	2.97	1.31	2.62
12–24	1000	2.35	2.35	4.70
24–48	2230	1.76	3.93	7.86
48–72	1270	1.81	2.29	4.58
72–96	1900	0.90	1.72	3.44
96–120	1490	0.85	1.27	2.54

extraction and cleanup steps with the chromatographic analysis, requires minimum sample handling and provides accurate and precise results in the range of chlorthalidone concentrations from 0.1 to 20 μg/ml. A regenerable CN precolumn is used for the preparation of the sample. Indeed, the same precolumn has been used for the analysis of more than 300 samples without showing any signs of deterioration.

An analysis time per sample of 15 min and the possibility of automation render the method very adequate for clinical laboratories, after testing the potential interference of other concomitant drugs, or for bioequivalence/bioavailability studies, where hundreds of samples must be analyzed in a short time.

References

[1] Food and Drug Administration, Office of Generic Drug, Guidelines for a Protocol to Conduct Bioavailability Study on Chlorthalidone Drug Products, Rockville, MD, USA, 1983.
[2] J. Mcainsh, W. Bastain, J. Young and D. Harry, Biopharm. Drug Dispos., 2 (1981) 147.
[3] D.C. Muirhead and R.B. Christie, J. Chromatogr., 416(1987) 420.
[4] S.-J. Park, H.-S. Pyo, Y.-J. Kim, M.-S. Kim and J. Park, J. Anal. Toxicol., 14 (1990) 84.
[5] A.J.M. Guelen, A.M. Baars and T.B. Vree, J. Chromatogr., 181 (1980) 497.
[6] R.L. Williams, C.D. Blume, E.T. Lin, N.H.G. Holford and L.Z. Benet, J. Pharm. Sci., 71 (1982) 533.
[7] P. Campíns-Falcó, R. Herraez-Hernández and A. Sevillano-Cabeza, J. Liq. Chromatogr., 16 (1993) 2571.
[8] P. Campíns-Falcó, R. Herraez-Hernández and A. Sevillano-Cabeza, J. Chromatogr., 619 (1993) 177.
[9] J. Bauer, J. Quick, S. Krogh and D. Shada, J. Pharm. Sci., 72 (1983) 924.
[10] Code of Federal Regulations, Bioavailability and Bioequivalence Requirements, Vol. 21, Part 320, 1991.