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Experimental design approach for the optimisation of a HPLC-fluorimetric method for the quantitation of the angiotensin II receptor antagonist telmisartan in urine

N. Torrealday [a], L. González [a], R.M. Alonso [a,*], R.M. Jiménez [a], E. Ortiz Lastra [b]

[a] *Departamento de Química Analítica, Facultad de Ciencias, Universidad del País Vasco, Apdo. 644, E-48080 Bilbao, Spain*
[b] *Departamento de Medicina Legal y Toxicología, Facultad de Medicina, Universidad del País Vasco, Apdo. 644, 48080 Bilbao, Spain*

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Abstract

A high performance liquid chromatographic method with fluorimetric detection has been developed for the quantitation of the angiotensin II receptor antagonist (ARA II) 4-((2-*n*-propyl-4-methyl-6-(1-methylbenzimidazol-2-yl)-benzimidazol-1-yl)methyl)biphenyl-2-carboxylic acid (telmisartan) in urine, using a Novapak C18 column 3.9 × 150 mm, 4 μm. The mobile phase consisted of a mixture acetonitrile–phosphate buffer (pH 6.0, 5 mM) (45:55, v/v) pumped at a flow rate of 0.5 ml min^{-1}. Effluent was monitored at excitation and emission wavelengths of 305 and 365 nm, respectively. Separation was carried out at room temperature. Chromatographic variables were optimised by means of experimental design. A clean-up step was used for urine samples consisting of a solid-phase extraction procedure with C8 cartridges and methanol as eluent. This method proved to be accurate (RE from −12 to 6%), precise (intra- and inter-day coefficients of variation (CV) were lower than 8%) and sensitive enough (limit of quantitation (LOQ), ca. 1 μg l^{-1}) to be applied to the determination of the active drug in urine samples obtained from hypertensive patients. Concentration levels of telmisartan at different time intervals (from 0 up to 36 h after oral intake) were monitorised.

Keywords: Telmisartan; Angiotensin II receptor antagonists; HPLC-fluorimetric; Urine

1. Introduction

High blood pressure is quantitatively the largest single risk factor for premature death and disability due to its high prevalence in Western society [1]. Despite the considerable success of treatments, hypertension still remains one of the greatest public health problems [2].

Telmisartan, 4-((2-*n*-propyl-4-methyl-6-(1-methylbenzimidazol-2-yl)-benzimidazol-1-yl)methyl)-biphenyl-2-carboxylic acid, is an angiotensin II receptor antagonist (ARA II) widely used in the treatment of hypertension. The therapy with these

* Corresponding author. Tel.: +34-94-601-2686; fax: +34-94-464-8500.
E-mail address: qapalror@lg.ehu.es (R.M. Alonso).

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drugs offers a good quality of life for hypertensive patients due to the absence of side effects and its once daily administration [3]. The key advantages of the non-peptide ARA II antagonists are related to their specificity of action and minimisation of untoward effects [4].

Telmisartan undergoes minimal biotransformation in the liver to form telmisartan 1-*o*-acylglucuronide [5], its major inactive metabolite. Maximum plasma concentration (C_{max}) occurs within about 3 h (T_{max}) after its oral administration, giving plasma levels of 50 μg l^{-1} for a 40 mg dose. Renal excretion is a minor elimination pathway for telmisartan, hence the small amount of the dose excreted in urine (less than 1%). The mean elimination half-life ($t_{1/2}$) is approximately 24 h, in contrast with the rapid absorption process of the drug. The rest of the parent compound (more than 98%) is excreted in the faeces [6].

Analytical methods for the direct determination of telmisartan are very scarce. Indeed, the only method found in literature was developed by one of the laboratories in charge of commercialisation of telmisartan. This unpublished method also deals with high performance liquid chromatography coupled to fluorescent detection, but it has only been applied to plasma and the clean-up step is based on the column-switching technique (Boehringer Ingelheim, internal data).

The existence of several fluorescent functional groups in its molecular structure, such as biphenyl and imidazole [7,8] (Fig. 1), makes fluorescent methods suitable for the determination of telmisartan in biological fluids, such as urine and plasma, after an initial chromatographic separation.

Plasma concentration levels and urinary excretion profiles (50 μg l^{-1} for a dose of 40 mg and < 1%) made us choose fluorescent detection due to the low limits of quantitation that can be achieved with this technique. Since most analytical methods developed for the determination of these antihypertensive drugs use liquid chromatography [9–24] the applicability of HPLC-fluorimetric to the quantitation of this novel ARA II, telmisartan, was investigated.

Thus, the aim of this work is to establish a chromatographic method for the separation and

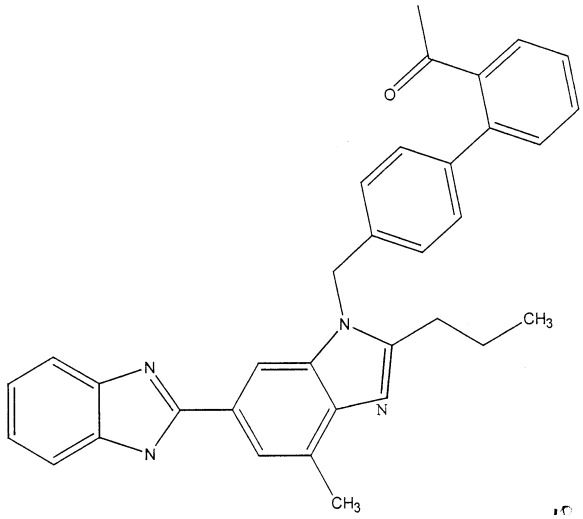

Fig. 1. Chemical structure of telmisartan.

determination of telmisartan in urine samples validating it in accordance to the summary report of the Conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies [25].

An optimisation study of the HPLC technique main variables: pH, buffer concentration (mM), percentage of organic modifier (%) and flow (ml min^{-1}) was carried out. The traditional sequential approach, although widely used, involves a large number of runs and does not enable us to establish the multiple interacting parameters; thus, experimental design methodology was used as optimisation criteria [26,27]. The main advantages of this methodology include the simultaneous screening of a large number of factors affecting response and the estimation of possible interactions. Factorial and composite central designs were used for approaching the response surface with regard to the optimal fluorescent signal. Fractional factorial design was used to determine which factors had an effect on the studied response. Afterwards, central composite design was used to optimise these influent parameters in the previously selected experimental domain.

Solid-phase extraction is a convenient tool for sample clean-up, achieving trace enrichment of the

analytes as well as removal of interfering components. Thus, prior to chromatographic separation, telmisartan was extracted from urine by a SPE procedure. The experimental variables that affect the extraction efficiency (recovery, %) such as the nature of the cartridge, the composition of washing and eluting liquids and the pH at which sample is applied were studied.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of two Waters Model 510 HPLC pumps, a Waters Model 717 Plus autosampler fitted with a 200 μl syringe and a Waters Model 474 Scanning fluorescence detector (Barcelona, Spain).

The fluorescence detector was set at an excitation wavelength of 305 nm and an emission wavelength of 365 nm. The other instrumental parameters, gain and attenuation, were set at normal values (10 and 256, respectively). Chromatograms were recorded by means of a computer and were treated with the aid of the software MILLENIUM 32 CHROMATOGRAPHY MANAGER from Waters.

A Waters Nova-pak C_{18} column, 3.9 × 150 mm I.D., 4 μm was used to perform separation. Prior to the analytical column, a Waters Nova-pak C_{18} guard column 3.9 × 20 mm I.D., 4 μm was placed to prevent column degradation. The column was kept at room temperature during analysis.

Urine samples were centrifuged in a 5804 R Eppendorf refrigerated centrifuge (Hamburg, Germany) prior to the solid extraction step. SPE columns were placed in a vacuum manifold system from Supelco (Bellefonte, PA, USA) coupled to a vacuum pump from Millipore (Bedford, MA, USA). The extracted urine samples were evaporated to dryness under a gentle nitrogen stream using a Zymark Turbovap Evaporator LV from Varian (Barcelona, Spain).

pH measurements were made using a Radiometer Copenhagen PHM84 pH-meter (Bargsvaerd, Denmark) using a Crison 5209 glass-combined electrode (Barcelona, Spain) with a reference system Ag/AgCl and electrolyte KCl 3 M sat. AgCl.

2.2. Reagents and solutions

Telmisartan, active substance, was kindly provided by the manufacturer Boehringer Ingelheim (Ingelheim, Germany).

Stock solution of telmisartan (1000 mg l^{-1}) was prepared in methanol and was stored at 4 °C in the dark to avoid degradation. Working solutions were prepared daily by appropriate dilution of this stock solution.

Acetonitrile, methanol, ethyl acetate and tetrahydrofuran were Lab-Scan HPLC grade (Dublin, Ireland). All reagents used were Merck pro-analysis (Darmstadt, Germany). Deionised distilled and ultrapure water used in all the experiments was obtained from Milli-RO and Milli-Q systems, respectively (Millipore, Bedford).

The buffer solutions were H_3PO_4/KH_2PO_4 (pH 2; 1 M), CH_3COOH/CH_3COONa (pH 4.5; 1 M), KH_2PO_4/K_2HPO_4 (pH 6; 1 M) and $Na_2B_4O_7$ (pH 9, 1 M). In order to achieve the required pH value, different volumes of 1 M HCl and KOH solutions were added to diluted buffered solutions.

Solid phase extraction was studied using non-endcapped 1 ml per 100 mg Varian Bond Elut C_8 columns (Barcelona, Spain). Some other packing materials were assayed such as C_{18} and C_2 (same characteristics).

2.3. Chromatographic conditions

The separation was performed using acetonitrile–phosphate buffer (pH 6, 5 mM) (45:55, v/v) as mobile phase. All eluents were filtered through a 0.45 μm type HVLP Durapore membrane filter (Millipore, Dublin, Ireland) and the residual air was removed from them by bubbling helium through.

The volume injected into the chromatographic system was 20 μl. The assay was performed at room temperature and the flow was fixed at 0.5 ml min^{-1}. The effluent was monitored at excitation and emission wavelengths of 305 and 365 nm, respectively.

2.4. Urine samples collection

Blank urine samples were obtained from several healthy female and male volunteers aged 20–50 years-old who were not under medical treatment.

Urine samples were obtained from hypertensive patients under treatment with Micardis® and Pritor® (telmisartan—80 mg). All samples were collected for 36 h at different time intervals (0–2, 2–4, 4–8, 8–24, 24–36 h after their daily oral dose).

All urine samples were collected in polypropilene tubes and frozen at −20 °C until analysis.

2.5. Solid-phase extraction of urine samples

Once thawed and vortex-mixed, 1 ml of human urine was mixed with 0.5 ml of phosphate buffer (pH 6, 0.1 M). The mixture was shaken and centrifuged for 5 min at 3500 rpm ($16.1 \times g$) at controlled temperature (4 °C) to avoid either decomposition or biological activity.

The Bond Elut C_8 cartridge (1 ml per 100 mg) was conditioned with 2 ml of methanol and 1 ml of phosphate buffer, (pH 6, 0.1 M). The cartridge was not allowed to dry before the application of 1 ml of the previously buffered sample. The application of the sample was carried out in a very low vacuum ($P < 5$ mmHg) which enabled slow passage of the sample through the cartridge. The column was then washed with 1 ml of a mixture of methanol–phosphate buffer (pH 6; 0.1 M) (30:70 v/v) and dried in a full vacuum ($P > 20$ mmHg) for 20 min. Telmisartan was eluted with 1 ml of pure methanol.

Afterwards, the eluate was dried under a stream of nitrogen at 40 °C. The remaining residue was dissolved in 0.5 ml of the mobile phase and this solution (20 μl) was injected into the chromatographic system under optimal conditions.

2.6. Assay validation

The validation was performed in accordance to the summary report of the conference on "Analytical Methods Validation: Bioavailabilty, Bioequivalence and Pharmacokinetics Studies" [25], which has provided guidelines for pharmacokinetics studies in humans and animals.

Calibration data were generated by spiking blank urine samples with the appropriate volume of stock solution of Telmisartan yielding concentrations of 1, 2, 5, 10, 50, 100 and 1000 μg l^{-1}. Calibration curves were represented by plots of the peak areas versus concentration and were fitted to the linear regression $y = a + bx$.

The limit of quantitation was defined as the lowest concentration in the calibration curve that can be measured with acceptable accuracy, precision and variability. A maximum intra-day coefficient of variation of 20% and a maximum deviation from the nominal value of 20% were allowed. It was determined by using at least five urine samples spiked with appropriate volume of Telmisartan stock solution.

The intra-day and inter-day accuracy and precision of the method were evaluated using quality control (QC) samples of different known concentration levels (1, 100 and 1000 μg l^{-1}). Intra-day accuracy and precision were determined by assaying five replicates of each concentration level of the above mentioned QC samples in a single run. Inter-day precision and accuracy was established over a 1-month-period. Precision was characterised by the coefficients of variation (CV, %) whereas accuracy was expressed as a percentage error of nominal versus measured concentration (RE, %). The limits of acceptable variability were set at 15% except for the limit of quantitation concentration level, which allows up to 20%.

Five different lots of urine donations from male and female volunteers were carefully evaluated for interference in the assay.

The stability of Telmisartan was investigated during one sample run in the autosampler at room temperature after several hours of reconstitution following urine clean-up. The stability of Telmisartan through several freeze–thaw cycles was also investigated.

Extraction efficiency was calculated by replicate extraction of urine samples spiked with Telmisartan at three concentration levels (1, 100 and 1000 μg l^{-1}).

Recoveries were estimated against the initial amount of analyte spiked on the matrix, using

linear calibration curves based on standard solutions of telmisartan.

3. Results and discussion

3.1. Chromatographic conditions

Experimental design was used to optimise the chromatographic variables that had an important influence on the fluorescent response. Two different kinds of designs were applied: firstly a fractional factorial design, to evaluate which of the studied variables had an influence on the response, and secondly, the central composite design to obtain the response surface from which the optimal conditions for the target response could be deduced.

3.1.1. Fractional factorial design

The chromatographic variables considered in the fractional factorial design were pH, buffer concentration (mM), percentage of organic modifier (%) and flow (ml min^{-1}). Temperature was not considered in this design because the separation of ARA II gave narrow and symmetrical peaks at room temperature. Detection wavelengths (excitation and emission at 305 and 365 nm, respectively) were fixed considering the fluorescence spectrum of telmisartan and a previous work dealing with pK_a determination by spectrofluorimetry of this antagonist [28].

When applying experimental design methodologies, it is advisable to keep the number of variables as low as possible in order to avoid very complex response models and large variability [29]. Thus, the 2^4 experiments needed to complete a whole factorial design were reduced by introducing a confounding and running the so-called fractional factorial design.

This design confounds some main effects with interactions or interactions among themselves, resulting in a smaller set of experiments and nevertheless, it is able to identify the influence of each parameter as well as first-order interactions between factors. Fractional factorial design involves 2^{k-p} experiments, where k is the number of factors studied and p accounts for the degree of fractionality of the fractional factorial design ($p < k$) [30]. In this case, the main effect estimate for factor flow was confounded with the estimate of the interaction effect for pH, buffer concentration and acetonitrile percentage as shown in Eq. (1). Number of runs was then reduced from 2^4 to 2^3. The design generator $I = 1234$ was defined in this way as pH, buffer concentration and acetonitrile percentage was expected to be a non-significant interaction.

$$\text{Flow} = \text{pH} \times \text{buffer conc} \times \%\text{ACN} \quad (1)$$

A two level fractional factorial design involving eight runs and three replicates of the central point was carried out. Matrix for fractional factorial design is shown in Table 1.

The value of the flow in the experiments was fixed according to the product in coded variables of the other three variables. Level codification (Table 2) was established taking into account the domain where experiments could be easily interpreted. pH interval included telmisartan pK_a value [28] to study variations related to ionic or non-ionic forms. Limit values for flow and buffer concentration were usual working parameters. Percentage of organic modifier, one of the theoretically most influent factors, was varied in order to get more strong or weak mobile phases. Acetonitrile was chosen as organic modifier considering some previously carried out randomised experiments. This solvent assured better peak shape compared with methanol, being both of them the most frequently used HPLC solvents.

A combined response was defined to evaluate the model built with these four parameters. The choice of quality criteria is problematic because the separation requirements are often unclear and difficult to express quantitatively. Theoretically, they should take into account information concerning the quality and time taken for the separation [31]. Analysis time, being a critical factor affecting chromatography, we intended to shorten it by minimising retention time variable. In terms of quantitation, high peak height or peak area (the last chosen in most of cases) is desirable, so maximisation was the target for this variable. Although less important, peak width value could not be very high in order not to compromise

Table 1
2^{4-1} fractional factorial design matrix for telmisartan

Experiment number	% ACN	pH	Buffer concentration (mM)	Flow (ml min^{-1})
1	−1	−1	−1	−1
2	+1	−1	−1	+1
3	−1	+1	−1	+1
4	+1	+1	−1	−1
5	−1	−1	+1	+1
6	+1	−1	+1	−1
7	−1	+1	+1	−1
8	+1	+1	+1	+1
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0

Table 2
Factors and levels of the 2^{4-1} fractional factorial design

	−1	0	+1
% ACN	30	50	70
pH	3	4.5	6
Buffer concentration (mM)	5	15	25
Flow (ml min^{-1})	0.8	1	1.2

resolution from matrix interferences in urine samples. Similar criteria were also corroborated by other authors [32–34]. By means of these criteria, a global response was defined as stated in Eq. (2).

$$R = A/(t_R \times w_{1/2}) \quad (2)$$

where A is peak area (mV s), t_R is the retention time (min) and $w_{1/2}$ peak width at medium height (min).

The results of the screening design are shown in Table 3.

Analysis of the results was performed using the non-linear regression analysis program (NLREG) [35]. The most general polynomial function for response and variables is shown in Eq. (3).

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_{ij} \beta_{ij} x_i x_j \quad (3)$$

where Y is the studied response, x_i, x_j the variables considered in the study and β_0, β_i, β_{ij} the numerical parameters to be calculated. The final estimation of these parameters is achieved when square sum of errors (U) is minimised (Eq. (4)).

$$U = \sum_i^n (Y_{exp} - Y_{calc})^2 \quad (4)$$

where n is the number of experiments Y_{exp} is the response measured and Y_{calc} is the response given by the program following the proposed regression model.

The analysis of the output was based on the evaluation of the prob (t) parameter associated with each β_i parameter. To accomplish t statistic probability NLREG uses the value of final estimate for the parameter and the standard error, carrying out a two-sided test based on t statistic (relation between estimated value of parameter and its standard error). Prob (t) indicates the probability of β_i being zero, thus discarding critical influence of variable x_i corresponding to that β_i upon a critical parameter. The parameters whose probability of being zero was greater than 10% (prob $(t) \geq 0.1$) were systematically eliminated.

The new regression model without the eliminated parameters was then re-evaluated by NLREG. This process was repeated until no β_i parameter had a prob (t) value higher than 0.1. When this happened, the last proposed model was accepted.

The initial regression model proposed to evaluate the response is shown in Eq. (5):

Table 3
Matrix of trials and responses in the fractional factorial design for telmisartan

Experiment number	% ACN	pH	Buffer concentration (mM)	Flow (ml min^{-1})	$R \times 10^{-4}$ (mV s min^{-2})
1	30	3	5	0.8	6.04
2	70	3	5	1.2	313
3	30	6	5	1.2	61.6
4	70	6	5	0.8	199
5	30	3	25	1.2	18.1
6	70	3	25	0.8	217
7	30	6	25	0.8	83.6
8	70	6	25	1.2	76.3
9	50	4.5	15	1	288
10	50	4.5	15	1	287
11	50	4.5	15	1	275

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (5)$$

where Y was the response, x_1 the percentage of acetonitrile (%), x_2 was the pH, x_3 was the buffer concentration (mM) and x_4 was the flow (ml min^{-1}). This was the most general allowed model function concerning this 2^{4-1} fractional factorial design. Design generator being $I = 1234$ (Rs IV design) set of aliases ($1 = 234$, $2 = 124$, $3 = 124$, $12 = 34$, $13 = 24$, $23 = 14$) should be taken into account and so, model parameters are limited as reflected in Eq. (5).

Different regressions were assayed from the most general function allowed for this design (Eq. (5)). The choice criterion was the best fit to the regression (percentage of variance explained). The final equation obtained for telmisartan response was Eq. (6).

$$Y = -1051.96 + 43.06x_1 + 67.15x_2 - 1.56x_1x_2 \quad (6)$$

From this function, it could be concluded that only pH value and percentage of acetonitrile affect the response. Both factors appear either in individual and interaction factors. The individual terms for both factors have a positive numerical parameter, while the interaction parameter is negative. Thus, it was decided to run a more complex experimental design to exhaustively set the influence of these factors on experimental response.

3.1.2. Central composite design

From the results of the fractional factorial design two experimental variables were excluded, since they had no influence on the studied response.

Next, a central composite design was built using the remaining two variables: pH (x_2) and percentage of acetonitrile (x_1). A central composite design consists of a full factorial design plus a orthogonal star design. In this case the full factorial design was a two-level one (2^2, experimental levels -1 and $+1$) and the codified levels for the star points were $-\sqrt{2}$ and $+\sqrt{2}$.

This central composite design involved eight runs plus three replicates of the central point. The design matrix for the experiments is given in Table

Table 4
Design matrix for $2^2+2\times2$ central composite design

Experiment number	% ACN	pH
1	-1	-1
2	$+1$	-1
3	-1	$+1$
4	$+1$	$+1$
5	$+\sqrt{2}$	0
6	$-\sqrt{2}$	0
7	0	$-\sqrt{2}$
8	0	$+\sqrt{2}$
9	0	0
10	0	0
11	0	0

4 and level codification for this design is shown in Table 5.

The two rejected variables from the fractional factorial design were fixed to carry out the central composite design. The buffer concentration was fixed at its lowest level (5 mM), so as to minimise precipitation, usually occurring at high phosphate buffer concentration in acetonitrile media. The flow was fixed at 1 ml min^{-1}. If faster analysis was needed, flow could easily be increased, but it was not necessary, since retention times were highly satisfactory. The assayed central composite design and the studied response values (the same than in the fractional factorial design) can be seen in Table 6.

The regression model proposed for this design is given in Eq. (7).

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{112} x_1^2 x_2 + \beta_{122} x_1 x_2^2 \quad (7)$$

where Y is the studied response, x_1 is the percentage of acetonitrile and x_2 is the pH.

The data collected in the Table 6 were analysed by NLREG. From the output data the following equation (Eq. (8)) could be deduced. Model choice criteria were the same as in fractional factorial design.

$$Y = 104.07 x_2 + 0.20 x_1^2 - 6.83 x_1 x_2 - 19.23 x_2^2 + 0.98 x_1 x_2^2 \quad (8)$$

The percentage of explained variance associated to this equation was 99.88%, which represents an excellent adjustment of the experimental values to the theoretical model. pH appears in four factors: an individual positive first-order factor, two quadratic terms and a negative interaction factor with acetonitrile. Response is maximised at the highest pH values of the experimental domain, as result of two contributions. Firstly, at high pH values

Table 5
Level codification for the central composite design

	$-\sqrt{2}$	-1	0	$+1$	$+\sqrt{2}$
% ACN	20	30	50	70	80
pH	2	3	4.5	6	7

Table 6
Design matrix and response in the central composite design of telmisartan

Experiment number	% ACN	pH	$R \times 10^{-4}$ (mV s min^{-2})
1	30	3	16.26
2	70	3	574.83
3	30	6	116.71
4	70	6	947.64
5	80	4.5	624.28
6	20	4.5	1.28
7	50	2	88.94
8	50	7	484.69
9	50	4.5	281.47
10	50	4.5	283.35
11	50	4.5	304.02

($pH > pK_a$) telmisartan is ionised and is not strongly retained in the column, so retention time is smaller and secondly, fluorescent intensity is very dependent on pH [28], becoming higher at basic pH values. Percentage of acetonitrile appeared in both positive terms, first-order and quadratic. The experimental behaviour is in accordance with the explained by reversed-phase mechanisms in chromatography. As acetonitrile percentage is increased, eluotropric strength is greater and the analyte is rapidly eluted so response R is higher. Thus, it seems as both variables (pH and acetonitrile percentage) should be set at their highest levels (7 and 80) in order to maximise the response. Nevertheless, chromatographic requirements made us set the organic modifier percentage at 45% in order to achieve good separation of telmisartan from matrix interferences. Finally, optimal analysis conditions were pH 6 (fluorescence of telmisartan is enhanced [28]) and 45% of acetonitrile in the mobile phase. As it can be seen on the three-dimensional plot of response surface (Fig. 2), these conditions also gave acceptable values of response.

3.2. Urine samples clean-up

Effect of sample pH was clearly stated with all the solvents and packings. Urine buffered at pH 6 gave the highest recoveries in all the cases, stronger ionisation of telmisartan at pH 9 produced important losses of analyte (REC < 50%) due to its

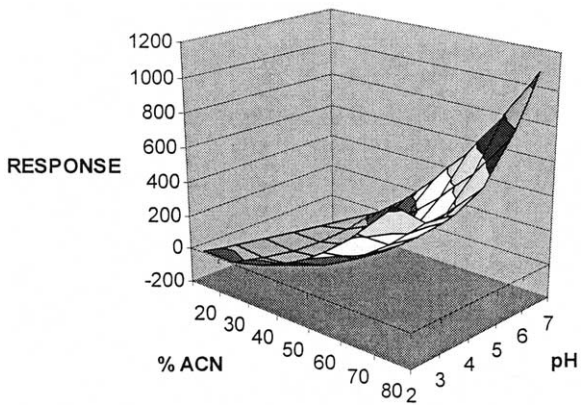

Fig. 2. Response surface for telmisartin chromatographic behaviour using the central composite design.

weak interaction with apolar cartridges. Consequently, the cartridge was conditioned at pH 6 with phosphate buffer and the urine sample was applied to the cartridge at the same pH. As selectivity is an important requirement for optimising an SPE procedure, THF was discarded because it produced important interfering peaks. Methanol provided better recoveries than ethyl acetate, whereas this last gave somewhat cleaner extracts. Extraction efficiency was initially preferred to selectivity and pure methanol was selected as eluting solvent. In order to choose an appropriate washing solvent to enhance selectivity, elution of telmisartan was examined as a function of the composition of mixtures methanol–phosphate buffer (pH 6; 0.1 M). The elution profiles obtained from plots of recovery against percentage of methanol in the mixture gave information about the best composition for the washing solvent (that containing the largest amount of methanol without eluting the drug) and also the best elution solvent (the lowest amount of methanol affording complete recovery of the drug). The washing solution chosen (methanol–phosphate buffer, pH 6; 0.1 M (30:70, v/v)) eliminated the endogenous compounds from the matrix without eluting the analytes, yielding a chromatogram free of interferences. Pure methanol was needed for total elution of telmisartan.

3.3. Assay validation

Figures of merit for the assay validation of HPLC-fluorimetric of telmisartan are shown in Table 7.

Linear range for the standard curve of Telmisartan in spiked urine was 1–1000 μg l^{-1}. The mean correlation coefficient was 0.999. The application of this SPE–HPLC-fluorimetric method to the quantitation of telmisartan in clinical samples from patients undergoing treatment with Micardis and Pritor 80 (telmisartan—80 mg) confirmed the appropriate concentration range of the calibration curve.

The limit of quantitation (LOQ) is 1 μg l^{-1} and allowed telmisartan quantitation in samples from the whole time interval (from 0 to 36 h). Thus, this method is sensed to quantitate telmisartan even though the small amount excreted unaltered in urine ($<1\%$) due to its low LOQ.

Intra- and inter-day found concentrations were within $\pm 6\%$ the nominal value, except for the LOQ level where accuracy was 88%. Intra- and inter-day precision in the matrix did not exceed the 8% CV, except for LOQ concentration level where relative standard deviation reached 15%. As it can be seen, statistical evaluation of the data established agreement with the guidelines on validation [25].

Extraction efficiency calculated from spiked urine samples at QC concentrations levels of 1,

Table 7
Validation assay of HPLC-fluorimetric determination of telmisartan in spiked human urine

	Telmisartan
Linear concentration range (μg l^{-1})	1–1000
Precision (CV)[a]	
Intra-day	<7.6
Inter-day	<5
Accuracy (%RE)[b]	−12–6
LOQ (μg l^{-1})	1.0

[a] Precision values were studied at three concentration levels: 1, 100 and 1000 μg l^{-1}. Data presented in this table are the maximum values found.

[b] Accuracy values were studied at three concentration levels: 1, 100 and 1000 μg l^{-1}. Data presented in this table are summarised for all concentration values.

100 and 1000 μg l^{-1} are similar (ca. 78%) and variability is included in the deviation associated to species variability.

Selectivity was guaranteed by studying influence of five different matrices on chromatogram endogenous peaks, none of these matrices showed interfering peaks for telmisartan determination by the proposed HPLC-fluorimetric method.

3.4. Application to real samples

The developed method was applied to the determination of Telmisartan in urine samples obtained from hypertensive patients. Fig. 3 shows the chromatograms of urine samples spiked with 1 μg l^{-1} of telmisartan compared with samples obtained from patient #1 undergoing treatment with Pritor 80 mg (24–36 h interval) (concentration 0.93 μg l^{-1}). Concentration values were interpolated from the daily calibration curves of this ARA II.

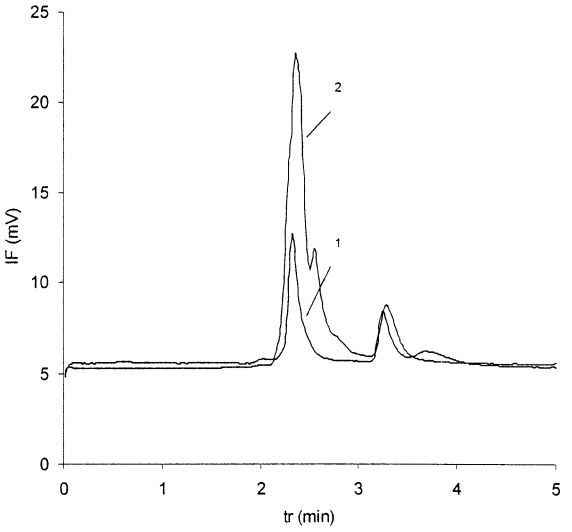

Fig. 3. Chromatograms of (1) a human urine sample spiked with 1 μg l^{-1} of telmisartan and (2) a human urine sample obtained from patient #1, 24–36 h after administration of one tablet of PRITOR 80 mg (concentration of telmisartan 1 μgl^{-1}).

4. Conclusions

HPLC with fluorimetric detection is specific, sensitive and precise enough for the determination of telmisartan in urine samples. The validation of the assay is adequate in terms of generally accepted guidelines for linearity, limit of quantitation, precision and accuracy in the bioanalytical laboratory. Chemometric approach allowed us to reduce the number of experiments needed for chromatographic optimisation, as well as the attainment of a true optimum set of conditions. Separation was accomplished in less than 5 min. Analysis time (including extraction step for 12 samples) can be carried out in 1 h.

The SPE procedure is very simple and effective and provided no interference peaks for endogenous components, then method was proved to be selective.

Fluorescence detection provided excellent LOQ value, thus allowing quantitation of telmisartan in urine despite the minor excretion pathway for this compound.

Future investigations would deal with application of the validated method to plasma samples.

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