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Use of teicoplanin stationary phase for the enantiomeric resolution of atenolol in human urine by nano-liquid chromatography–mass spectrometry

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Abstract

Nano-liquid chromatography (nano-LC) was used for the enantiomeric resolution of atenolol employing a teicoplanin modified silica stationary phase prepared in our laboratory. Experiments were carried out in a fused silica capillary of 75 μm i.d. packed with chiral modified silica particles of 5 μm diameter. Separated enantiomers were revealed by on-line UV detector at 205 nm or electrospray-ion-trap mass spectrometer (ESI-MS). Atenolol enantiomers were eluted utilizing a mobile phase with the following composition: 500 mM ammonium acetate pH 4.5/methanol/acetonitrile 1:60:39 (v/v/v) allowing to achieve good enantioresolution in a reasonable analysis time (about 8 min) with a flow rate of about 900 nL/min. After comparing the sensitivity of the nano-LC method using a conventional UV detector for capillary electrophoresis, a zeta cell (3 nL volume) employed in nano-LC and the ion-trap MS the method was validated with the MS detector offering the highest sensitivity (limit of detection (LOD) = 50 ng/mL; limit of quantification (LOQ) = 400 ng/mL for each atenolol enantiomer). (−)-Ψ-Nor-ephedrine was used as the internal standard. The method was successfully applied to the analysis of atenolol enantiomers present in human urine samples of a patient under atenolol therapy.

Keywords: Nano-liquid chromatography; Enantiomers; Chiral; Drugs; Teicoplanin

1. Introduction

In the last two decades, great attention was paid by several researchers to the development of separation methods capable to resolve chiral compounds with the aim to use them for both analytical and preparative purposes. The developed analytical methods have been widely applied to the analysis of enantiomers in different fields such as biological, pharmaceutical, biomedical, environmental, etc. where such type of molecule need to be monitored in order to carry out studies, e.g., pharmacokinetic, chiral purity control, etc. [1].

Several analytical methods have been employed for the separation of enantiomeric compounds making use of a chiral environment interacting with the two analytes either before or during the separation process. Among these techniques it is noteworthy to mention gas chromatography (GC), thin layer chromatography (TLC), high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC) [2–10]. Using HPLC due to the advantages of this technique over others, e.g., excellent precision, sensitivity, robustness, etc., have carried out most of the enantiomeric separations. In spite of the above-mentioned advantages several groups investigated the possibility of using miniaturized methods such as CZE, CEC and recently capillary/nano-liquid chromatography (CLC/nano-LC) for the separation of chiral compounds [6–14].

The separation of chiral compounds with miniaturized methods has been achieved using several chiral selectors and

Abbreviations: Nano-LC, nano-liquid chromatography

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among them teicoplanin, a glycopeptide antibiotic, resulted to be very effective for the separation of a wide number of enantiomers [7,15–17].

4-(2-Hydroxy-3-isopropylaminopropoxy)phenylacetamide (atenolol, ATN) is a β-adrenaceptor antagonist also known as β_1-blocker mainly used for the treatment of heart pathologies. As a drug, is commonly used in order to treat angina pectoris, hypertension, cardiac arrhytmia, myocardial infarction, etc. [18–20]. It has been reported that mainly (*S*)-atenolol exhibits the pharmacological activity and therefore analytical methods capable to separate and analyze ATN enantiomers are mandatory in order to easily carry out studies of biological fluids such as urine and serum [18]. The enantioselective analysis of (*R*)- and (*S*)-atenolol in urine samples was demonstrated by several groups. Recently, Lamprecht et al. separated the two enantiomers by HPLC with a column-switching setup and a teicoplanin stationary phase [19] while Iha et al. analyzed the ATN enantiomers in both human urine and plasma comparing liquid–liquid and solid-phase extraction methods [20] employing HPLC and a Chiralcel OD-H column.

To our best knowledge, the analysis of ATN enantiomers in urine, by using nano-liquid chromatography, has not been reported. Therefore, in this study a new separation method is presented with the aim to use a laboratory assembled nano-LC instrumentation and a fused silica capillary packed with teicoplanin stationary phase both prepared in our laboratory for the separation of these compounds.

In order to demonstrate the applicability of the method to practical analysis of ATN enantiomers present in human urine, the extracted samples were analyzed and revealed with an UV detector and an ion-trap mass spectrometer. The capillary was connected with the MS through an ESI-nano-spray interface acquiring ion-chromatograms, MS and MS–MS spectra as well.

2. Experimental

2.1. Reagents and chemicals

Ammonium acetate, methanol (MeOH), acetonitrile (MeCN), all of HPLC analytical grade, were purchased from BDH (Poole, England). Racemic atenolol, *S*-(−)-, *R*-(+)-atenolol and (−)-Ψ-nor-ephedrine were purchased from Sigma (St. Louis, MO, USA) while ammonia solution (30%) and acetic acid were from Carlo Erba (Milan, Italy).

Mobile phases were daily prepared by mixing the appropriate volumes of organic solvents, water and buffer solutions.

LiChrospher diol silica phase 5 μm particle diameter, sodium cyanoborohydride and sodium periodate were from Merck (Darmstadt, Germany). Stock standard solutions (1 mg/mL) of analyte and internal standard (I.S.) were prepared by dissolving suitable amounts of pure substances in methanol. Standard solutions were prepared by diluting, at the desired concentrations, the stock mixtures with methanol and injected for the nano-LC experiments.

2.2. Instrumentation

The instrumentation used for nano-LC experiments was prepared in our laboratory employing apparatus used for either HPLC or CE properly modified. The mobile phase was delivered at a flow rate of about 900 nL/min by using a Spectrasystem P2000 HPLC pump (Thermo Separation Product, Fremont, CA, USA) splitting the flow originally at 1000 μL/min. A stainless steel T piece (Valco, Houston, TX, USA) was connected to the pump through a PEEK capillary 50 cm × 127 μm i.d.; connection to the injector and to waste was done using a stainless steel tube 5 cm (100 μm i.d.) and 50 cm (50 μm i.d.) fused silica capillary, respectively. The split ratio using 75 μm i.d. packed capillary was about 1/1100. A 60 nL injection valve (Vici Valco Instruments, Houston, TX, USA) was used for sampling while UV detection at 205 nm was done employing a multiwavelength UV–vis Spectra Focus detector (Thermo Separation Product, Fremont, CA, USA) or an UV detector with U–Z View Flow Cell LC Packings (Amsterdam, the Netherlands). Data were acquired on-line with a Compaq 486 computer with an appropriate software (Thermo Separation Product) or with a laboratory assembled computer with a Pentium 4 and a Chromeleon, respectively. MS and MS–MS spectra of the separated enantiomers were acquired with a LCQ mass spectrometer, ThermoFinnigan (St. Josè, CA, USA); the teicoplanin CSP capillary was connected to the MS through a nano-electrospray ion source (nano-ESI), Thermo-Finnigan.

A Pico Tip™ emitter (New Objective, Inc., Cambrige, MA, USA), standard coated silica Tip (360 μm/75 μm o.d. × 30 μm i.d. × 10.7 cm) was positioned at 1–2 mm from the MS orifice. Separated enantiomeric zones were detected in the positive ion mode; capillary voltage was 46 V while the ion-spray voltage was 1.7 kV; capillary temperature, 160 °C.

The marker used to determine the dead volume (t_0) was methanol or acetone and the perturbance of the baseline observed in the chromatogram. Retention and enantioresolution factors were calculated by using the following equations:

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

$$\alpha = \frac{k_2}{k_1} \quad (2)$$

where t_R, t_0 are the retention time of analytes, dead time of enantiomer 1 and 2 first and second eluted, respectively.

2.3. Capillary column and chiral stationary phase preparation

Fused silica capillaries of 75 μm i.d. and 375 μm o.d. were purchased from Composite Metal Services (Hallow, UK) and

were packed in our laboratory with chiral stationary phase (CSP) (diol-silica particles modified with teicoplanin). The synthesis of the teicoplanin CSP was done using a method employed for the preparation of vancomycin CSP previously published by our group [21].

Briefly, 400 mg of LiChrospher DIOL silica 5 μm 100 Å particles were suspended in 30 mL of water:methanol 4:1 (v/v)–60 mM of $NaIO_4$ mixture and sonicated for 1 h in order to oxidize the diol to aldhehyde groups. The mixture was centrifuged at 4000 rpm for 5 min and the solution (of periodate) was eliminated. The silica phase was washed three times with 20 mL of water. One hundred and seventy two milligrams of teicoplanin (M_w: 1885 Da) was dissolved in a mixture of 50 mM NaH_2PO_4 titrated with NaOH to 7.04 and 3 mM of $NaCNBH_3$. The final concentration of teicoplanin was 3 mM. The solution was added to oxidated diol silica, sonicated for 1 h, centrifuged and the phase washed three times with water. Finally, the modified phase was treated with 30 mL of 50 mM phosphate buffer pH 3.1 and $NaCNBH_3$; sonicated for 1 h, centrifuged and the stationary phase recovered. The teicoplanin-CSP was washed (three times) with water, methanol and dried under vacuum at 30 °C.

The fused silica capillary was coupled with a 10 cm × 4.1 mm i.d. stainless steel HPLC pre-column, filled with the slurry (LiChrospher 100 RP18 5 μm particles in acetone) and connected to the HPLC pump. A mechanical HPLC frit (Valco) was used to retain the packed particles. The reservoir was removed, cleaned, filled with water and connected again to the capillary that was flushed for 30 min; the frit was prepared with the heated wire (about 1100 °C × 6 s).

The capillary was cut close to the end frit, connected to the pump and flushed with water in order to eliminate the excess of the stationary phase. The capillary was again connected through the open end with the pre-column containing the slurry of teicoplanin stationary phase suspended in a mixture of acetone:water (1:1, w/w) and packed for 22.8 cm. Finally, the capillary was packed with the RP18 particles (5 cm) and after flushing with water the frit prepared and the free stationary phase eliminated.

2.4. Analysis of atenolol enantiomers in human urine

Urine samples were collected from a patient under atenolol therapy at different times, t_0 before the intake of 12.5 mg of (*R*,*S*)-atenolol and after 90, 180 and 300 min.

In order to recover atenolol present in the urine samples we used a method similar to that one previous published by Iha et al. [20]. Briefly, 1 mL of urine was mixed with 4.5 mL of water and 0.25 mL of 8 M sodium hydroxide and the mixture extracted twice with 2.5 mL chloroform-2-propanol (4:1, v/v). The two aliquots were mixed, centrifuged at 2500 × *g* and evaporated using a nitrogen stream. The residues were dissolved in 2 mL of methanol and injected for the analysis.

3. Results and discussion

For the chiral separation of racemic atenolol we used a teicoplanin stationary phase packed in a fused silica capillary and different mobile phases containing a mixture of methanol–acetonitrile–ammonium acetate. Several parameters such as the pH of the buffer, its concentration and the content of organic modifier were changed in order to find the optimum experimental conditions allowing the highest enantiomeric resolution of the studied analytes. Optimum conditions were obtained eluting the racemic compounds with a mobile phase containing the above reported mixture in the ratio: 60:39:1, v/v/v; the pH and the concentration of the buffer were 4.5 and 500 mM, respectively.

Fig. 1 shows a typical chromatogram achieved analyzing racemic atenolol in presence of (−)-Ψ-nor-ephedrine (I.S.).

As can be observed the two enantiomers were baseline resolved in 8.3 min. Limit of detection (LOD) and limit of quantification (LOQ) were measured analyzing standard mixtures at different concentrations and measuring the signal-to-noise ratio (S/N 3 and 10, respectively) finding 3 and 10 μg/mL for each enantiomer, respectively. The same experiments were done utilizing the UV detector with a zeta-cell observing a higher sensitivity with S/N = 1.5 and 5 μg/mL of LOD and LOQ, respectively.

The nano-LC system was coupled with an ESI-MS nanospray in order to use a more sensitive and specific detector useful in order to analyze and characterize compounds present in complex matrices such as biological ones.

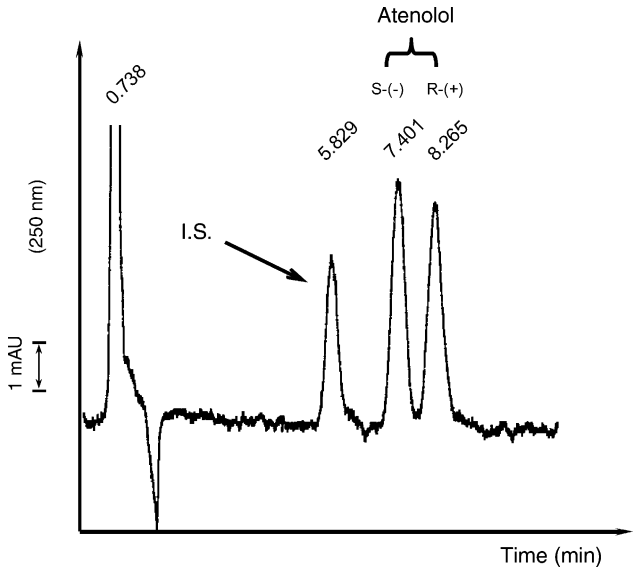

Fig. 1. Chromatogram of the enantiomeric separation of racemic atenolol in presence of (−)-Ψ-nor-ephedrine used as an I.S. *Experimental conditions*: Packed capillary with teicoplanin stationary phase (75 μm i.d.; 23.0 packed); mobile phase: methanol–acetonitrile–500 mM ammonium acetate pH 4.5 (60:39:1, v/v/v); UV detection at 205 nm; flow rate: 900 nL/min; injection 60 nL of extracted urine sample; concentrations of I.S. and racemic atenolol were 20 and 40 μg/mL, respectively.

Table 1
Comparison of relative standard deviation (R.S.D.%) observed by UV–vis detector and nano-spray ionisation-MS (NSI-MS)

	R.S.D.%						
	t_{R1}	t_{R2}	k_1	k_2	α	$A_1/A_{I.S.}$	$A_2/A_{I.S.}$
UV–vis	0.2	0.3	2.8	2.9	0.1	5.3	5.5
NSI-MS	0.5	0.8	1.9	2.3	0.9	7.2	6.3

For experimental conditions, see Fig. 1 and text.

Attention was paid to resolve some technical problems related to the use of the tip spraying because the commercial one provided by the producer of the nano-spray interface exhibited drawbacks, e.g., high costs and limited use because the metal coating of the capillary. When the tip was used connecting the spray voltage with the metal coating we were able to use this tip for only a few experiments, therefore the spray voltage of the MS was connected to the T-junction (capillary-column). Using this setup, the tip was used for at least 100 nano-LC runs. Baseline resolution of the atenolol enantiomers was achieved also using the MS detector.

The nano-LC-UV and -MS systems were compared analyzing a standard mixture containing atenolol enantiomers and I.S. and measuring the intra-day repeatability of retention times, retention factors, enantioselectivity, peak area ratios and enantioresolution. Concentrations of standard mixture were: UV, 20 and 40 μg/mL I.S. and racemic atenolol, respectively; MS, 50 and 2 μg/mL I.S. and racemic atenolol, respectively.

The results depicted in Table 1 clearly show that when using UV detector better repeatability of the various parameters studied than when using MS detector. However, the former data were quite satisfactory advising the use of MS for further studies because offering a higher sensitivity. The longer analysis time observed when the nano-LC system was coupled with the MS can be explained with the longer distance between the end of the CSP and the detector.

Based on the above discussed results experiments were carried out verifying the inter-day precision (three days, three replicate measurements) finding a good within day precision with R.S.D. of retention times, enantioselectivity (1.1 and 0.7%, respectively) while R.S.D. of peak area ratios were about 4.5%.

The elution order of atenolol enantiomers was verified by injecting the single enantiomers and the racemic mixture spiked with the *S*-(−)-atenolol; the *S*-(−)-isomer was firstly eluted in our nano-LC setup. The same elution order was reported by Lamprecht et al. [19] using a teicoplanin stationary phase in HPLC.

The atenolol enantiomers calibration graphs were linear in the concentration range 0.1–10.0 μg/mL for each enantiomer; the linearity data are reported in Table 2.

Considering the interesting results obtained, the optimized nano-LC method was applied to the assay of the two enantiomers present in the biological fluid of a patient under intake of 25 mg/day dose of racemic atenolol.

Table 2
Calibration data obtained separating the two enantiomers of atenolol

Features	Analyte	
	S-(−)-Atenolol	*R*-(+)-Atenolol
Regression equation	$y = 0.2626x - 0.0897$	$y = 0.2673x - 0.0788$
Correlation coefficient (R^2)	0.9988	0.9985
Standard deviation for the slope	2.538×10^{-4}	2.942×10^{-4}
Standard of deviation for the intercept	0.0130	0.0151
Linear range (μg/mL)	0.1–10.0	0.1–10.0

I.S. (−)-Ψ-nor-ephedrine 50 μg/mL. For experimental conditions, see Fig. 2 and text.

Fig. 2 shows the nano-LC–ESI-MS chromatogram of the analysis of (I) blank urine sample, (II) urine sample collected after 5 h intake, (a) MS spectra of the I.S. and (b) mass spectra of the two atenolol enantiomers. The two atenolol enantiomers clearly exhibited the same mass spectra with $m/z = 267.1$. The presence of atenolol was confirmed acquiring the MS–MS spectra finding three additional peaks at m/z 190.1, 225.0 and 249.8; these spectra showed the same m/z peaks obtained analyzing a standard racemic mixture of atenolol by infusion.

Accuracy and precision were assessed analyzing three extracted urine samples spiked with the racemic atenolol standard mixture at three concentration levels included in the linearity range; three replicates at each level were carried out. The results are reported in Table 3.

Several human urine samples were collected at different time from a volunteer under atenolol therapy (25 mg racemic atenolol for each day) and after liquid–liquid extraction analyzed in order to verify the urinary excretion of atenolol enantiomers at different times. As can be observed in Fig. 3 the concentration of atenolol enantiomers increased by increasing the time. Some difference of the enantiomeric ratio was noticed, but it was not remarkable; this can be explained considering that atenolol is metabolized very slightly [19].

Table 3
Accuracy and precision data for the assay of atenolol enantiomer in human urine by using nano-liquid chromatography with a teicoplanin capillary column

Analyte	Concentration (μg/mL)	Accuracy (recovery, %)	Repeatability (R.S.D.%), $n = 3$
S-(−)-Atenolol	1.0	104.7 ± 6.5	3.0
	3.0	97.0 ± 5.3	3.0
	5.0	96.4 ± 7.3	4.5
R-(+)-Atenolol	1.0	99.2 ± 6.0	2.4
	3.0	100.7 ± 5.9	3.5
	5.0	96.4 ± 4.7	3.0

For experimental conditions, see Fig. 2.

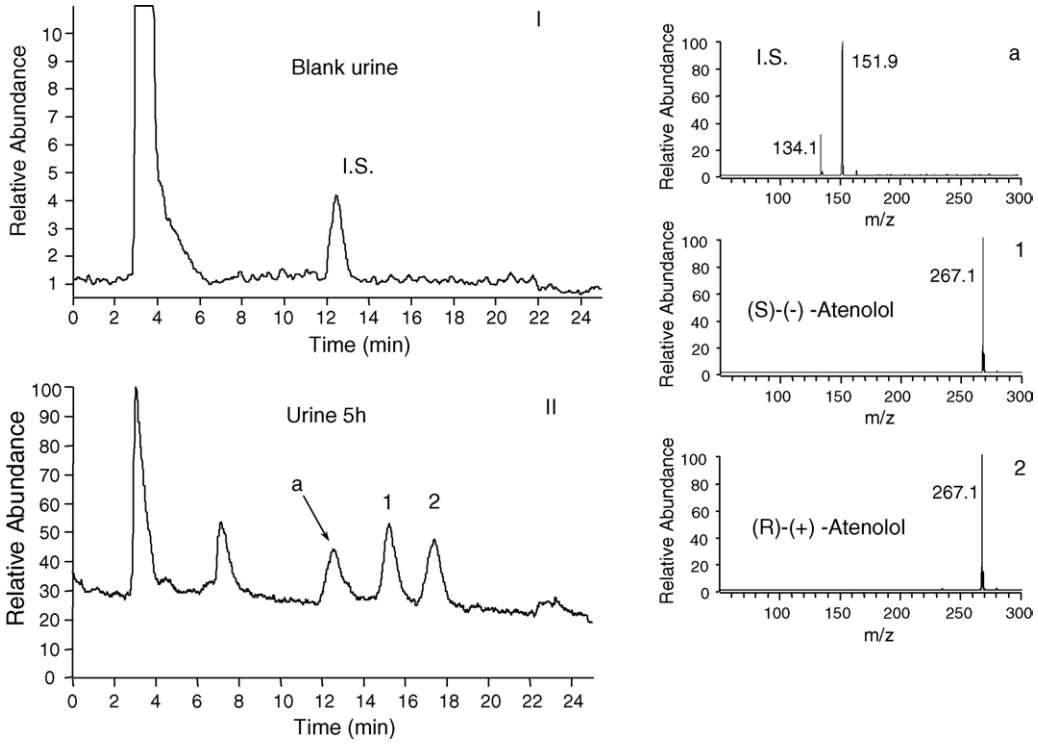

Fig. 2. Nano-LC–ESI-MS chromatogram of the analysis of human urine extract containing atenolol enantiomers and the internal standard (−)-Ψ-nor-ephedrine. (I) Blank urine with I.S. 50 μg/mL; (II) urine under atenol therapy with I.S. 50 μg/mL. (a) and (1) and (2) MS spectra of I.S., *S*-(−)- and *R*-(+)-atenolol, respectively. Mass range 50–300 *m/z*. Experimental conditions as reported in Fig. 1 and text.

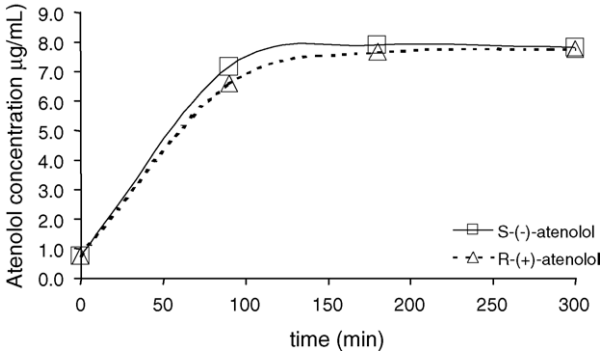

Fig. 3. Urinary excretion of atenolol enantiomers vs. time after oral administration of 25 mg racemic atenolol to a patient under therapy. For experimental conditions, see Fig. 2 and text.

4. Conclusions

Atenolol enantiomers were separated by nano-LC in a 75 μm i.d. packed capillary with teicoplanin stationary phase. Analytes zone were detected by an UV detector operated at 205 nm and with an ion-trap mass spectrometer acquiring ion chromatograms, MS and MS–MS spectra. Comparing the results obtained with in-column UV detector, 3 nL zeta cell and MS the best results concerning sensitivity were recorded when the MS detector was used (LOD = 50 ng/mL for each enantiomer). The method was tested studying several validation parameters finding good repeatability and sensitivity. The method was successfully applied to the analysis of atenolol enantiomers present in human urine samples of a patient under ATN therapy. The use of the MS detector allowed the measurement of the mass and MS–MS spectra of the studied compounds that allowed to confirm their presence in urine samples. The kinetic of ATN excretion in urine was followed and our data are in accord with those previously reported by other groups.

The nano-LC method allows achieving good results at low costs and minor environmental impact due to the minute amount of chiral stationary phases and lower volumes of mobile phases, respectively. Besides we achieved good results analyzing ATN enantiomers in urine samples, we did not apply the method to serum samples because not available in our laboratory.

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