Quantitative Analysis of Torsemide in human plasma by High Performance Liquid Chromatography with ultraviolet detection

Key words: Torsemide, liquid chromatography, ultraviolet detection, HPLC, human plasma.

Palabras clave: Torasemida, cromatografía líquida, detección ultravioleta, HPLC, plasma humano.

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Abstract

This report describes a specific and precise HPLC method for the quantification of torsemide in human plasma. The procedure included an efficient solid phase extraction, and the HPLC analysis used a phosphate buffer/acetonitrile mobile phase with a C18 column and ultraviolet detection at 288 nm. The mean absolute recovery ranged between 91.9% and 118.0%. The limit of detection was determined to be 0.010 μg/mL. The method described is suitable for torsemide plasma monitoring and pharmacokinetic studies. Data of three healthy volunteers are presented.

Resumen

Este informe describe un método de cromatografía líquida de alta resolución (HPLC) específico y preciso para la cuantificación de torasemida en plasma humano. El procedimiento incluye extracción eficiente en fase sólida y el análisis con HPLC, utilizando como fase móvil un tampón fosfato/acetonitrilo en una columna C18 y detección ultravioleta a 288 nm. La recuperación absoluta media osciló entre 91.9% y 118.0%. El límite de detección se determinó en 0.010 μg/mL. El método descrito es adecuado para el monitoreo y estudios farmacocinéticos de torsemida en plasma. Se presentan los datos de tres voluntarios sanos.

Introduction

Torsemide (1-isopropyl-3-[(4-m-toluidino-3-pyridyl) sulfonyl]urea) is a high ceiling loop diuretic indicated in the treatment of edema associated with congestive heart failure (CHF) and hepatic or renal disease.[1-4]

This diuretic acts from within the lumen of the thick ascending portion of the loop of Henle, where it inhibits the $Na^+/K^+/2Cl^-$-carrier system[1,5-7] increasing salt and water excretion.

Torsemide can be administered both orally and intravenously. It is rapidly absorbed when administered orally, and its bioavailability ranges from 70 to 96%.[1,2,6,8] Approximately 80% of total torsemide is cleared by hepatic metabolism and the remaining 20% by renal clearance (in patients with healthy renal function).[2,5]

At present, a comparison is being made between torsemide and other diuretics in the treatment of CHF. The most important differences between torsemide and other diuretics (e.g. furosemide and hydrochlorthiazide) are that the former has a lesser kaliuretic effect, and its bioavailability is less variable.[2,7,9] This improves treatment outcomes[10] and reduces health care costs.[10,11] Moreover, Murray et al.[10] have described that patients treated with torsemide were less likely to be readmitted because of heart failure or any other cardiovascular conditions, and also were less fatigued.

In order to make a more suitable evaluation of torsemide plasma levels and a better comparison between torsemide and other diuretics, it is necessary to use analytical methodologies that allow the quantification of this drug in biological fluids. Nowadays, many methods to quantify furosemide and other diuretics in human plasma, [12-17] but not so many methods to quantify torsemide,[18] are available.

The aim of this investigation was to develop and validate a method to quantify torsemide in human plasma by HPLC-ultraviolet (UV) detection.

Experimental

Chemicals. Torsemide was kindly donated by Boehringer Mannheim (Argentina). Acetonitrile and methanol used during the extraction and drug analysis were HPLC-grade and purchased from Sintorgan® (Buenos Aires, Argentina). Anhydrous potassium phosphate monobasic and anhydrous potassium phosphate dibasic were p.a. and purchased from Mallinckrodt (Mallinckrodt Chemical Works, USA). Hydrochloric acid was p.a. and purchased from Merck Química Argentina (Buenos Aires, Argentina). Bumetanide was kindly donated by Gramon Laboratories (Buenos Aires, Argentina). Double distilled water (pH 5) was obtained with a Figmay® distillatory system (Córdoba, Argentina).

Bond Elut-C18 cartridges (500 mg of sorbent mass, 6 mL column reservoir volume) were supplied by Varian (Harbor City, CA, USA).

Chromatography. The HPLC system (JASCO Corporation, Tokyo, Japan) was equipped with an intelligent HPLC pump (PU-980) with a ternary gradient unit (LG-980-02), an automatic sampler injector (AS-950), a UV/VIS detector (UV-975) and an on-line degassing system (Alltech, Deerfield, IL, USA). Data and chromatograms were collected and analyzed using a Borwin Chromatography Software

Table 1. Chromatographic conditions

Mobile phase	A: 28 mM phosphate buffer, pH 6.8 B: acetonitrile	
Gradient	Time (min)	% B
	0 – 7.5	25
	8 - 10	40
	10.5 - 14	25
Flow rate	0.8 ml/min	
Detector wavelength	288 nm	
Injection volume	20 µl	
Column temperature	Room temperature	
Run length	14 minutes	

(JMBS Developments, France). The C_{18} reverse phase column was a Lichrospher 100 5μm (Merck KGaA, Germany). The length of the separation column was 125 mm x 4 mm. Chromatographic conditions are shown in *table I*.

Standard solution and plasma samples. A torsemide stock solution was prepared in methanol to a final concentration of 1 mg/mL. A working standard solution of 100 μg/mL in methanol was prepared from the stock solution.

A bumetanide stock solution was prepared in methanol to a final concentration of 1 mg/mL. A working standard solution of 100 μg/mL in methanol was prepared from the stock solution.

Human plasma, adjusted to pH 5 with 2M HCl, was spiked with torsemide to reach final concentrations of 0.05, 0.1, 0.25, 0.5, 1, 3, 6 and 12 μg/mL and bumetanide as an internal standard in a concentration of 3 μg/mL. Blood samples were collected from young healthy volunteers. Plasma was separated by centrifugation for 10 minutes at 3000 rpm.

All solutions and plasma samples were stored at 4 ºC until analysis

Sample preparation and extraction procedures. Aliquots (1 mL) of spiked plasma samples were loaded onto a Bond Elut-C18 cartridges, previously conditioned with 5 mL of methanol and 5 mL of water. The columns were washed twice with 2 mL of water and dried. The elution was performed with 3 mL of methanol. The eluate was evaporated to dryness under a nitrogen stream. The residue was reconstituted in 100 μl of methanol and filtrated through a 0.45 μm pore diameter filter before the injection. Five blank unspiked samples were prepared using the same extraction procedure.

Method validation

Recovery and precision. The absolute recovery of torsemide was assessed on plasma specimens at three concentration levels (0.5, 3 and 12 μg/mL) by triplicate analysis. Intra-day precision involved three measurements of the same sample within a single run. Inter-day precision involved the estimation of the run to run variation of the same sample for three consecutive days. The extraction efficiency was determined by comparing the detector responses obtained for fortified sample peak areas with the peak areas resulting from direct injections of equivalent quantities of standard solutions. The recovery rate and coefficient of variation (%CV) were calculated under those conditions.

Linearity of standard curves. Three sets of eight 1.0 mL plasma samples were spiked with torsemide working solution resulting in final concentrations of 0.05, 0.1, 0.25, 0.5, 1, 3, 6 and 12 μg/mL and with bumetanide working solution resulting in a final concentration of 3 μg/mL. The linearity was tested under these conditions.

Limit of detection (LOD) and limit of quantification (LOQ). Both the LOD, defined as the lowest concentration that the analytical process can reliably differentiated from background noise, and the LOQ, defined as the lowest concentration that can be measured with stated level of confidence, were calculated as described by Quattrochi[19] and Shah et al,[20] respectively. Briefly, LOD was estimated by the injection of five blank unspiked plasmas and the integration of baseline noise of the system in the area covering the mean retention time of torsemide (between 4 and 6 minutes). The LOD was calculated as three times the mean baseline noise. For LOQ estimation, five replicated of three concentrations (0.05, 0.1, 0.25,) were tested. The LOQ was estimated as the lowest concentration which showed a coefficient of variation (CV) less than 20%.[20]

Application of the method. Torsemide plasma profile was evaluated using the method developed. Three healthy volunteers received a 5 mg torsemide single oral dose. Written agreement was obtained from each volunteer who did not take any other medication for 1 week before and throughout the study. Peripheral heparinized

blood samples were obtained from each volunteer before and 2 and 4 hours after drug administration. Intake of food was delayed for 4 h after medication. Plasma samples were separated by centrifugation at 3000 rpm and they were stored at 4 ºC until analysis.

Results and discussion

A simple, fast and reliable reverse-phase HPLC method was developed to quantify torsemide in human plasma. Chromatographic separation was performed using a gradient of solvents. The mean retention time for torsemide was 5.08 minutes (SD: 0.1), and for bumetanide was 6.86 minutes (SD: 0.18) *(figure 1B)*. In order to test for the absence of endogenous interference a plasma blank extract was injected. No chromatographic peaks, which could interfere with the resolution of torsemide and bumetanide, were observed *(figure 1A),* but a delayed plasma peak was present in some of the plasma specimens *(figure 2)*.

The chromatographic analysis was preceded by a highly effective solid-phase extraction procedure. The mean absolute recovery values assessed at three different concentration levels (0.5, 3 and 12 μg/mL) (triplicate determinations) ranged between 91.9% and 118% *(table II)*. Intra-day and inter-day coefficients of variation (CV%) are shown in *table II*. Bumetanide mean recovery was 108.8% and the CV was 2.5%. Despite of the high efficiency and reproducibility established in the torsemide extraction procedure the addition of bumetanide was necessary to prevent any recovery variation due to possible water pH fluctuations. A calibration curve was constructed by least squares linear regression analysis. The linearity of the method was tested with eight calibration points within the concentration range of 0.05-12 μg/mL. The squared correlation coefficient (r^2) was 0.9910.

The LOD for torsemide in human plasma was found to be 0.010 μg/mL, and the LOQ was 0.10 μg/mL.

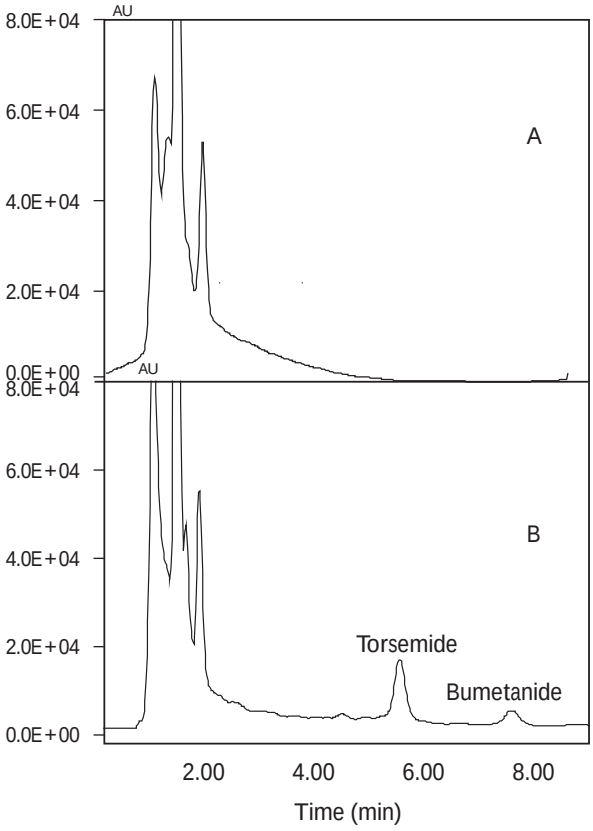

Figure 1. Chromatogram for an extracted blank plasma (A) and for an extracted plasma spiked with 1.0 μg/ml of torsemide and 3.0 μg/ml of internal standard bumetanide(B). Au: absorbance units.

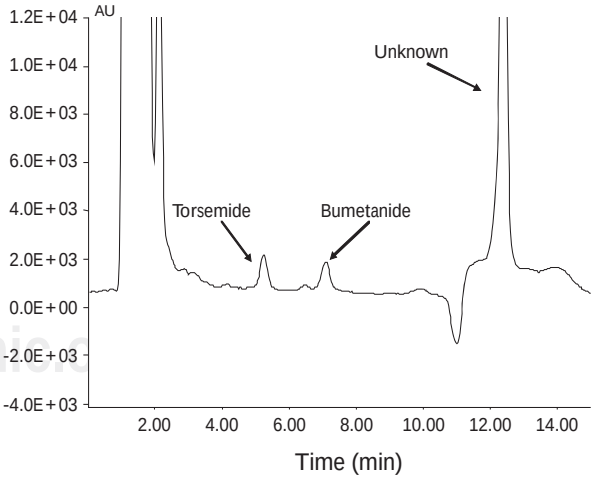

Figure 2. Extended chromatogram of an spiked plama. After 12 minutes an unknown plasma peak was eluted.

Table II. Torsemide intra and inter assay recovery and precision at three plasmatic concentrations

	0.5 μg/ml	3 μg/ml	12 μg/ml
Intra-day mean recovery (%)	96.6	109.2	117.3
Intra-day precision (%CV)	2.3	6.5	0.23
Inter-day mean recovery (%)	91.9	101.8	118.0
Inter-day precision (%CV)	10.1	11.3	2.2

CV: coefficient of variation

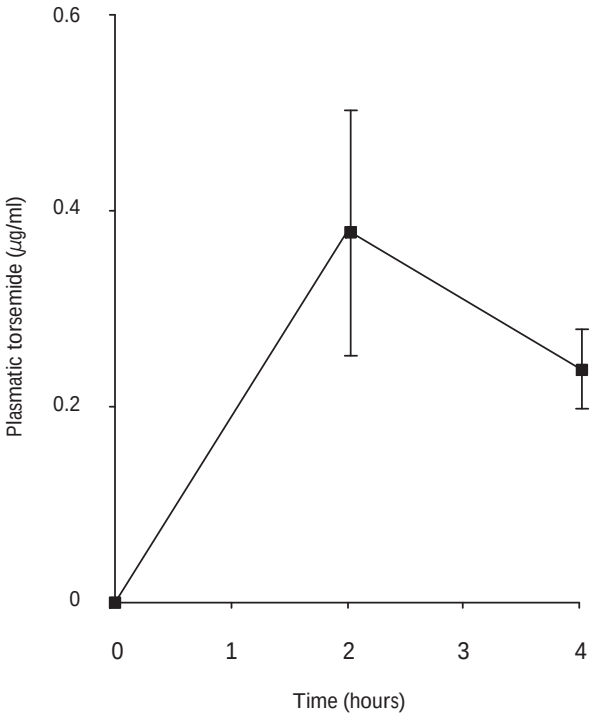

Figure 3. Plot of mean plasma torsemide concentration *vs* time after a single 5 mg oral dose.

In a first attempt to extract torsemide from human plasma, the extraction was performed according to the procedure described by Olmos et al[21] for the qualitative investigation of furosemide, hydrochlorothiazide and other diuretics in urine by TLC. Two plasma pH conditions (pH: 7.00 and 5.00) were tested (data not shown). The best extraction efficiency was obtained at pH 5.00 because the torsemide recovery at this pH resulted four times higher than at pH 7.00. Several elution solvents (methanol, acetone, ethyl acetate and chloroform) were tested (data not shown). Methanol was selected as the elution solvent because of its best extraction performance and lower cost as compared with the others solvents.

Mobile phase and flow rate conditions were optimized to achieve the best resolution between torsemide and interfering bands from plasma components, and between torsemide and the internal standard bumetanide. The solvents gradient was necessary to avoid the interference of an unknown plasma peak that eluted after 12 minutes. These conditions resulted in an optimal run length of fourteen minutes. The concentration range for the calibration curve was selected according to that previously described for plasma levels[2,22-25] and the broad spectrum of different indications.[26] Torsemide showed to have excellent linearity in the concentration range selected.

As a group of drugs, diuretics are an essential tool in the treatment of cardiovascular disorders today. They represent one of the cornerstones in the treatment of various edematous conditions, mainly those secondary to CHF. Originally designed for the control of fluid retention, they are widely used in the treatment of essential hypertension due to their antihypertensive effect. In comparison with other diuretics (e.g. furosemide, the most widely prescribed diuretic), torsemide seems to have many advantages: it shows a lesser kaliuretic effect, higher oral bioavailability and a longer half-life.[9,10,22,27] Nevertheless, the administration of torsemide must be carefully monitored, mainly in relation to high-risk patients, such as those with liver disease, because a hydroelectrolytic imbalance could contribute to make their healthy-state steadily worse.[5] For this reason, in order to carry

out a more complete evaluation of this drug it is necessary to apply suitable analytical methods to quantify the drug in biological samples.

Three healthy volunteers received 5 mg of torsemide orally. Blood samples were collected before, and two and four hours after torsemide administration. Mean torsemide concentration profile for the three volunteers is shown in *figure 3.* The torsemide plasmatic concentration observed were comparable with those reported by other authors.[25]

The present method allows quantify plasma torsemide levels with sensitivity and precision using standard laboratory HPLC equipment. In conclusion, the method described above is useful for the quantification of torsemide in human plasma, and it would be suitable for torsemide plasma monitoring and pharmacokinetic studies.

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