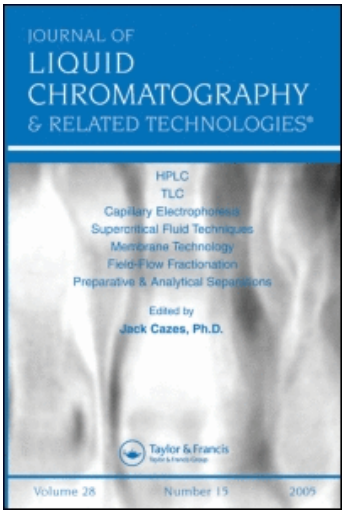

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Chatterjee, D. J. , Li, W. Y. , Hurst, A. K. and Koda, R. T.(1995) 'High-Performance Liquid Chromatographic Method for Determination of Atenolol from Human Plasma and Urine: Simultaneous Fluorescence and Ultraviolet Detection', Journal of Liquid Chromatography & Related Technologies, 18: 4, 791 — 806
To link to this Article: DOI: 10.1080/10826079508009273
URL: http://dx.doi.org/10.1080/10826079508009273

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(4), 791-806 (1995)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF ATENOLOL FROM HUMAN PLASMA AND URINE: SIMULTANEOUS FLUORESCENCE AND ULTRAVIOLET DETECTION

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ABSTRACT

A rapid, reliable analytical method was required to study the disposition of atenolol following oral administration in subjects in various stages of pregnancy. Available methods showed wide variability due to matrix interference. A simple HPLC method is reported for the determination of atenolol in human blood and urine. Atenolol and the internal standard, albuterol, were isolated using solid phase extraction and separated isocratically on a C-18 analytical column with a mobile phase consisting of a mixture of an aqueous solution of mono-basic ammonium phosphate and N,N-dimethyloctylamine, and acetonitrile (93:7 v/v). Atenolol and the I.S. were monitored in the effluent using both fluorescence (228/310 nm, excitation/emission) and ultraviolet (224 nm) detection. Within-run and between-run precision showed a c.v. <5% for concentration of 50-400 ng/ml with an error <5%. Recovery following solid phase extraction ranged from 72.8% at 50 ng/ml to 95.5% at 500 ng/ml. The method is linear over a range of 50-

750 ng/ml. The assay has been applied for quantification of atenolol plasma levels for the determination of pharmacokinetic parameters following oral dosing.

INTRODUCTION

Atenolol, a selective ß-1 adrenergic blocking agent, is currently used for the treatment of hypertension, angina pectoris and certain types of arrhythmias (1-3). Several methods have been previously reported for the determination of atenolol in biological fluids. These include gas-chromatographic methods requiring derivatization (4-5), and high performance liquid chromatographic (HPLC) techniques using either solid phase or liquid extraction with ultraviolet or fluorescence detection (6-8). Since the UV maximum for atenolol occurs at 224 nm, it is often difficult to obtain HPLC chromatograms free from interference because of substances having high UV absorbance at similar wavelengths in the matrix. Atenolol shows high native fluorescence at excitation/emission wavelengths of 228/310 nm, respectively. Although HPLC chromatograms using fluorescence detection are generally free from interference relative to UV detection, there has been a lack of a suitable internal standard for analysis of atenolol. Moreover, methods of analysis of atenolol in biological specimens using fluorescence detection from several previously reported methods show wide variability due to interfering substances.

This paper describes a HPLC method for determining atenolol in human plasma and urine using albuterol as an internal standard. The sample is prepared by solid phase extraction for sample cleanup and the reconstituted sample is injected into an HPLC and separated on a C_{18} reversed-phase analytical column. The effluent is monitored with a fluorescence detector at an excitation/emission wavelength of 228/310 nm, respectively. Before the conditions of the method were optimized, the UV chromatogram showed evidence of interfering substances that were co-eluting with atenolol and the internal standard. To avoid any quenching or enhancement of the fluorescence of atenolol or albuterol due to co-eluting endogenous subtances that could affect the accuracy and reproducibility of the method, conditions were modified until complete resolution of all peaks was accomplished by monitoring the effluent using UV detection at 224 nm. The method described gives excellent chromatographic separation and is rapid, sensitive and reproduceable, with a sensitivity limit of 50 ng/ml.

EXPERIMENTAL

Materials and Reagents

Atenolol, albuterol (internal standard), and human serum were purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium phosphate monobasic and *N,N*-

dimethyloctylamine (DMOA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Octadecyl (C-18) solid phase extraction columns (100 mg) were obtained from Baxter Healthcare Co. (Muskegon, MI).

Instrumentation

The HPLC system consisted of the following components: Perkin Elmer, Series 410 LC solvent delivery pump, LC 90 UV spectrophotometric detector, LS-4 fluorescence spectrophotometic detector, LCI-100 laboratory computing integrator (for fluorescence detector), Hitachi AS-4000 auto sampler, and 2000-D computing integrator (for UV detector). Atenolol was separated on a C-18 reverse phase, 250 x 4.6 mm I.D., 5 μ analytical column, preceeded by a C-18 guard column, both Adsorbosphere C-18, Alltech Associates, Inc, (Deerfield, IL). Samples were eluted isocratically at a mobile phase flow rate of 1.5 ml/min. The effluent was monitored using a variable wavelength UV detector at an analytical wavelenth of 224 nm placed in series with a fluorescent detector with an excitation/emission wavelenth of 228 nm and 310 nm, respectively.

Mobile Phase

The mobile phase consisted of a mixture of an aqueous solution containing 25 mM mono-basic ammonium

phosphate and 1 mM *N,N*-dimethyloctylamine (DMOA) with acetonitrile, 93:7 v/v, adjusted to pH 3.0 with phosphoric acid 85%. The mobile phase was filtered through a Nylon 66 membrane filter (Sartolon, Sartorius, Germany) and deaerated with helium gas.

Preparation of Standard Solutions:

Accurately weighed atenolol was dissolved in an appropriate volume of distilled water to obtain a stock solution containing 1 mg/ml. This solution was further diluted to obtain working standard solutions with concentrations of 1 and 10 μg/ml. Pooled normal human serum was spiked with appropriate volumes of these standards to achieve standard solutions containing 50, 100, 200, 250, 500 and 750 ng/ml, that were used to obtain a standard curve. A standard curve of atenolol in urine was prepared in a similar manner. The internal standard solution was prepared by dissolving an appropriate amount of albuterol in water to give a concentration of 1 mg/ml. This solution was further diluted to obtain an internal standard solution with a concentration of 5 μg/ml.

Sample Preparation

To 500 μl of spiked serum or patient serum sample was added 50 μl of internal standard solution. The mixture was vortexed for 30 seconds then loaded on a preconditioned 100 mg, C-18 solid-phase extraction

column. Extraction columns were preconditioned by passing through one column volume of methanol followed by two column volumes of water. Serum samples containing the internal standard were passed through the solid phase columns and the loaded columns washed with 3 x 0.2 ml of water. Atenolol and the internal standard were eluted from the column using 2 x 0.5 ml of methanol. The combined methanol extracts were evaporated to dryness under a stream of dry, filtered air at 40-45° C. The residue was reconstituted with 150 µl of water, vortexed for 30 seconds, then centrifuged at 14,000 g for 4 minutes. The supernatant was transferred into injection vials and 50 µl was injected into the HPLC using an autoinjector.

Spiked standards and patient serum specimens were treated similarly. However, urine specimens required dilution in which 10 µl of urine was diluted to 500 µl with distilled water. Internal standard solution was added and the identical procedure as previously described was followed.

RESULTS AND DISCUSSION:

Both UV absorbance and fluorometric methods have been widely used for the detection of atenolol following HPLC separation (6,7). UV spectra reported by Verghese *et al.* (7) and confirmed in our laboratory indicate that the molar absorbance of atenolol is

sufficiently intense for detection at concentrations as low as 15-25 ng/ml from small sample volumes (500 μl). Atenolol also displays high intrinsic fluorescence. Albuterol proved to be an ideal internal standard for this assay since it was found to migrate closely to atenolol, showed a UV maximum at 224 nm and a fluorescence excitation/emission at 228/310 nm, respectively, and their peaks could be completely resolved under the conditions of the method. Samples were detected by passing the HPLC effluent serially through a variable wavelength UV detector at 224 nm followed by a fluoresence detector at excitation/emission wavelengths of 228 nm and 310 nm, respectively, as shown in Fig 1. Typical HPLC chromatographs of atenolol and the internal standard, albuterol, in plasma using UV absorbance and fluorescence detection are shown in Fig 2. Chromatographs using UV detection indicated the presence of at least two potentially interfering peaks, one that eluted in close proximity to atenolol and the other close to the internal standard. Although endogenous interfering substances in human plasma may show only weak or complete absence of fluorescence, co-elution of peaks may cause quenching or enhancement of fluorescence resulting in variability in accuracy and reproducibility of the analytical method. Modifications in mobile phase composition and pH, and sample cleanup

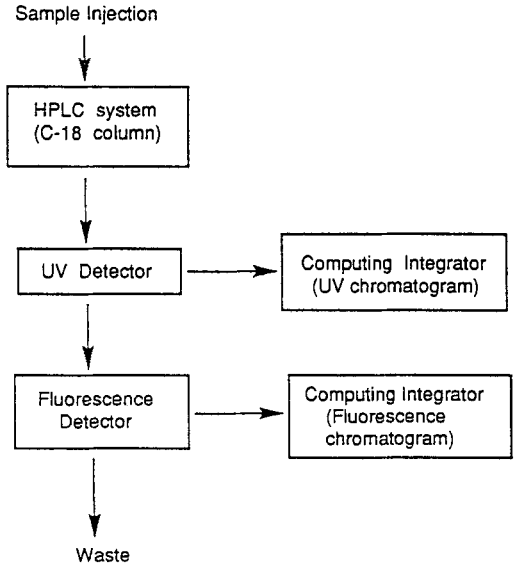

Figure 1. Schematic of the HPLC system.

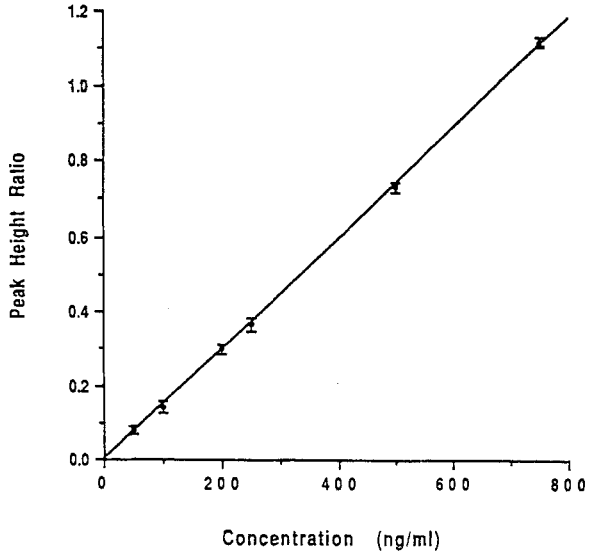

Figure 2. Standard curve of peak height ratio of atenolol to internal standard versus atenolol concentration (shown as means ± s.d. of five determinations at each concentration).

procedures, were made until optimal conditions were achieved whereby all UV and fluoresence peaks could be completely resolved. The retention times for the internal standard and atenolol using UV detection were 7.1 and 10.4 minutes, and for fluorescence detection, 7.3 and 10.6 minutes, respectively. Since the UV and fluorescence detectors were coupled in series, the fluorescence peaks lagged their respective UV peaks by approximately 0.2 minutes. The assay cycle period was approximately 30 minutes.

All results reported herein have used fluorescence detection since this method was relatively free from interference compared to UV detection. Depending on the excitation/emission wavelengths used, 228/310, 228/606 or 278/606, the limit of detection for the method was 5 ng/ml, 10 ng/ml or 25 ng/ml, respectively. However, when the assay conditions were optimized to achieve separation of all essential peaks, the results using UV detection were comparable to fluorescence. Thus, the method is equally applicable to quantitate atenolol using either fluorescence or UV detection.

Variability of the method was determined by assaying six known serum concentrations on five separate days. Results of this study are shown in Table I. A standard curve plot of peak height ratio of atenolol to internal standard versus atenolol concentration is

TABLE I. Standard curve and day-to-day variability of Atenolol assay in serum at six concentrations determined on five consecutive days.

Atenolol Concentration ng/ml	Peak Height Ratio Mean ± S.D.
50	0.076 ± 0.010
100	0.142 ± 0.016
200	0.299 ± 0.014
250	0.368 ± 0.018
500	0.731 ± 0.013
750	1.117 ± 0.013

Standard Curve (five consecutive days)
Slope = 1.48×10^{-3}
y-Intercept = -1.98×10^{-3}
Correlation coefficient = 0.9995

shown in Figure 3. The standard curve shows that the method is linear over a concentration range of 50 - 750 ng/ml, with a mean slope of 1.48×10^{-3}, a *y*-intercept of -1.98×10^{-3}, and a mean correlation coefficient of 0.9995.

Within-run and between-run precision and accuracy were determined at four different concentrations. Within-run precision was determined at 20, 50, 150 and 400 ng/ml, each sample being assayed six times within the same run. The results shown in Table II demonstrate that the method is precise with a coefficient of variation of less than 5 percent for concentrations of 50 - 400 ng/ml with a percent error of less than 5 percent. Although the accuracy of the method was

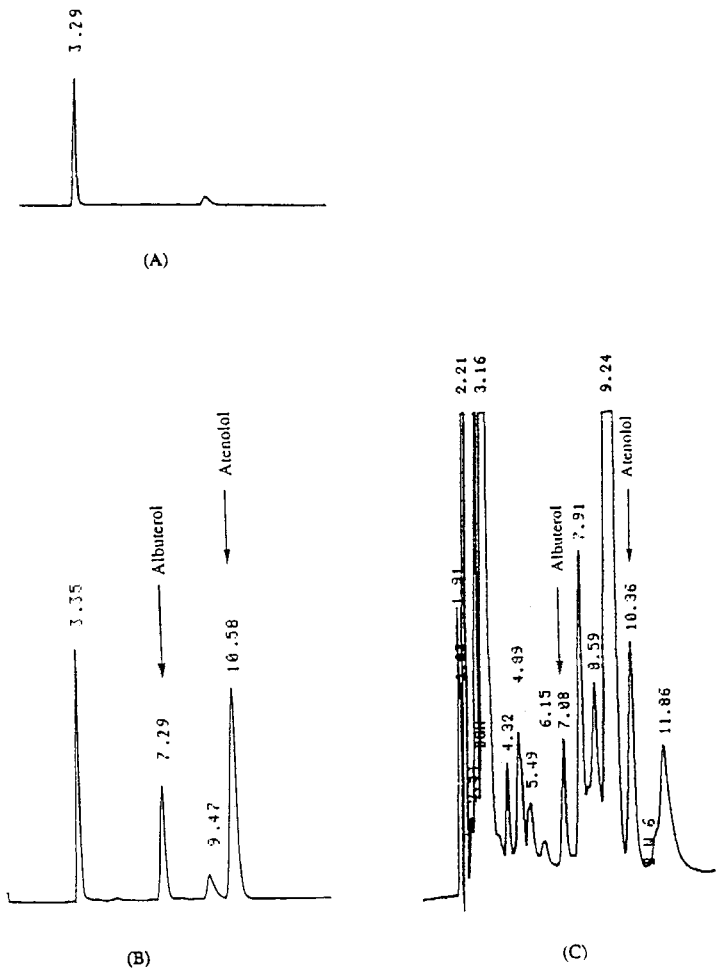

Figure 3. Typical HPLC chromatograms using fluorescence detection showing (A) human plasma blank; (B) human plasma containing atenolol, 22.3 ng and I.S., 22.3 ng; (C) HPLC chromatogram using UV detection obtained from the same injection shown in (B) above, indicating clear separation of atenolol and I.S. from interfering peaks from the matrix.

TABLE II. Within-run precision and accuracy for determination of atenolol in serum.

Atenolol Concentration, ng/ml Actual	Measured* ± S.D.	C.V.%	% Error
20	19.6 ± 3.0	15.3%	2.0%
50	52.3 ± 2.2	4.1%	4.6%
150	154.7 ± 3.7	2.4%	3.1%
400	389.1 ± 6.8	1.7%	2.7%

*Mean of 6 assays

excellent for the 20 ng/ml sample, the assay variability was wide, with a coefficient of variation of 15.3%. Between-run precision was determined by repeated assays of four spiked serum samples containing 150, 250, 400 and 700 ng/ml of atenolol on six separate days. The results of this study are presented in Table III. The coefficient of variation for all samples tested ranged from 0.82 - 6.45%, with a percent error of 0.72 - 4.50%.

Recovery (extraction efficiency) following solid phase extraction was determined on serum specimens spiked with atenolol at four known concentrations (50, 100, 250 and 500 ng/ml) and corresponding concentrations in water. The percent recovery was determined by comparing the peak height of the extracted plasma samples to the peak height for the non-extracted aqueous solution of equal concentration. Table IV

TABLE III. Between-run precision and accuracy for determination of atenolol in serum.

Atenolol Concentration, ng/ml Actual	Measured* ± S.D.	C.V.%	% Error
150	147.5 ± 9.5	6.5%	1.7%
250	251.8 ± 2.1	0.8%	0.7%
400	382.0 ± 15.3	4.0%	4.5%
700	706.2 ± 10.1	1.4%	0.9%

*Mean of 6 assays

TABLE IV. Recovery of atenolol and internal standard from spiked serum samples following C_{18} solid-phase extraction.

Plasma Concentration (ng/ml)	Mean Recovery* ± S.D. % Atenolol	I.S.
50	72.8 ± 14.5	
100	77.8 ± 10.9	
250	88.1 ± 2.6	
500	95.5 ± 3.1	
I.S., 250 ng		92.3 ± 5.1

shows a mean recovery ranging from 72.8 ± 14.5% for the 50 ng/ml sample to 95.5 ± 3.1% for the 500 ng/ml sample. The mean extraction efficiency for the internal standard was 92.3 ± 5.1%.

The stability of atenolol in plasma was determined since samples are frequently stored frozen for a period of time prior to analysis. It has been recommended

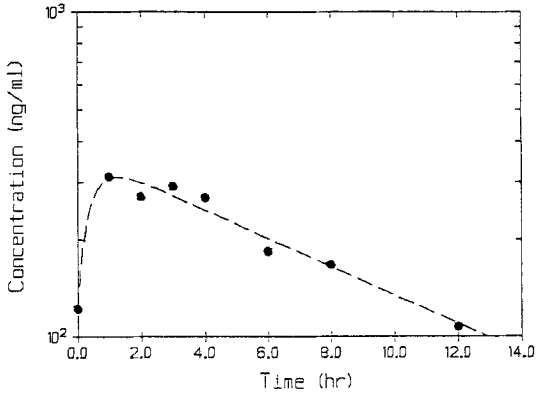

Figure 4. Plasma concentration-time profile of atenolol following administration of a 50 mg oral dose to a subject in the third trimester of pregnancy. The data is fitted a one-compartment oral absorption model. Fitted parameters are k_{el} = 0.10 hr^{-1}; k_a = 2.20 hr^{-1}; AUC = 3.55 $\mu g \cdot hr/ml$, V_d = 34.9 L.

that samples should not be stored for a long period prior to analysis because of instability (6). Samples containing known concentrations of atenolol were maintained at ambient temperature (23°), 4° and -20° C for a period of seven days. The samples were all analyzed within the same run. No significant trend in sample concentration change were seen following storage at the different temperatures over a seven day period.

The method was applied to an investigation of changes in the pharmacokinetic profile of atenolol in women throughout pregnancy. The pharmacokinetics of atenolol was studied in 14 patients in their first,

second and third trimesters of pregnancy. A typical plasma concentration-time curve over the 12 hour dosing interval following chronic oral dosing of 50 mg twice a day is shown in Figure 4. The data were fitted to a one-compartment model with extravascular dosing. All model-defined parameters (k_{el}, k_a, V_d, AUC) were obtained and the total clearance was calculated.

The analytical method described is reliable and sensitive, and is applicable for the determination of atenolol in blood or urine.

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Received: April 22, 1994
Accepted: October 19, 1994