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A micro liquid chromatographic assay for the determination of plasma-unbound atenolol[1]

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Abstract

An improved high performance liquid chromatographic assay for plasma-unbound atenolol is described. The assay has a wide range (10–5000 ng ml^{-1}) of linearity and a detection limit of 5 ng ml^{-1} (or 0.1 ng per injection) with acceptable intra- and inter-assay reproducibilities using small volumes of plasma (100 μl). Following administration of a single dose of atenolol to the rat, nine blood samples were collected over a period of 8 h. These samples were analyzed for atenolol concentrations by a sensitive and specific microbore high performance liquid chromatograph with a photodiode-array detector. This multi-channel detector was used to acquire spectral information on atenolol and demonstrated a superior performance in comparison to all other techniques in that both qualitative and quantitative information were acquired with the system. Because of it sensitivity and applicability to plasma analysis, the assay can be used for pharmacokinetic studies and is valuable in therapeutic drug monitoring.

Keywords: Atenolol; Liquid chromatography; Plasma

1. Introduction

Atenolol, 4-(2-hydroxy-3-isopropylaminopropoxy)-phenylacetamide, is a selective adrenergic blocking agent that competitively blocks β-adrenergic receptors within the myocardium and vascular smooth muscle [1–5]. Many analytical procedures have been devised for the determination of atenolol in plasma or urine [6–13]. Published methods were based on fluorescence spectrophotometry [6], gas chromatography (GC) with electron-capture detection [7,8], high performance liquid chromatography (HPLC) [9–12] and mass spectroscopy [13]. Among the methods used, GC determination of atenolol is specific and sensitive to 10 ng ml^{-1}, but is relatively complex and requires lengthy prederivatization steps. Fluorescence spectrophotometry requires an elaborate extraction procedure and a large volume of plasma [6]. HPLC with fluorescence or ultraviolet detection is popular and is considered to be a sensitive

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method in the determination of atenolol, because of its reasonable simplicity, sensitivity, and specificity [9,10]. The majority of these HPLC assays have relied on the initial denaturation of the plasma proteins by the addition of sodium hydroxide solution or organic solvents followed by extraction of atenolol into an immiscible organic solvent [14,15]. Such procedures are laborious and require about 1–2 h for the preparation of samples. Relatively expensive apparatus is required with these potentially hazardous solvents, especially when the solvent is being evaporated to dryness. More recently, the use of a solid-phase extraction technique has been described for the determination of atenolol in plasma, but these procedures also suffer problems when a large number of samples are handled [16,17]. A direct injection of a plasma sample using coupled columns and switching valves provides an improved analytical tool to solve the above difficulties [18]. Nevertheless, the novel LC assay may cause some errors which arise from the complexity of operation, column deterioration, and difficulty in automation.

The ultrafiltration technique has been applied to plasma processing for drug and biogenic amine monitoring assays [19,20]. The ultrafiltration procedure offers the advantages of simplicity and high reproducibility, eliminating problems associated with the precipitation procedures (e.g. sample dilution, incomplete protein precipitation, drug coprecipitation, acid-catalyzed degradation etc.). However, ultrafiltration is not recommended for the determination of highly bound drugs. In fact very little, approximately 5–15%, of atenolol is bound to plasma protein. Hence the ultrafiltration allows the determination of the unbound atenolol concentration in plasma. Alternatively, the total atenolol concentration can be obtained by addition of sodium dodecyl sulfate to displace plasma protein-bound atenolol. To the authors' knowledge, this clean-up procedure using ultrafiltration has not been applied in assaying plasma atenolol. The role of the membrane in ultrafiltration is to act as a selective barrier, enriching certain compounds and depleting others [21]. This is a very attractive method for the isolation of small and hydrophilic molecules by discriminating against high-molecular-weight substances, such as proteins and suspended matters, on the basis of molecular size. Ultrafiltration samples (ultrafiltrates) can also be applied directly onto the HPLC column without further purification. Nevertheless, a more important advantage of the ultrafiltration technique over conventional liquid-extraction proocedures is faster and more efficient separation of the plasma proteins. In addition, the plasma volume required in ultrafiltration is relatively small compared to that of the conventional procedures. The remaining plasma can be used for other assays. In the present study the ultrafiltration procedure, instead of conventional extraction procedures was thus applied for the pretreatment of plasma samples.

In general, the anti-hypertensive effect of a single dose of atenolol persists for 24 h. Hence there is a need to analyze plasma samples at very low concentrations at which conventional HPLC systems have difficulties or have no place in many pharmacokinetic studies. Much of the interest in microbore HPLC has been the result of increased mass sensitivity and lower detection limits in its applications. Compared with conventional columns of 4.6 mm i.d., a microbore column of 1.0 mm i.d. leads to a 20-fold increase in detection sensitivity and achieves better detection limits. In addition, preliminary HPLC identifications were based on the comparison of capacity factors (k') (or retention times) for sample components and standard compounds [22]. Therefore, a reliable assignment of peak identity requires the determination of additional component characteristics. This can be solved by dual or multiple detectors, such as a dual-channel or a photodiode-array detector, based on the drug's absorption properties [23,24]. Therefore, the atenolol peak can be reliably identified and discriminated from others based on its retention time and UV absorption characteristics.

2. Experimental

The HPLC system comprises an Ultra-Plus UP200M pump (Micro-Tech Scientific, Sunnyvale, CA), a CMA-200 microautosampler with 20

μl sample loop (CMA/Microdialysis, Stockholm, Sweden), a Beckman 168 photodiode-array detector, a microbore reversed-phase column (Inertsil-2, 5 mm ODS, 1.0 × 150 mm i.d., G.L. Sciences, Japan), a Beckman I/O 406 interface, and Beckman System Gold Data Analysis Software (version 8.10, Beckman Instruments Inc., Taiwan, ROC). The Beckman 168 photodiode-array detector permits the scanning of chromatographic and spectral data (200–350 nm). The mobile phase consists of acetonitrile–phosphate buffer (pH 3.0, 10 mM) (5:95, v/v), filtered with a 0.22 μm nylon filter under reduced pressure and degassed by helium for 20 min. The flow rate was 70 μl min^{-1}.

Triethylamine, (±) atenolol, and monosidium dihydrogen orthophosphate were purchased from RBI (Research Biochemicals International, Natick, MA). HPLC-grade acetonitrile was purchased from Merck (Merck-Schuchardt, Darmstadt, Germany). All reagents were of analytical quality unless otherwise stated. Standard stock solutions of atenolol prepared at a concentration of 50 μg ml^{-1} in doubly-distilled water were stored at −70°C in the dark prior to preparation of a standard mixture. Atenolol was prepared every day from a portion of the stock solution after appropriate dilution.

Male SD rats ($n = 4$, 250–300 g) were used in the present assay. An indwelling venous cannula was inserted in one of the femoral veins. Samples of about 200 μl of blood each were collected at 0 min, 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 4.5 h, 6 h, and 8 h after administration of 2 mg kg^{-1} atenolol (i.p.). Blood samples were collected into prechilled polypropylene tubes, with Na_2EDTA as an anticoagulant, and centrifuged (15 min, 1200 g at 4°C) immediately to separate the plasma. Frozen plasma samples were stored at −70°C prior to the day on which atenolol concentration was determined. After thawing at 4°C, 100 μl of plasma sample was transferred to a Millipore Ultrafree-MC unit (PLGC, Ultrafree-MC with 10 000 nominal molecular weight cut-off (NMWC), Millipore Co., Bedford, MA) and then centrifuged at 15 000 g for 40 min. The Ultrafree-MC unit is a 400 μl sample cup with a regenerated cellulose membrane sealed to the bottom, which sits inside a 1.5 ml microcentrifuge tube. 20 μl of the ultrafiltrate was injected onto the microbore HPLC system. Plasma atenolol concentration is calculated by comparing each peak area with the calibration curve and is corrected for recovery volume. The identity of the peaks in the chromatogram is confirmed by their retention times, standard addition, and spectra (System Gold Data Analysis Software, Version 8.10).

3. Results and discussion

Good volume recoveries ($n = 6$, 98 ± 2%) of standard mixtures in the ultrafiltration procedure were found as in the previous study. Under the same conditions, typically more than 50% ($n = 12$, 54 ± 4%) of rat plasma sample volume was recovered. Under the described chromatographic conditions, Fig. 1 shows a well-resolved chromatographic peak of atenolol with a retention time at 6.88 min. Fig. 1A is a typical chromatogram of an authentic atenolol (1000 ng ml^{-1}). A blank chromatogram did not demonstrate peaks corresponding to atenolol. Figs. 1B and 1C are chromtograms of rat plasma ultrafiltrates containing 1000 and 50 ng ml^{-1} atenolol respectively. The spectral scans (200–350 nm) of atenolol at the selected interval of 6.10–7.05 min were also shown in the plasma ultrafiltrates in Figs. 1B and 1C. An analysis was completed within 10 min. Comparison between the spectrum of the authentic atenolol and that of the atenolol in plasma ultrafiltrate was also useful for the identification of atenolol. Occasionally, each peak in the plasma ultrafiltrates was also verified by spiking with the authentic atenolol, to see if the addition increased its peak height proportionally. In addition, a superimposed-alignment technique was used if the chromatographic peaks differed slightly in elution times between runs or co-eluted with other undetermined interferences.

The known amounts of atenolol were prepared at various concentrations (range: 10–5000 ng ml^{-1}) in a blank plasma filtrate to create a calibration curve for chromatographic determination of plasma atenolol levels. Standard curves and correlations (R^2) for atenolol responses are y

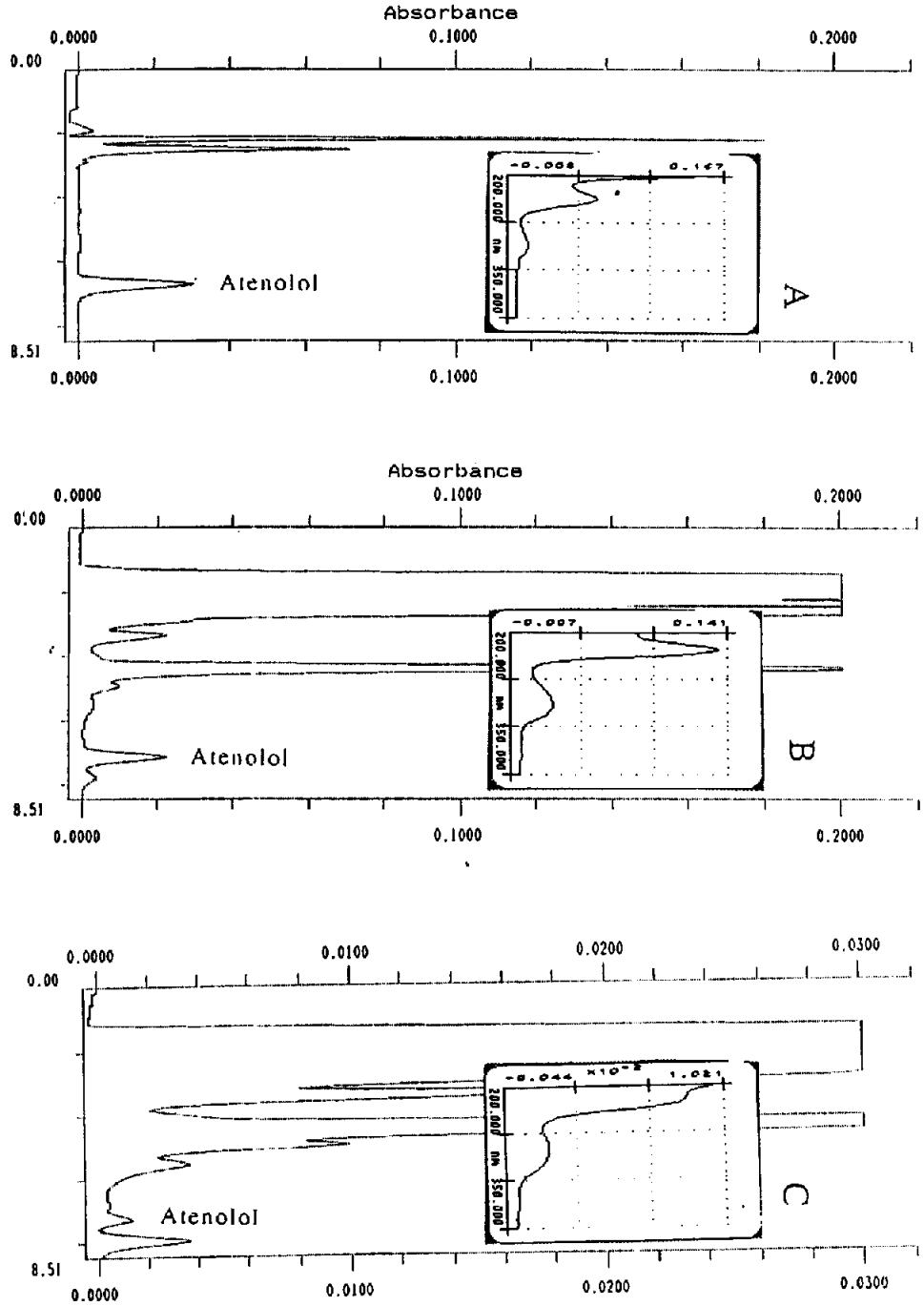

Fig. 1. Typical chromatograms of (A) atenolol (1000 ng ml^{-1}), (B) atenolol (1000 ng ml^{-1}) in rat plasma and (C) atenolol (50 ng ml^{-1}) in rat plasma by the HPLC–DAD system.

Table 1
Inter- and Intra-assay reproducibilities of the HPLC–DAD assay

Reproducibility	Atenolol concentration (ng ml^{-1})	n	RSD (%)
Intra-assay			
authentic standard	20	16	2.8
	100	16	1.2
pooled plasma	20	16	3.8
	100	16	1.2
Inter-assay			
authentic standard	100	6	3.4

$= 5.403x - 0.102$, $R^2 = 1.000$ at 223 nm, and $y = 0.506x + 0.001$, $R^2 = 1.000$ at 275 nm. The higher response of atenolol at 223 nm was used to determine plasma atenolol concentration. However, this determination may cause lower accuracy at very low concentration levels because of a relatively larger intercept of the standard curve. The detection limit (signal-to-noise ratio = 3) for atenolol was 5 ng ml^{-1} (or 0.10 ng per injection) at 223 nm in this study. The superior linearity, mass sensitivity and detection limits enable one to determine a wide range of atenolol levels by direct injection of ultrafiltrates without preconcentration of samples.

The precision of the assays was tested using standard mixtures and ultrafiltrates of pooled human plasma containing 20 and 100 ng ml^{-1} authentic atenolol. The intra- and inter-assay variabilities were assessed and are expressed as relative standard devaition (%RSD) in Table 1. The intra-assay variabilities were determined with 16 replicates at 1 h intervals in the standard and the plasma ultrafiltrates. They were less than 4% for either concentration. The inter-assay variability of the same standard mixture was assessed on six consecutive working days. The RSD value of the inter-assay variability was 3.4%.

An application of the present method was made to determine the plasma-unbound (or free) atenolol levels in rats after a single dose (2 mg kg^{-1} i.p.) of atenolol administration. The levels of atenolol in rats are shown in Table 2. The kinetic variables were: peak plasma concentration, 804 ng ml^{-1}; time of peak, 30 min; elimination half life, 1.85 h; AUC, 1.677. These data are not totally in agreement with those reported by others [25,26]. The differences may be due to the i.p. administration of atenolol and a relatively short blood collection period (8 h). Non-retainment of the drug in the digestive tract may also favor such occurences. Since the aim of this investigation was not the determination of pharmacokinetics, the applicability of the present assay to a small animal experiment was deemed to be satisfactory.

4. Conclusion

Although many studies are routinely carried out using conventional extraction procedures, the variations in recovery, precision and accuracy of different extraction procedures are still problems in inter-laboratory comparisons. When comparing the present method with these conventional procedures, it can be seen that the plasma pretreatment

Table 2
Plasma concentration (ng ml^{-1})–time profile and some pharmacokinetic parameters of atenolol in rats after administration of a dose of 2 ng kg^{-1} (i.p.)

Rat	0 min	15 min	30 min	60 min	2 h	3 h	4 h	6 h	8 h	$t_{1/2}^{a}$	AUC[b]
1	N.D.[c]	0.806	0.806	0.518	0.262	0.157	0.075	0.069	0.045	1.848	1.607
2	N.D.	0.910	0.908	0.543	0.288	0.157	0.107	0.067	0.055	1.888	1.770
3	N.D.	0.674	0.516	0.514	0.231	0.174	0.089	0.034	0.017	1.375	1.251
4	N.D.	0.664	0.758	0.523	0.280	0.150	0.119	0.061	0.048	2.075	1.621

[a] Elimination half life (h).
[b] Area under peak.
[c] Area under peak.

of the present method took less than 30 min for 16 samples, whereas the conventional extraction procedures took 8 h for 8–10 samples in routine assays. Because of the very low hold-up volume (< 5 μl) of the Millipore Ultrafree-MC units and the precision of the CMA-200 microautosampler (< 1 μl), only 100 μl plasma samples are required to achieve excellent inter- and intra-assay precision as shown from this study. Furthermore, the present assay is fast and requires a relatively simple pretreatment of plasma samples. A large number of plasma samples can be processed daily (≈ 144). In addition, when available, pharmacokinetic data derived from plasma-unbound drugs are more appropriate than from plasma total drugs. In the present assay, the determination of unbound drugs with a high sensitivity and low detection limits in very small volumes of plasma has a great analytical potential for studies requiring repeated blood sampling, such as pharmacokinetic studies in small animals and in pediatric and clinical research.

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