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Short communication

Efficient assay for the determination of atenolol in human plasma and urine by high-performance liquid chromatography with fluorescence detection

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Abstract

An efficient method for the determination of atenolol in human plasma and urine was developed and validated. α-Hydroxymetoprolol, a compound with a similar polarity to atenolol, was used as the internal standard in the present high-performance liquid chromatographic analysis with fluorescence detection. The assay was validated for the concentration range of 2 to 5000 ng/ml in plasma and 1 to 20 μg/ml in urine. For both plasma and urine, the lower limit of detection was 1 ng/ml. The intra-day and inter-day variabilities for plasma samples at 40 and 900 ng/ml, and urine samples at 9.5 μg/ml were <3% (n=5).

Keywords: Atenolol

1. Introduction

Atenolol, 4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetamide, is a water soluble, β_1 selective (cardioselective) adrenoceptor antagonist used in the treatment of angina and hypertension. It is primarily eliminated renally with minimal hepatic metabolism [1,2]. As part of our pilot study to investigate the relationship between the pharmacokinetic and pharmacodynamic characteristics of atenolol following oral and intravenous drug administration in the Chinese population, it is necessary to monitor the drug concentrations in both plasma and urine. A number of assay procedures have been reported in the literature. Due to the high hydrophilicity of atenolol, methods employing gas chromatography demand more elaborate sample extraction and derivatization procedures [3–5]. Methods employing high-performance liquid chromatography are more efficient, and higher detection sensitivity and selectivity are achieved by coupling with fluorescence detection [6–13]. Among these literature methods, various sample preparation procedures were employed, for example, solvent–solvent extraction [6–9], solid-phase extraction [10–12] and protein precipitation coupled with column switching to remove endogenous compounds [13]. For the HPLC assays, the internal standards employed were either not well resolved from atenolol (procainamide [6] or sotalol [7]), or eluted with an excessively long retention time (metoprolol [8]). When the retention time of the internal standard, metoprolol, was reduced, atenolol

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was eluted too close to the solvent front [9]. Internal standard was not employed in some reports [12,13]. In this report, we describe an efficient and validated assay using a simple 2-step solvent extraction sample preparation procedure, isocratic HPLC conditions and α-hydroxymetoprolol as the internal standard.

2. Experimental

2.1. Materials

Atenolol was purchased from Sigma (St. Louis, MO, USA), and the internal standard, α-hydroxymetoprolol, was obtained from Astra Hassle (Mölndal, Sweden). HPLC grade cyclohexane, *n*-butanol and methanol were purchased from Mallinckrodt (Chesterfield, MO, USA). Analytical grade triethylamine (TEA) was purchased from Riedel-de Häen (Seelze, Germany), and phosphoric acid, sodium hydroxide and sulphuric acid were from BDH (Poole, UK). All reagents were used without further purification. Stock solutions of atenolol and α-hydroxymetoprolol were prepared in distilled and deionised water at 200 μg/ml and stored at 4°C. The working solutions for spiking plasma and urine were freshly prepared daily. Blank human plasma samples were obtained from the Red Cross of Hong Kong.

2.2. Chromatography

A Hewlett-Packard series 1050 HPLC system equipped with an HP 1046A programmable fluorescence detector and ChemStation software package was used (Hewlett-Packard, Wilmington, DE, USA). The HPLC mobile phase was composed of 18% methanol in 0.5% w/v TEA adjusted to pH 3.5 with phosphoric acid. Separation was achieved using an Alltech Spherisorb ODS-2 column (5 μm, 4.6×250 mm) at a flow-rate of 1 ml/min. The eluent was monitored by fluorescence detection at 229 nm (excitation) and 298 nm (emission).

2.3. Sample preparation

2.3.1. Plasma samples

The plasma samples (1 ml) were treated in glass centrifuge tubes with the internal standard (50 μl, 4 μg/ml), water (250 μl), sodium hydroxide (200 μl, 2 *M*), and a mixture of cyclohexane–*n*-butanol (5 ml, 55:45, v/v). The mixture was shaken (10 min), centrifuged (1000 *g*, 5 min) and the separated organic layer was transferred to a clean centrifuge tube. The organic phase was back-extracted with dilute sulphuric acid (100 μl, 0.1 *M*) by vortex mixing (30 s). The mixture was chilled (4°C, 10 min), centrifuged (1000 *g*, 5 min) and the acidic aqueous phase (bottom layer) was withdrawn and an aliquot (70 μl) was analysed by the HPLC assay. The standard samples for plasma calibration curve were prepared by spiking blank plasma with 250 μl of the appropriate working solutions to give plasma concentrations of atenolol at 2, 5, 10, 50, 100, 250, 500, 1000, 2500 and 5000 ng/ml.

2.3.2. Urine samples

A procedure similar to that for plasma samples was employed. Urine samples (0.5 ml) were mixed with water (0.5 ml), sodium hydroxide (200 μl, 2 *M*), and the internal standard (50 μl) was added to give a concentration of 20 μg/ml. The volume of sulphuric acid for back-extraction was 200 μl and an aliquot (60 μl) was used for HPLC analysis. The standard samples for urine calibration were prepared by spiking urine blank with 500 μl of the appropriate working solutions to give atenolol concentrations of 1, 2, 4, 8, 16 and 20 μg/ml.

2.3.3. Validation assay

The extraction recoveries of the internal standard and atenolol at low to high concentrations (10, 500 and 5000 ng/ml) were determined in replicates ($n=5$) by comparing the respective peak areas of the chromatograms of the extracted samples relative to the untreated standards containing an equivalent amount of the compounds. The reproducibilities of the assay were determined by analysing multiple spiked blank plasma samples at 40 and 900 ng/ml and blank urine samples at 9.5 μg/ml ($n=5$) over three separate days using the procedures described above in Sections 2.2 and 2.3. The intra-day and inter-day variability were calculated as percentage standard deviation of the mean.

3. Result and discussion

3.1. Chromatography

The sample preparation was adapted from a literature method [8]. The basic analytes were extracted into the organic phase from alkalinised biological fluid, followed by back extraction into a small volume of aqueous acidic medium. This procedure is fast, reproducible and cost-effective. However, employing the reported HPLC conditions, the retention time (t_R) of the internal standard, metoprolol, was found to be too long (t_R>13 min, k'> 4.1) while the analyte, atenolol (t_R, 4.5 min; k', 1.4), was eluted too close to the solvent front and co-eluted with polar plasma components. As anticipated, efforts to increase the retention time of atenolol were counteracted by further lengthening of the elution time of metoprolol, and shortening of the retention time of metoprolol reduced the retention time of atenolol even further (this work and reported in Ref. [9]). Further work revealed that a metabolite of

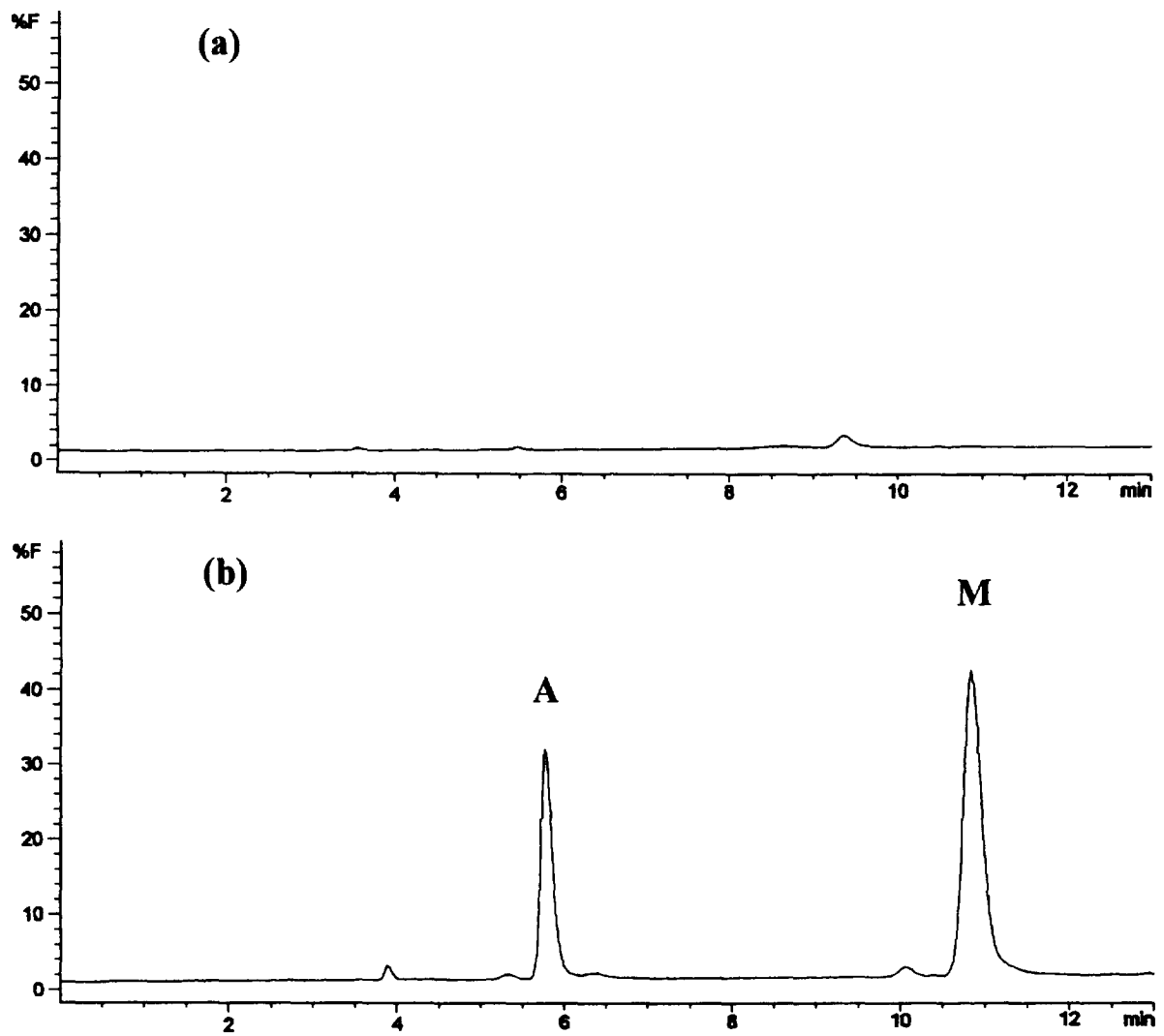

Fig. 1. Representative chromatogram of plasma samples: (a) plasma blank; (b) plasma blank spiked with atenolol (100 ng/ml) and internal standard, α-hydroxymetoprolol, (200 ng/ml). Peaks: (A) atenolol; (M) α-hydroxymetoprolol.

metoprolol, α-hydroxymetoprolol and atenolol has a similar polarity. They were well resolved and were eluted within 13 min. Under the current assay conditions using isocratic elution, atenolol and α-hydroxymetoprolol were eluted with retention times of 5.5 and 11 min, respectively (Figs. 1 and 2). Interference from naturally occurring plasma and urine components was not encountered.

3.2. Linearity and quantification limit

Peak plasma levels of less than 1 μg/ml following oral administration and less than 5 μg/ml following intravenous administration of 50 mg atenolol in healthy adults were reported [14]. Thus, due to the wide range of plasma concentrations anticipated, the assay for plasma concentration was calibrated from 2 to 5000 ng/ml. The standard curves were constructed by plotting the peak-area ratio of atenolol/internal standard versus atenolol concentration. In order to avoid undue bias to the lower concentrations of the standard curve by the higher concentrations, the calibration was split into two concentration ranges: 2 to 100 ng/ml and 100 to 5000 ng/ml. For urine analysis, a calibration range of 1 to 20 μg/ml was established. Linear standard curves, with standard errors, for both plasma and urine samples obtained over three independent runs were described by the following regression equations:

Plasma concentrations 2–100 ng/ml; $y=0.076(\pm0.001)x+0.057(\pm0.071)$ $r^2=0.9972$; plasma concentrations 100–5000 ng/ml; $y=0.064$

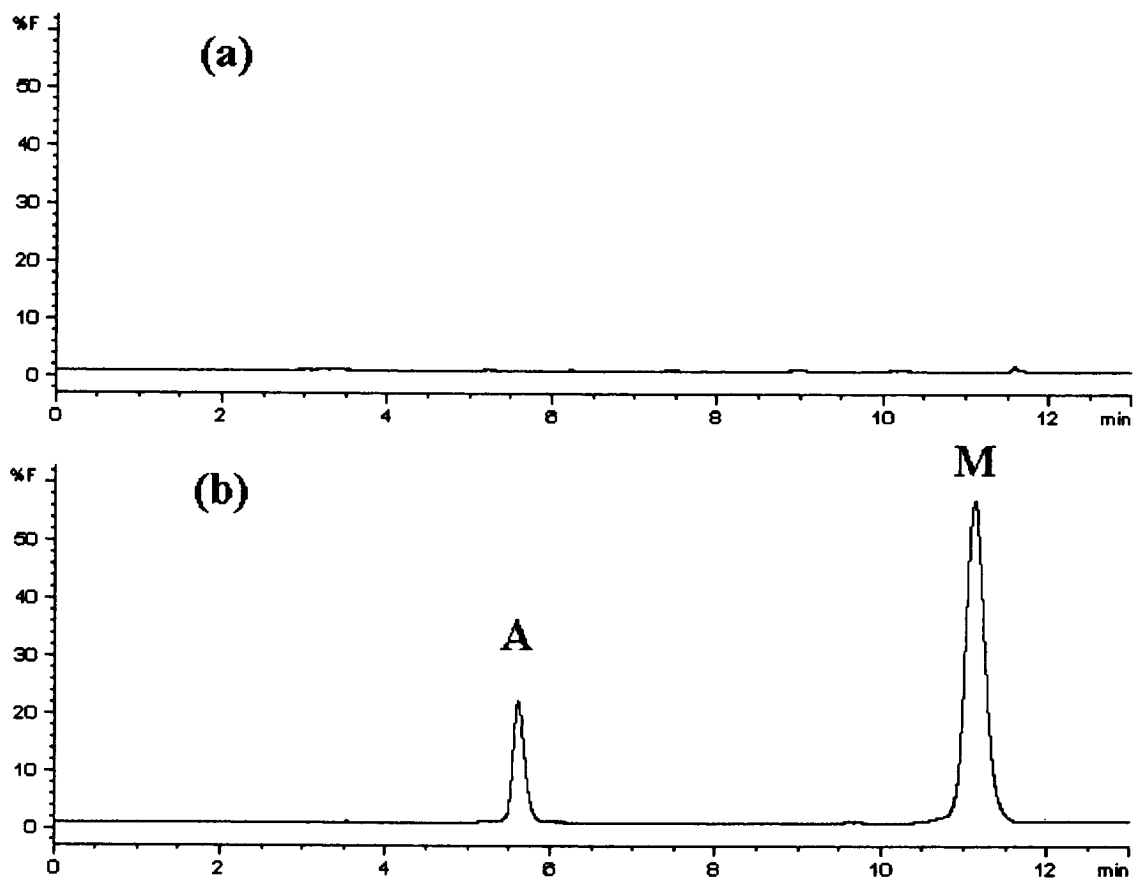

Fig. 2. Representative chromatogram of urine samples: (a) urine blank; (b) urine blank spiked with atenolol (4.0 μg/ml) and internal standard, α-hydroxymetoprolol, (20 μg/ml). Peaks: (A) atenolol; (M) α-hydroxymetoprolol.

($\pm 0.001)x+3.76(\pm 1.55)$ $r^2=0.9989$ and urine concentrations 1–20 μg/ml; $y=0.428(\pm 0.003)x+0.11(\pm 0.03)$ $r^2=0.9993$.

The detection limit for atenolol in plasma and urine was at 1 ng/ml with a S/N ratio of 5:1.

3.3. Recovery

Recovery studies were performed in replicates ($n=5$). Recoveries of the internal standard from both plasma and urine were identical (83%). The recoveries of atenolol from plasma and urine were 74 and 79%, respectively. The standard errors of the mean were 1.5% in all the recovery studies.

3.4. Precision and accuracy

In plasma, the intra-day variations at both concentrations were between 0.5 to 3%, and the inter-day variations were 2.4% and 1.2% at 40 and 900 ng/ml, respectively. In urine, the intra-day variation at 9.5 μg/ml was between 0.5 to 3% and the inter-day variation was 2%.

4. Conclusion

The assay described in this report is highly efficient. The analytes were extracted into the organic phase from alkalinised human samples and back-extracted into a small volume of aqueous acid for direct injection onto the HPLC. The choice of α-hydroxymetoprolol as the internal standard enabled the chromatographic run to be completed within 13 min. Coupled with fluorescence detection, a low detection limit of 1 ng/ml was achieved.

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