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DETERMINATION OF ATENOLOL IN PLASMA BY DUAL-COLUMN LIQUID CHROMATOGRAPHY AND FLUORIMETRIC DETECTION

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SUMMARY

A simple and rapid liquid chromatographic method for the determination of atenolol in plasma is described. Plasma proteins were precipitated with zinc sulphate and sodium hydroxide prior to injection onto a precolumn, which was connected to the analytical column by a switching valve. When atenolol was eluted onto the analytical column, the precolumn was cleaned by backflushing to eliminate strongly retained endogenous compounds. The atenolol fluorescence was measured after excitation at 197 nm. The limit of quantitation in plasma was 15 ng/ml. The within-day precision of atenolol was 1.6% at a level of 210 ng/ml, 5.0% at 25 ng/ml and the between-day precision was 3.3% at 50 ng/ml.

INTRODUCTION

Atenolol (Fig. 1) is a hydrophilic β-receptor blocking drug. Only 5% of the drug is metabolized, and the degree of plasma protein-binding is ca. 3% [1].

Some gas chromatographic methods for the determination of atenolol in plasma have been described [2–4]. Owing to the high hydrophilicity of the drug, reversed-phase liquid chromatography (LC) may be a simpler and faster technique, as the derivatization step can be avoided. Previously reported LC methods include sample work-up using liquid–liquid extraction and evaporation, both time-consuming steps [5–14]. Sample work-up using liquid–solid distribution with [10] and without [14] evaporation has been used, but gave in one case [14] low absolute recoveries.

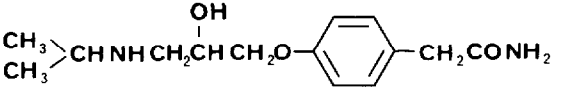

Fig. 1. Chemical structure of atenolol.

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Coupled columns have been used to reduce the analysis time; late-eluting peaks are directed from the precolumn to waste [15,16]. Only the zone containing the analytes is transferred to the analytical column for further separation. During the separation on the analytical column the precolumn is cleaned in the foreflush or the backflush mode.

This report describes a fast and simple reversed-phase LC method in which plasma proteins were precipitated before injection onto a dual-column system. Only slightly retained compounds were introduced onto the analytical column for separation. Strongly retained endogenous compounds were eliminated from the precolumn by backflushing.

EXPERIMENTAL

Apparatus

The LC system consisted of a Waters M45 pump (Waters Assoc., Milford, MA, U.S.A.), a Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and a Waters WISP 710B automatic injector. The fluorimetric detector was a Schoeffel FS 970 (Schoeffel Instruments, Westwood, NJ, U.S.A.) with an excitation wavelength of 197 nm and no emission filter. The integrator was an SP 4270 (Spectra Physics, San Jose, CA, U.S.A.). The centrifuge was an IEC Centra-7R refrigerated centrifuge (International Equipment Company, Needham, MA, U.S.A.).

Chemicals

Atenolol was kindly supplied by STADA (Bad Vilbel, F.R.G.). Zinc sulphate, sodium hydroxide and buffer substances were all of analytical grade. Acetonitrile was of HPLC grade (Merck, Darmstadt, F.R.G.). N,N-Dimethyl-N-octylamine (DMOA) was obtained from ICN Pharmaceuticals (Plainview, NY, U.S.A.).

Chromatographic system

The precolumn (30 mm×4.6 mm I.D.) was a Pecosphere C_{18}, 3 μm (Perkin Elmer, Beaconsfield, U.K.) and the analytical column (100 mm×4.6 mm I.D.) was a Microsorb C_{18}, 3 μm (Rainin Instruments, Woburn, MA, U.S.A.). The precolumn was inserted in a switching valve, Kontron Tracer MCS 670 (Kontron, Zürich, Switzerland). The valve was controlled by a Tracer timer 210 (Kontron). The mobile phase contained 0.1 m*M* DMOA in 4% (v/v) acetonitrile in phosphate buffer (pH 2, μ=0.05). The precolumn was cleaned in the backflush mode by disconnection from the analytical column by the switching valve (Fig. 2). The mobile phase was used as the washing solvent.

Sample preparation

Plasma (1.00 ml) was mixed with 0.70 *M* zinc sulphate (0.5 ml) containing 0.5 m*M* DMOA and 1.0 *M* sodium hydroxide (0.5 ml). After centrifugation at 600 *g* for 15 min, the supernatant was filtered through a piece of cotton inserted into the tip of an Eppendorf pipette, and 500 μl of the clear solution were injected onto the precolumn.

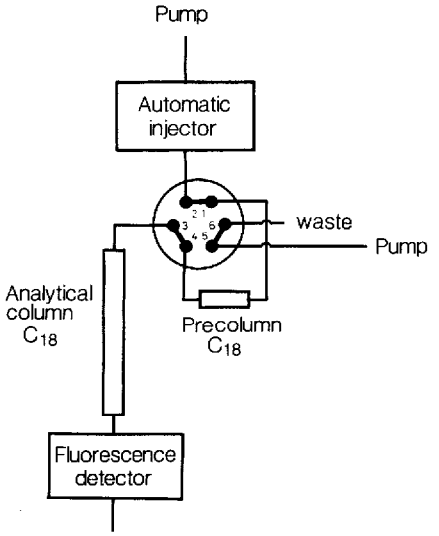


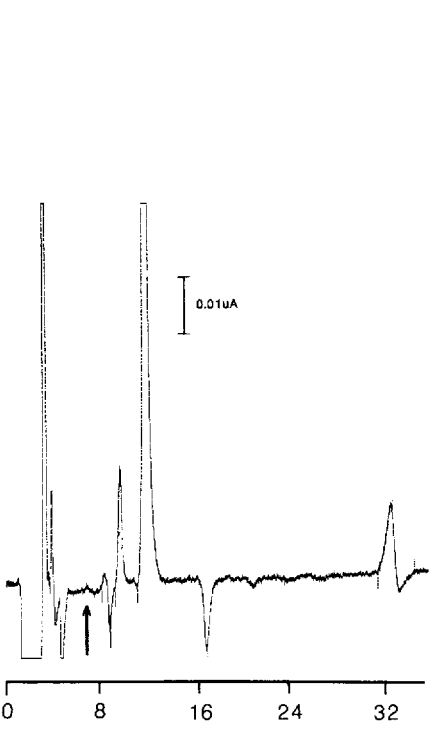
Fig. 2. Scheme of the dual-column system. Chromatographic conditions: precolumn, Pecosphere C_{18}; analytical column, Microsorb C_{18}; mobile phase to both columns, 0.1 m*M* DMOA in 4% (v/v) acetonitrile in phosphate buffer (pH 2); detection, excitation at 197 nm without emission filter.

RESULTS AND DISCUSSION

Sample preparation

Atenolol is a hydrophilic compound: the octanol–buffer partition coefficient is 0.04 [17]. Extraction of more than 99% was obtained when the aqueous phase was adjusted to pH 12, saturated with sodium chloride and extracted with 6% (v/v) heptafluorobutanol in dichloromethane [4].

Using reversed-phase LC, with the possibility of injecting aqueous solutions, protein precipitation was a more straightforward sample preparation. Different methods to precipitate plasma proteins were tested: (1) 0.35 *M* perchloric acid (0.1 ml) containing 0.5 m*M* DMOA and (2) 0.70 *M* zinc sulphate containing 0.05 m*M* DMOA and 1.0 *M* sodium hydroxide (0.5 ml + 0.5 ml). The concentration of atenolol in plasma was 100 ng/ml. Perchloric acid was a more efficient protein precipitant, resulting in less dilution, compared with zinc sulphate and sodium hydroxide [18]. However, the final concentration of the analyte in the injected solutions from the two different precipitation methods was about the same, as the pH of the supernatant of the perchloric acid had to be adjusted to stabilize atenolol. When the pH was increased to 5, atenolol was stable for at least 15 h, otherwise atenolol was degraded by ca. 30% within 6 h. Precipitation with zinc sulphate and sodium hydroxide gave a pH of 7.0 in the supernatant and, in this environment, atenolol was stable for at least 48 h. The absolute recovery of atenolol through protein precipitation by perchloric acid was 100% ($n=2$) compared with a directly injected aqueous solution mixed with perchloric acid. The absolute recovery from plasma, precipitated by zinc sulphate and sodium hydroxide, was 110% ($n=2$) compared with atenolol added to the supernatant. The absolute



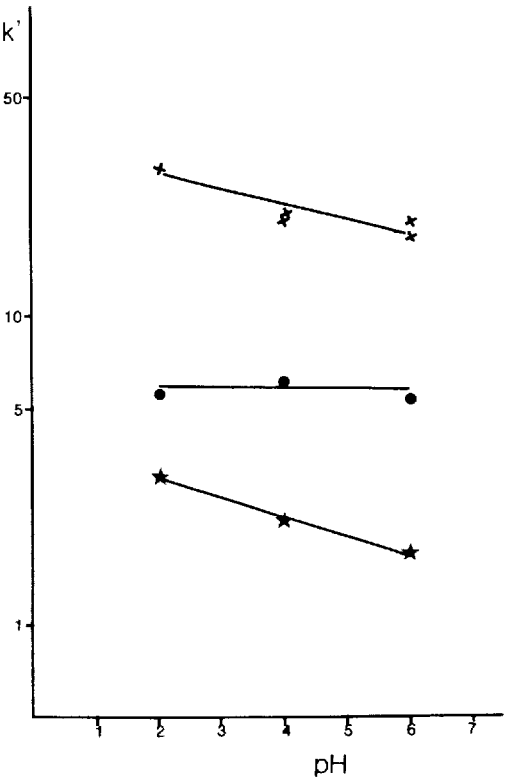


Fig. 3. Chromatogram from blank plasma. Chromatographic conditions: precolumn, Pecosphere C_{18}; analytical column, Microsorb C_{18}; mobile phase, 0.1 m*M* DMOA in 4% (v/v) acetonitrile in phosphate buffer (pH 2); detection, excitation wavelength at 197 nm without emission filter. The arrow indicates the retention time of atenolol.

Fig. 4. Influence of pH on the retention of atenolol and the endogenous compounds. Chromatographic conditions: precolumn, Nucleosil C_8, 5 μm; analytical column, Microsorb C_{18}; mobile phase, 2.6 m*M* DMOA in 1% (v/v) acetonitrile in phosphate buffer (μ=0.05). (⋆) Atenolol; (●) endogenous compound; (×) endogenous compound.

recovery of atenolol from an aqueous solution mixed with zinc sulphate and sodium hydroxide was 102% (n=2) compared with atenolol added to the supernatant.

Recoveries of over 100% were probably due to volume differences, which occur when the proteins are precipitated. If the analyte only is distributed in the clear supernatant, the concentration increases when the volume decreases due to a voluminous precipitate. As the blank plasma chromatogram was cleaner when precipitation was performed with zinc sulphate and sodium hydroxide than when perchloric acid was used, the former method was selected.

Chromatography

Atenolol, an aminoalcohol, was separated from plasma components on two reversed-phase C_{18} columns. Several studies have shown that the peak symmetry

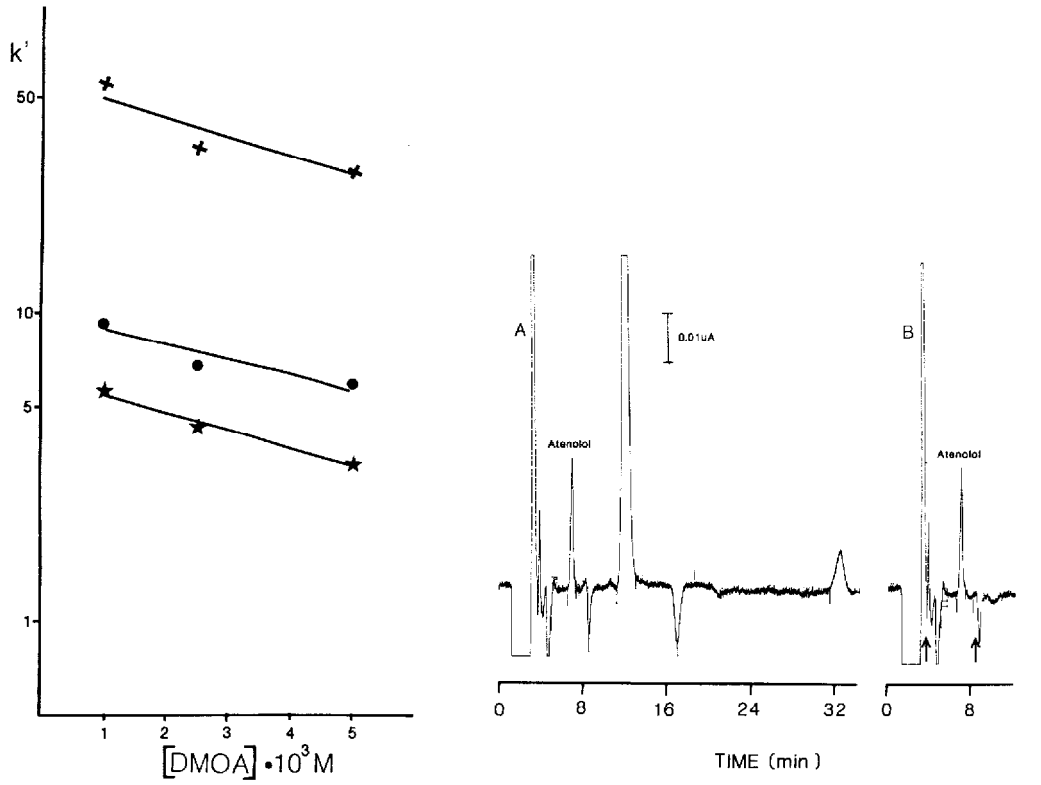


Fig. 5. Influence of the concentration of DMOA on the retention of atenolol and the endogenous compounds. Chromatographic conditions: precolumn, Pecosphere C_{18}; analytical column, Microsorb C_{18}; mobile phase, DMOA in 1% (v/v) of acetonitrile in phosphate buffer (pH 2). (★) Atenolol; (●) endogenous compound; (×) endogenous compound.

Fig. 6. Plasma chromatogram (A) without and (B) with the column-switching technique. Chromatographic conditions as in Fig. 3. The arrows indicate where the column-switching was activated. The plasma sample contained 151 ng/ml atenolol.

of amines is improved by the addition of an amine in the mobile phase [19–21]. For atenolol the peak asymmetry factor was reduced from 2.5 to 1.1 by the addition of 0.1 m*M* DMOA [k' was regulated to 4.3 in both cases by adjusting the acetonitrile content to 7% (v/v) and 3% (v/v), respectively, in phosphate buffer, pH 2]. Injection of the plasma supernatant samples resulted in a higher chromatographic efficiency compared with injection of atenolol in the mobile phase, owing to the trace enrichment effect [22]. Variation of the injection volume between 25 and 500 μl of the supernatant gave the same chromatographic efficiency.

In blank plasma chromatograms two large peaks with long retention times appeared (Fig. 3). Only one of the endogenous compounds was affected by changing the pH of the mobile phase between 2 and 6 (Fig. 4). When the concentration of DMOA was increased the retention of the endogenous compounds decreased to the same extent as that of atenolol (Fig. 5), indicating that the endogenous com-

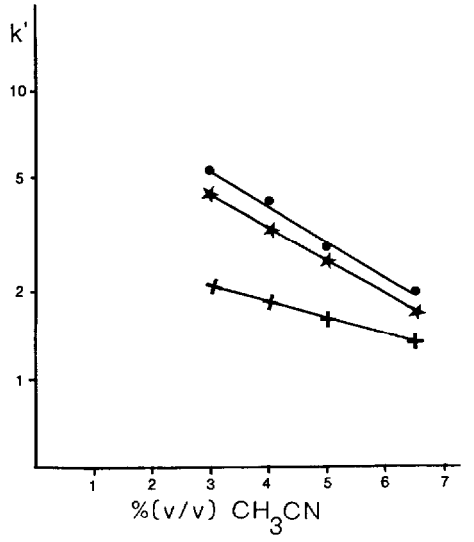


Fig. 7. Influence of the content of acetonitrile (CH_3CN) on the retention of atenolol and the negative peaks. Chromatographic conditions: precolumn, Pecosphere C_{18}; analytical column, Microsorb C_{18}; mobile phase, 0.1 m*M* DMOA in various amounts of acetonitrile in phosphate buffer (pH 2). (★) Atenolol; (●) negative peak; (+) negative peak.

TABLE I

RELATIVE STANDARD DEVIATIONS (R.S.D.) AND ABSOLUTE RECOVERIES OF ATENOLOL ON TWO LEVELS

Added amount (ng/ml)	R.S.D. (%)	Absolute recovery (%)
210	1.6	110
25	5.0	112

pounds are cationic. However, the strongly retained endogenous compounds and atenolol were separated on a 30-mm C_{18} column. The late-eluting peaks could then be eliminated by coupling the short C_{18} column in series with the analytical column. When atenolol was eluted onto the analytical column the precolumn was disconnected by a switching valve and cleaned by backflushing with a solvent of the same composition as the mobile phase. With this front-cut technique the analysis time was reduced significantly. The switching event and the chromatogram obtained are shown in Fig. 6.

The plasma chromatogram also contained two early-eluting negative peaks, which probably are system peaks due to equilibrium disturbances in the mobile phase when precipitated plasma was injected (cf. refs. 23 and 24.). The retention of the negative peaks decreased to a different extent than that of atenolol with increasing content of acetonitrile, which means that the content of acetonitrile could be used to obtain chromatographic selectivity (Fig. 7). In the method 0.1 m*M* DMOA in 4% (v/v) of acetonitrile in phosphate buffer (pH 2, μ=0.05) was used.

Stability of the dual-column system

Injections of 100 precipitated plasma samples (500 μl) reduced the number of theoretical plates of the dual-column system only slightly (ca. 8%). Both the retention time and the back-pressure were unaffected. Replacing the precolumn restored the initial plate value.

Sensitivity and precision

A high detection selectivity towards endogenous compounds was obtained by using fluorescence detection. UV detection at 228 nm gave the same limit of detection as fluorescence detection at an excitation wavelength of 228 nm. The limit of detection (defined as a signal three times the background noise level) was ca. 15 ng. When the excitation wavelength was decreased from 228 to 197 nm the limit of detection was 6 ng, i.e. 2.5 times better. Emission filters (320, 370 and 580 nm) reduced the limit of detection considerably.

Calculations of atenolol were performed by construction of a calibration curve of peak heights against known added concentrations of atenolol to blank plasma. The absolute recoveries and the intra-assay precisions are shown in Table I. The absolute recoveries were compared with the response obtained by direct injection of atenolol dissolved in the supernatant of zinc sulphate and sodium hydroxide. Plasma levels as low as 15 ng/ml were determined. The day-to-day variation at a level of 50 ng/ml atenolol was 3.3% (relative standard deviation, $n=9$). Plasma samples were stable for at least three months when stored at -20°C, which is in accordance with previous findings [9].

CONCLUSION

Atenolol is determined in deproteinized plasma by reversed-phase LC and fluorimetric detection. The sample throughput was increased by using two columns separated by a switching valve.

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