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Simultaneous determination of atenolol and chlorthalidone in plasma by high-performance liquid chromatography Application to pharmacokinetic studies in man

Claudio Giachetti*, Anna Tenconi, Sergio Canali, Giovanni Zanolo

Istituto di Ricerche Biomediche 'A. Marxer', RBM S.p.A, P.O. Box 226, 10015 Ivrea (TO), Italy

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Abstract

An HPLC method developed to detect in a single run both atenolol and chlorthalidone, extracted from plasma, using two detectors (UV for chlorthalidone and fluorometric for atenolol) connected in series, is described. The drugs were separated on an ODS column at room temperature using a 0.05 *M* sodium dodecyl sulphate in phosphate buffer (pH 5.8)–*n*-propanol (95:5, v/v) solution, delivered at a flow-rate of 1.3 ml/min. Having ascertained the sensitivity (10 ng/ml of both drugs) and the intra-day reproducibility (pre-study validation), the reliability of the method was verified by inter-day assays (within-study validation) carried out during the analysis of plasma samples collected from healthy volunteers after single-dose treatment with atenolol+chlorthalidone tablets (pharmaceutical preparations containing 100+25 mg and 50+12.5 mg of the two drugs, respectively). © 1997 Elsevier Science B.V.

Keywords: Atenolol; Chlorthalidone

1. Introduction

Atenolol and chlorthalidone are well-known drugs widely used in the treatment of hypertension, the first exerting selective action on β_1 receptors and the second diuretic activity. Their pharmacological action and pharmacokinetics, following daily oral doses of 50–100 mg of atenolol [1–5] or 25–200 mg of chlorthalidone [6–9], are nowadays well explained and documented. In recent years, pharmaceutical preparations containing both drugs have also been marketed, their combination having the advantage of providing greater therapeutic effects than with either drug alone and permitting once a day administration only, the two drugs not interacting pharmacokinetically with each other or presenting synergic toxic effects. Several works have reported atenolol determination in plasma, adopting gas chromatographic techniques with an electron capture detector [10–12] or HPLC, using reverse phase columns and UV or fluorometric detection [13–19]. Similarly, chlorthalidone has been measured in biological fluid extracts both by GC and nitrogen detection [20–22] or HPLC using analogous columns and UV detection [23–27]. With these methods, sensitivity levels ranging from 5 to 10 ng/ml of plasma for atenolol and from 25 to 200 ng/ml for chlorthalidone were obtained. In the last few years the monitoring of these drugs has been extended to the separation and determination of their enantio-

*Corresponding author.

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mers, since the two different optical forms can show different biological activities in man. Enantio-selective fluorometric methods, developed for atenolol assay in plasma samples, involved the use of immobilised chiral β-cyclodextrine derivative [28] or α_1-acid glycoprotein [29] columns. Alternatively, its (−)-menthyl chloroformate enantiomer derivatives were separated adopting common ODS columns [30,31]. Determination of chlorthalidone enantiomers has been also reported in analysis of serum samples after separation on a bonded β-cyclodextrine column [32]. Since our purpose was the evaluation of the bioequivalence of two formulations containing both drugs by measuring only their racemates in plasma, we attempted to create an alternative, and equally sensitive, HPLC method able to simultaneously detect both of them. Our aim was to develop a common extraction procedure and a successive chromatographic separation suited to both drugs, but selective for each one, with the result of reducing sample preparation and analysis time, also taking into consideration the suggestions proposed by Dadgar and Kelly [27]. The idea of connecting UV and fluorometric detectors permitted simultaneous monitoring of atenolol and chlorthalidone on independent lines, with optimised responses for each one. The procedure adopted and the method validation are described below, along with the results obtained.

2. Experimental

2.1. Chemicals

Atenolol, chlorthalidone and salbutamol were supplied by Sigma-Aldrich, (Milan, Italy). Xipamide was extracted in ethyl acetate from a commercially available pharmaceutical preparation. Its identity and purity were verified on the basis of UV spectra recorded in aqueous acid and alkaline solutions. Reagents and solvents, all of analytical or LiChrosolv reagent grade were purchased from E. Merck (Darmstadt, Germany). Mobile-phase solution was prepared by diluting 100 ml of 0.1 *M* $NaH_2PO_4 \cdot H_2O$ solution with MilliQ water up to 1 l, followed by addition of 1 *M* NaOH solution to reach pH 5.8; 14.42 g of sodium dodecyl sulphate (SDS) were then carefully added and magnetically stirred up to complete dissolution.

2.2. Standard solutions

Each analyte was dissolved in methanol to yield 1 mg/ml stock solutions. Working solutions of 100, 10 and 1 μg/ml of atenolol+chlorthalidone were prepared by serial dilution of the stock solutions in methanol. The working solution of 10 μg/ml of salbutamol+xipamide (internal standards) was similarly prepared. All solutions were stored refrigerated in the dark when not in use.

2.3. Instrumentation and chromatographic conditions

The analytes were separated on a Supelcosil LC-18, 5 μm, 250×4 mm I.D. column (Supelchem, Milan, Italy) connected to a LiChrocart 4-4 manufix containing a LiChrospher 100 RP-18, 5 μm, guard column (E. Merck), operating at room temperature. The mobile phase was 0.05 *M* SDS in phosphate buffer (pH 5.8)–*n*-propanol 95:5 (v/v), delivered at the flow-rate of 1.3 ml/min by a System Gold programmable solvent module (Model 126, Beckman Analytical, Milan, Italy). The flow entered a System Gold programmable UV-VIS detector module (Model 166, Beckman) connected in series to a fluorescence spectrophotometer (Model F-1050, Merck-Hitachi, Milan, Italy). Both detectors were linked to a System Gold analog interface module (Model 406, Beckman) and the two signals simultaneously monitored overlapped by a System Gold HPLC ChemStation (Beckman). UV absorption was set at 225 nm for chlorthalidone and xipamide monitoring. Fluorometric detection of atenolol and salbutamol was done setting the excitation wavelength at 222 nm (emission at 300 nm). Injection of the samples was by autosampler (Model AS 2000-A, Merck-Hitachi).

2.4. Sample preparation

Plasma samples (1 ml) were spiked with 250 ng of xipamide and 250 ng of salbutamol. After brief

whirl-mixing (10 s), 10 ml of a dichloromethane–2-propanol solution (75:25 v/v) were added to the samples, followed by extraction on a rotary shaker for 10 min. After centrifugation (3500 rpm, about 2000 *g*, 10 min, 4°C) the organic phases were transferred to other tubes and taken to dryness under a nitrogen stream in a water bath at 50°C. The residues were redissolved in 200 μl of mobile phase, whirl-mixed (10 s) and transferred to polypropylene vials. After ultracentrifugation (10 000 rpm, about 6500 *g*, 10 min), the supernatants were transferred to glass vials and inserted in the autosampler rack for automatic injection (40 μl) into the HPLC system.

2.5. Calibration and calculation

Evaluation of the assays was performed by seven-point calibration curves at the nominal concentration range of 10–1000 ng/ml of atenolol and chlorthalidone in human plasma (Avis, Turin, Italy). Slope and intercept of the calibration lines were determined through weighted (1/nominal concentration^2) linear regression of each drug to internal standard peak-height ratios vs. drug concentration. Unknown samples were quantified by reference to these standard curves.

2.6. Analytical method validation

The following parameters of analytical method validation were checked and evaluated according to the acceptance limits proposed by Shah et al. [33].

2.6.1. Intra-day assays

2.6.1.1. *Extraction recovery*. The percentage of recovery was obtained by relating the peak height values of the analytes obtained after spiking plasma samples and mobile phase with equivalent amounts of the drugs (25, 100 and 500 ng/ml; $n=5$/concentration/drug) and internal standards (250 ng/ml; $n=15$/I.S.).

2.6.1.2. *Limit of quantitation (LOQ) (plasma-spiked samples)*. Replicate analyses ($n=5$) of plasma samples spiked at 10 ng/ml of both drugs were carried out.

2.6.1.3. *Accuracy and precision (plasma-spiked samples) and short time stability test on extracts*. Replicate analyses of plasma samples spiked at 25, 100 and 500 ng/ml of both drugs ($n=5$/concentration/drug) were carried out upon preparation. Aliquots of the same samples, extracted, remained capped in the autosampler (room temperature, 24 h) before injection. All results were then compared.

2.6.2. Inter-day assays

2.6.2.1. *Linearity and accuracy and precision (plasma calibration samples)*. Intercept, slope and coefficient of correlation (r) were evaluated for each daily calibration curve. Mean, S.D. and C.V. values of the slope and r parameters were calculated. Concentrations of all plasma calibration samples, analysed daily along with the unknown plasma samples, were back-calculated from the relative calibration curve.

2.6.2.2. *Accuracy and precision (plasma QC samples)*. Before unknown plasma samples analysis, separate aliquots of blank plasmas, spiked at the concentrations of 25, 100 and 500 ng/ml of both drugs, were prepared and stored frozen. Two replicates/concentration of these quality control samples were thawed, processed and analysed daily within each analytical run along with a complete calibration curve.

2.7. Pharmacokinetic studies design

2.7.1. Drug dosing and sampling

Eighteen volunteers received a first pharmaceutical preparation of Tenoretic tablet (Zeneca, Milan, Italy), containing 100 mg atenolol+25 mg chlorthalidone. Similarly, 18 other volunteers received a second pharmaceutical preparation of Tenoretic Mite tablet (Zeneca), containing 50 mg atenolol+12.5 mg chlorthalidone. Blood samples were withdrawn from a forearm vein, at the following times: 0 (pre-dose), 0.5, 1, 2, 3, 4, 6, 9, 12, 24, 72 and 120 h and plasma obtained. Duplicate samples of the separated plasma were transferred to polypropylene vials and stored frozen (−20°C) until analysis.

3. Results and discussion

3.1. Preliminary trials and analytical method validation

It is widely known that chiral forms of a drug can display different pharmacological and toxicological effects when administered to animals and man as a result of their different biological activities on the living structure. Successful analytical methods have therefore been developed, including also some for atenolol and chlorthalidone, and applied to separately analyse individual enantio forms in biological matrices, aimed at investigating their resulting different pharmacokinetic profiles. However, when for regulatory purposes the bioavailability of pharmaceutical preparations has to be compared, as in bioequivalence studies, only the racemates are generally required to be analysed [34]. This was our case, in which two pharmaceutical preparations, containing atenolol+chlorthalidone, were administered in man, and our purpose was to compare the relative plasma profiles, obtained by monitoring both enantiomers of the drugs, and to evaluate their bioequivalence. The work of Dadgar and Kelly [27] was essentially a source of useful information on the separation and simultaneous detection of atenolol and chlorthalidone in plasma using reversed-phase micellar liquid chromatography. On the other hand, the method did not explore concentrations below 50 ng/ml or above 800 ng/ml of either drug, or test alternative, and more sensitive, detection systems other than the UV one. By reproducing the analytical procedure suggested, we ascertained the effectiveness of the separation of the analytes using the same mobile phase, but the limits of detectability demonstrated by the method, mainly for atenolol, at the wavelength of 235 nm, as suggested, in addition to the interferences extracted from the matrix, were unsuitable for our aim. The original and effective idea of monitoring the eluates by connecting UV and fluorometric detectors in series allowed us to overcome this problem and to gain the immediate advantage not only of having both drugs simultaneously detected, but also of improving the search for the best sensitivity conditions for each one, independently for each channel. Selectivity and sensitivity were increased by using a longer column with ODS coating and containing particles of reduced size (a 5-μm, instead of a 10-μm octyl column, without a pre-column [27]), and monitoring chlorthalidone+xipamide (I.S.) under UV set at 225 nm and atenolol+salbutamol (I.S.) under fluorometric detection at λ_{ex}=222 nm and λ_{em}=300 nm. Under these conditions, the analytes were well separated, the matrix interferences were noticeably reduced and the sensitivity for atenolol improved. As examples, Figs. 1 and 2 show some chromatographic profiles obtained. As can be seen, all four compounds are detectable at the wavelength of 225 nm (Fig. 1), but the responses for atenolol and salbutamol with fluorometric detection are greatly increased (Fig. 2). The final improvement to the method was aimed at increasing the extraction yields (about 70%, declared for chlorthalidone only, using diethylether–2-propanol (95:5 v/v) as extraction mixture [27]), using, preferably, a system solvent effective for both main drugs in order to have a common extraction procedure. Mixtures of a chlorinated solvent with a consistent percentage of an alcohol appeared the most powerful alternative, giving rise to the final extraction solvent consisting of dichloromethane–2-propanol, mixed 75/25 v/v. As a result, the recovery for atenolol from plasma was, on average over the three concentrations tested, 81.4% (C.V. 9.0%) and that for chlorthalidone 92.7% (C.V. 7.2%). Lower, but reproducible, recoveries were found for their internal standards salbutamol (64.5%, C.V. 8.0%) and xipamide (70.4%, C.V. 8.2%), probably reflecting their greater hydrophilic properties than the other two compounds. In addition, also the quantitation limit was lowered to 10 ng/ml for both drugs. In fact, at this nominal concentration atenolol and chlorthalidone showed concentration levels of 10.3 (C.V. 6.3%) and 10.4 ng/ml (C.V. 2.8%), respectively. Intra-day assays confirmed the reproducibility of the method. On average, accuracy values ranged from 97.1 to 98.7% (for atenolol) and from 95.9 to 98.8% (for chlorthalidone) and precision values from 1.7 to 5.4% (for atenolol) and from 0.9 to 3.5% (for chlorthalidone). Analogous data were acquired from extracted samples stored in the autosampler, confirming their stability in solvent at room temperature for 24 h.

The studies involved the analysis of a total of

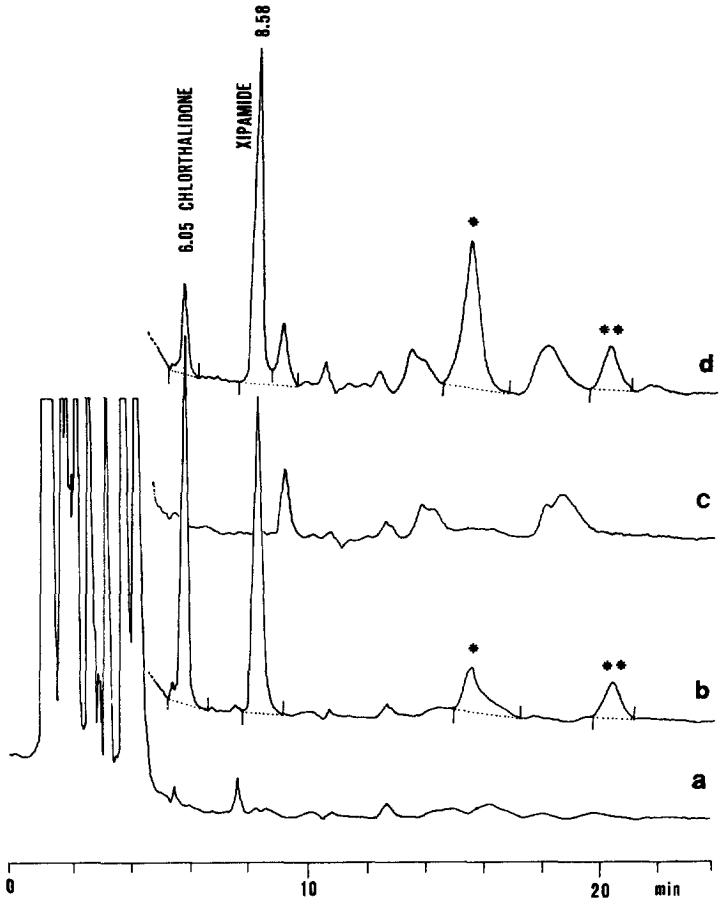

Fig. 1. Chlorthalidone plasma analysis (UV detection). Chromatographic traces recorded monitoring (a) unspiked and (b) spiked (250 ng/ml of the drug+250 ng/ml of xipamide, I.S.) calibration samples compared with authentic samples taken from a subject (c) before treatment and (d) 4 h after a p.o. dose of 100+25 mg of the two drugs (containing chlorthalidone at a concentration of 50 ng/ml). The asterisks * and ** indicate atenolol and salbutamol peaks, detected under the UV conditions selected.

more than 1000 samples, during which the linearity of the responses was verified over the entire range of the calibration curves (10–1000 ng/ml). The coefficient of correlation (r) obtained ranged from 0.99500 to 0.99968 (n=26) for atenolol and from 0.99592 to 0.99989 (n=28) for chlorthalidone, all acceptable at the significance level assumed (P=0.01). In addition, quite low C.V. values (8.0%, atenolol; 12.6%, chlorthalidone) were observed for the slopes. The limit of quantitation (LOQ) was confirmed to be 10 ng/ml (accuracy, 100.6 and 99.8%; precision, 3.2 and 2.4%; for atenolol and chlorthalidone, respectively). At concentrations higher than LOQ, the accuracy values ranged from 98.2 to 101.7% (atenolol) and from 98.6 to 100.9% (chlorthalidone), and those for precision from 3.6 to 8.3% (atenolol) and from 2.5 to 5.8% (chlorthalidone). No analytical runs were rejected, the inter-day evaluation of the quality control samples showing accuracy ranging from 91.6 to 98.0% for atenolol and from 98.1 to 101.5% for chlorthalidone, with precision ranges from 4.0 to 11.2% (atenolol) and from 4.9 to 9.0% (chlorthalidone). Atenolol concentrations in QC samples were all found to be within the acceptance intervals in 22 of the 26 runs, while those for chlorthalidone were all within the intervals considered in all runs (n=28),

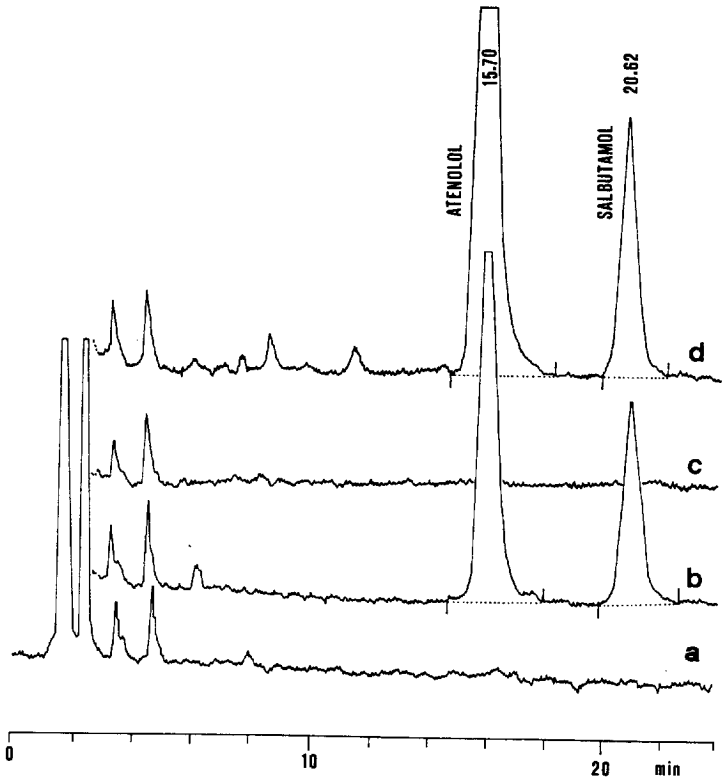

Fig. 2. Atenolol plasma analysis (fluorometric detection). The same chromatographic traces as Fig. 1 showing (a) unspiked and (b) spiked (250 ng/ml of the drug+250 ng/ml of salbutamol, I.S.) calibration samples compared with authentic samples taken from a subject (c) before treatment and (d) 4 h after a p.o. dose of 100+25 mg of the two drugs (containing atenolol at a concentration of 297 ng/ml).

fully satisfying the acceptance limits [33]. Finally, stability tests, carried out on plasma samples spiked at 25 and 500 ng/ml of both drugs and frozen for about 30 weeks, indicated that no degradation of either drug occurred.

3.2. Application to drug analysis in pharmacokinetic studies

The plasma concentrations observed after administration of these formulations combining the two drugs were in agreement with the literature data [1–10] obtained from testing atenolol and chlorthalidone singly at these doses. The mean plasma profiles obtained ($n=18$) are illustrated in Fig. 3.

4. Conclusion

With the aim to evaluating the bioavailability of reference pharmaceutical preparations versus new ones, containing atenolol+chlorthalidone, the monitoring of plasma concentrations of the two drugs, as racemates, involved the development of a method able to measure both drugs in a single HPLC run. Starting with the effective expedient of monitoring the column eluates through two different detectors connected in series, we derived the advantage of reducing the extraction to a single procedure and obtaining optimised responses for each drug independently. The successive improvement in the extraction solvent and column permitted a gain in extraction recoveries and in resolution from endogenous peaks,

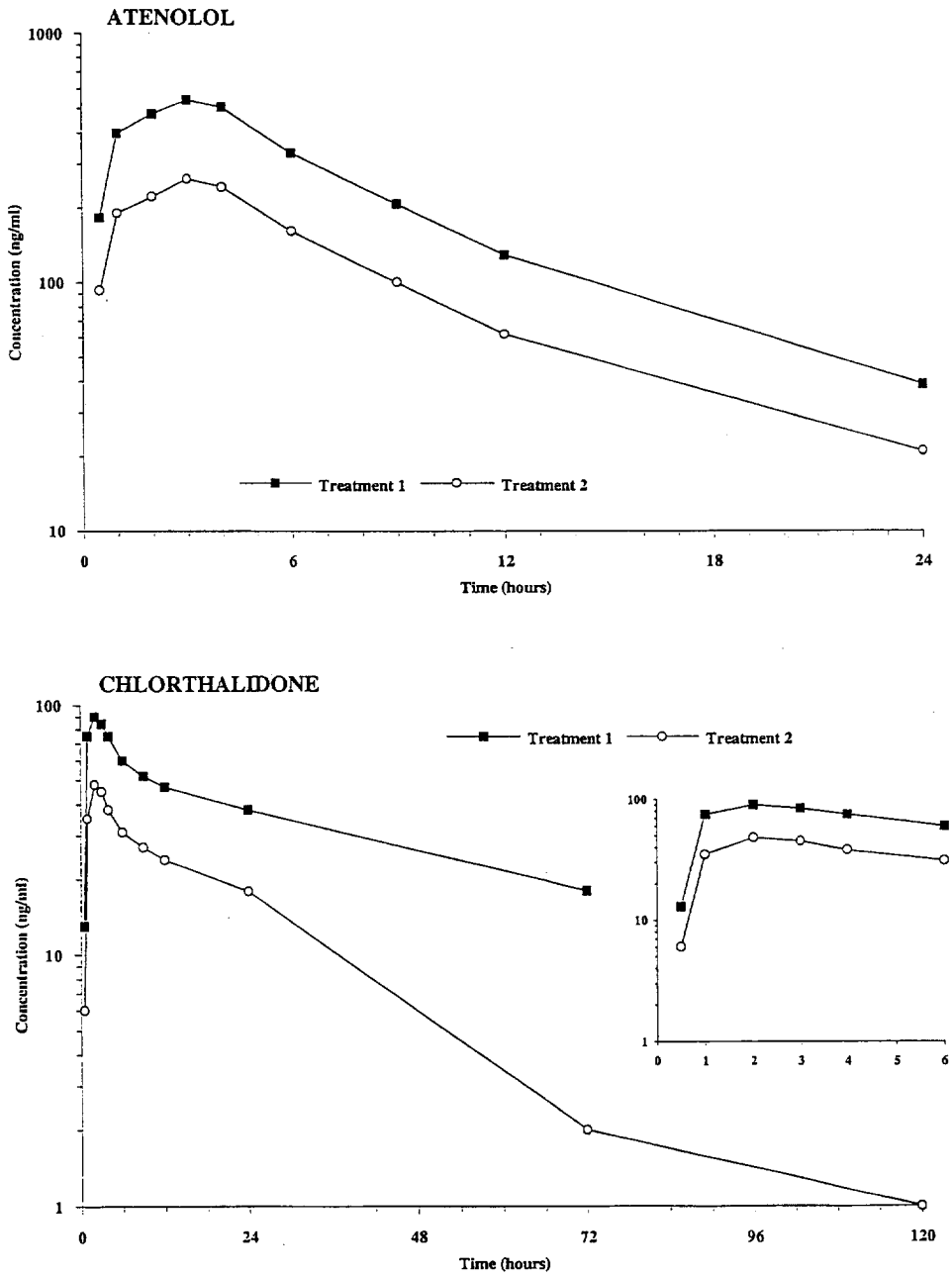

Fig. 3. Mean plasma concentration profiles of atenolol and chlorthalidone obtained from the two pharmacokinetic studies in healthy volunteers ($n=18$). Treatment 1 refers to administration of Tenoretic (100 mg atenolol+25 mg chlorthalidone, tablet), while Treatment 2 refers to that of Tenoretic Mite (50 mg atenolol+12.5 mg chlorthalidone, tablet).

reducing the run time to about 30 min. The method demonstrated to be sensitive (the LOQ was 10 ng/ml for both atenolol and chlorthalidone), precise and accurate. Its practical application in the analysis of unknown plasma samples was a further confirmation of its reliability.

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