Quantitative Determination of Atracurium in Human Plasma Using High-Performance Liquid Chromatography

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The authors have established a new method for extraction and determination of atracurium in human plasma that employs a reversed phase high-performance liquid chromatography (HPLC). This method made use of a fluorescent spectrophotometer at an excitation wavelength of 240 nm and an emission wavelength of 310 nm. The mobile phase was made of a phosphate buffer, distilled water and acetonitrile (20V:30V:50V). The analytical column used was a Little Champ C_{18}.

In a Bond Elute C_{18} extraction column, which had been prewashed with a phosphate buffer and a 50% methanol solution, atracurium was extracted from acidified plasma samples using a mixture of methanol and phosphate buffer. A standard curve was prepared by the internal standard method using metocurine. A high linear correlation between atracurium concentration and the ratio of the atracurium peak height to the metocurine peak height was observed ($r = 0.9994$). The lowest threshold for detection of atracurium was 15 ng/ml. When the plasma concentrations of atracurium were determined in 2 clinical cases, $t_{1/2\alpha}$ was 2.10 and 1.73 min and $t_{1/2\beta}$ was 15.57 and 21.57 min, respectively. These results indicate that this method of extraction and determination is appropriate for studying the pharmacokinetics of atracurium because it allows a high reproducibility, and provides an extremely accurate, simple and quick analysis. (Key words: atracurium, metocurine, a reversed phase high-performance liquid chromatography)

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Atracurium besylate (hereinafter atracurium) is a new, nondepolarizing muscle relaxant. This agent is short-acting[1–3] and is not affected by the function of the kidneys[4–7], liver[7–9] or by age in patients[10]. Instead, atracurium is metabolized by spontaneous decomposition under normal pH and body temperature conditions (Hofmann elimination)[11,12] and by hydrolysis with nonspecific esters[13,14]. Thus, the pharmacokinetics of this agent is of great interest. Until now, however, few reports have described the method for determination of atracurium concentrations in plasma[4,15,16]. The authors have established a method for extraction and determination of atracurium in human plasma, and using this method the pharmacokinetics of atracurium was studied in 2 clinical cases.

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Methods

1. *Analytical instruments*

High-performance liquid chromatography (HPLC) employed a Waters M-45 pump, a Valcovalve VISF-2 injector and fluorescent detector (Model FS 970, Schoffel Instruments). The analytical column used was a Little Champ C_{18} (grain size: 3 μm). The data was recorded by a Linear Instruments 252A recorder at a paper forward speed of 10 mm/6 min.

2. Reagents

A standard curve was drawn using atracurium, marketed in the United States by Burroughs Wellcome Co., with metocurine (Eli Lilly Co.) serving as an internal standard. For preparation of the mobile phase and the extraction fluid, the following reagents were employed: acetonitrile (Backer Chemical), NaH_2PO_4 (Mallinckrodt), H_3PO_4 for HPLC (Alfa Product), tetramethyl ammonium hydroxide-25% in methanol (THAM, Alfa products), KH_2PO_4 (Mallinckrodt), NaCl (Mallinckrodt), deionized double distilled water (hereinafter called DDW).

3. Conditions for separation and determination

The mobile phase was made of a phosphate buffer – a mixture of 30 mM NaH_2PO_4, 0.16% H_3PO_4 and 1% THAM, with its pH ajusted to 3.0 by H_3PO_4 – acetonitrile, DDW and acetonitrile (20V:30V:50V). The flow rate of the pump was 0.6 ml/min. The analysis was done at room temperature.

4. Extraction methods

Immediately after taking arterial blood samples, 20 μl of 3N-HCl was added to each sample and centrifuged at 15000g for 30 seconds using an Eppendorf 541S microcentrifuge. Keeping each plasma sample at a low temperature (0–4°C), the following extraction procedures were performed as quickly as possible. 0.5 ml of plasma was passed through a Bond Elut C_{18} column (Analytichem International) which was packed with 100 mg of octadecyl and had been washed in advance with methanol and a phosphate buffer – made of 10 mM KH_2PO_4, 50 mM NaH_2PO_4, 150 mM NaCl and 10 mM EDTA-2Na. Then, after washing with 2 ml of the above-mentioned phosphate buffer and 2 ml of 50% methanol, atracurium and metocurine were slowly extracted by suction using 800 μl of a mixture (80V:20V) of methanol and the other phosphate buffer – made of 100 mM NaH_2PO_4 and 30 mM H_3PO_4.

5. Preparation of a standard curve

Plasma was drawn from volunteers. Blank plasma (to which neither atracurium nor metocurine was added) and plasma samples treated with 25, 250, 500, 1000, and 2500 ng of atracurium, and 8000 ng of metocurine each (for use as an internal standard for correction of the recovery rate) were subjected to the above-mentioned extraction procedures. The extracted samples were then analyzed by HPLC at least three times, followed by calculation of the ratio of the atracurium peak height to the metocurine peak height and preparation of a standard curve. The same procedures were repeated 21 times on different days in order to study the accuracy of extraction and determination, i.e., coefficients of intra-assay and inter-assay variances (CV), recovery rates, etc.

6. Time course of plasma atracurium concentrations in anesthetized patients

From two adults (ASA risk 1 and 2), arterial blood was obtained before and 2, 4, 6, 8, 10, 15, 20, 25, 30, 45, 60, 75, 90 and 105 min after intravenous injection of 0.5 mg/kg of atracurium and also 120 min after the injection in one case, followed by determination of blood atracurium concentrations. In addition, neuromuscular function was measured by recording the force of thumb adduction with a Grass FT10 transducer after single twitch stimulation delivered via a Grass S88 stimulater at 0.15 Hz through 22 G needle electrodes to the ulnar nerve at the wrist and was recorded by a polygraph. One of the two subjects received only a single rapid intravenous injection of atracurium, while the other received, in addition to an initial injection, continuous subsequent doses which were administered upon 75% recovery from muscular relaxation so as to maintain 95% muscular relaxation.

7. Pharmacokinetic analysis

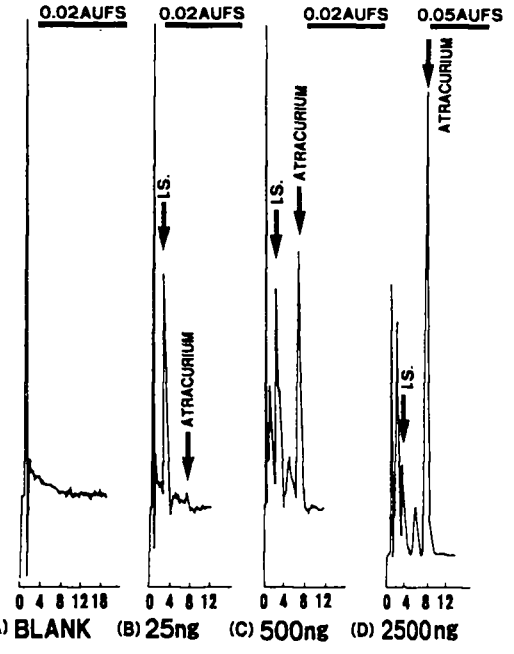

Fig. 1. Chromatograms of HPLC assay of atracurium in human plasma. (A) Blank plasma. Extracted plasma samples containing, (B) 25 ng, (C) 500 ng, (D) 2500 ng of atracurium, and 1000 ng of metocurine (I.S.) each. Retention times is ca. 4.0 min for IS, and ca. 9.5 min for atracurium at a flow rate of 0.6 ml/min.

The experimental plasma concentration-time data from both cases were fitted according to a two-compartment open model[17] using a nonlinear least-squares regression program (Super Application System, NLIN). The data points were weighted; Wi = 1/Yi×Yi, where Wi is the weight and Yi is the value of its observation.

Results

Figure 1 shows chromatograms for the extracted samples. The blank samples showed no peak which might interfere with atracurium or metocurine. The retention time was ca. 4.0 min for metocurine and ca. 9.5 min for atracurium respectively. Between the two peaks, the chromatogram returned to the baseline, indicating a good separation. The intra-assay CV of the peak height ratio was within 2.5% and the inter-assay CV was

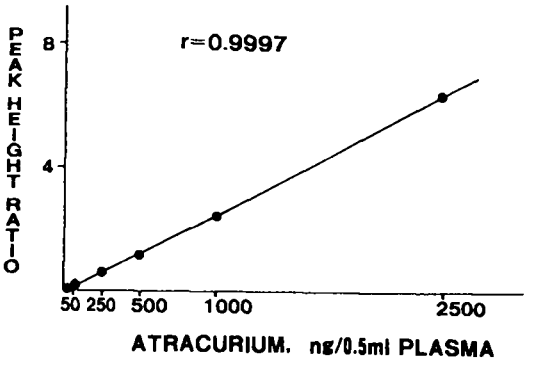

Fig. 2. A standard curve of atracurium. A very high coefficient of correlation (r) was observed.

within 9%. Recovery rate averaged 87.5% (n = 398) for atracurium and 88.7% (n = 446) for metocurine. The lowest threshold for the detection of atracurium was 15 ng/ml at an AUFS of 0.02 and signal-noise ratio of 3. Figure 2 shows an example of a standard curve. A very high correlation, represented by a first-degree equation, was obtained, with a coefficient of correlation (r) being 0.9938–0.9999 (mean ± S.D. = 0.9994 ± 0.0014; n = 21). Figures 3 and 4 show the time course of plasma atracurium levels and pharmacodynamic parameters according to twitch response of the adductor pollicis muscle.

Table 1 shows the pharmacokinetic data in 2 subjects; the half life of distribution ($t_{1/2\alpha}$) was 2.10 and 1.73 min, the half life of elimination ($t_{1/2\beta}$) was 15.57 and 21.57 min, and peak plasma levels (reached 2 min after intravenous injection) were 5612.1 and 5229.3 ng/ml, respectively.

Full (100%) recovery from muscular relaxation was noted ca. 130 ng/ml. To achieve 95% muscular relaxation, continuous intravenous injection of atracurium was necessary at a rate of 5–10 μg/kg/min corresponding to a plasma level of 650–910 ng/ml.

Discussion

Determination of plasma atracurium using a HPLC combined with a fluorescent detection has been reported by several investigators[4,15,16]. However, all of the conventional determination methods have many

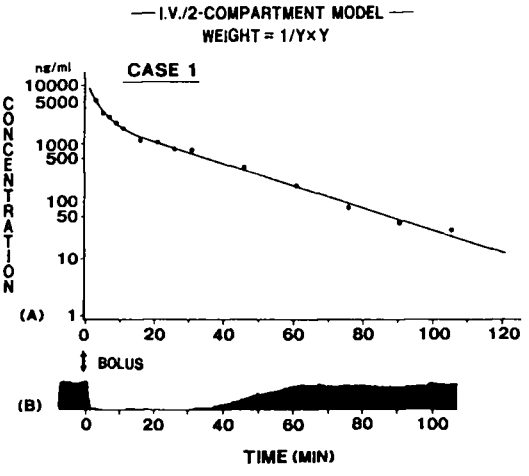

Fig. 3. (A) Representative, semi-logarithmic plot of atracurium plasma concentrations versus time for a patient after a single intravenous bolus dose of 0.5 mg/kg. Experimental datas (•) were fitted to a two compartment open model. The computer predicted curve was given by the solid line. (B) Single twitch responses of the adductor pollicis muscle following atracurium injection.

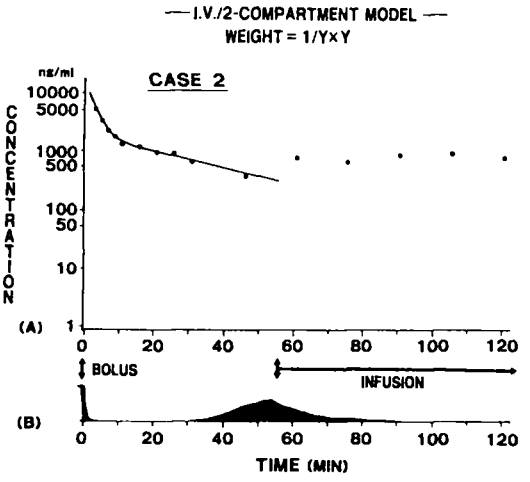

Fig. 4. (A) Representative, semi-logarithmic plot of atracurium plasma concentrations versus time for a patient after a single intravenous bolus dose of 0.5 mg/kg and maintenance infusion regimen (5–10 μg/kg/min). Experimental datas (•) were fitted to a two compartment open model. The computer predicted curve was given by the solid line. (B) Single twitch responses of the adductor pollicis muscle following atracurium injection.

Table 1. Pharmacokinetic parameters
—I.V./2-Compartment model— Weight = 1/Y×Y

	A ng/ml	B ng/ml	α 1/ml	β 1/ml	$t\frac{1}{2}\alpha$ min	$t\frac{1}{2}\beta$ min	Cl ml/kg/min	Vdcc L/kg	Vdss L/kg
Case 1	6067.81	2502.53	0.3303	0.0445	2.10	15.57	6.702	0.058	0.119
Case 2	8024.03	1821.79	0.4005	0.0321	1.73	21.57	6.516	0.015	0.154

Abbreviations: A = zero time intercept, α = rate constant (distribution phase), B = zero time intercept, β = rate constant (elimination phase), $t_{1/2\alpha}$ = distribution half-life, $t_{1/2\beta}$ = Elimination half-life, Cl = Clearance, Vdcc = Volume of central compartment, Vdss = Volume of distribution at steady-state.

inadequacies. Naill & Jones analyzed plasma atracurium levels without using any internal standard (IS) and at a high flow rate (3.5 ml/min) of the mobile phase. Obtaining stable data using this method is very time consuming. Furthermore, omission of an IS may be the cause of discrepancies in data between laboratories. Analysis at a high flow rate results in deterioration of the separating effciency of the analytical column. Furthermore, this analysis is uneconomical because it requires a great deal of mobile phase. Fahney et al.[4] adopted d-tubocurarine as a new IS in order to compensate for the above-mentioned deficiencies. However, the method adopted by them was essentially the same as the conventional method. The stationary phase of the column which they used was a cation – exchange material. Muscle relaxants made of quarternary ammonium have a polarity; hence, a reversed column is more appropriate for their analysis[16,18,19]. In general, reversed phase columns provide better analytic efficiency and greater sta-

bility of the stationary phase compared to ion-exchange columns. For these reasons, reversed phase column has begun to be widely used in recent years. Stiller et al.[16] analyzed atracurium using a reversed phase column and alcuronium as an IS. However, alcuronium emits very little fluorescence, and it emitted hardly any fluorescene when studied using our technique. The authors used a reversed phase column and metocurine (which emits sufficient fluorescene) as an IS. In this study, retention time was ca. 4.0 min for metocurine and ca. 9.5 min for atracurium. Between the two peaks, the chromatogram returned to the baseline, indicating sufficient separation. The extraction technique which we employed was a simple and quick one which used only DDW, methanol and phosphate buffer. No peak which might interfere with metocurine or atracurium was disclosed by chromatography of the blank samples, thus attesting to the efficiency of our plasma washing and extracting procedures.

In the analysis of the standard curve, a good correlation was noted ($r = 0.9994$ from 25 to 2500 ng). In addition, intra-assay CV (within 2.5%), inter-assay CV (within 9%) and recovery rates (87.5% for atracurium and 88.7% for metocurine) exhibited high reproducibility and accuracy, as was the case in conventional reports[4,15,16].

In the analysis of the pharmacokinetics of 2 clinical cases, peak concentration, $t_{1/2\alpha}$ and $t_{1/2\beta}$ were consistent with those reported in previous studies[4,5,7,8,20,21]. Thus, the reliability of this technique was also endorsed clinically. However, the lowest detection threshold of this technique (15 ng/ml) was slightly inferior to that reported by Neil (1 ng/ml)[15] and Fahney (10 ng/ml)[4].

Since the plasma atracurium level in clinical cases was 130 ng/ml when 100% recovery from muscular relaxation was observed, it remained higher than the lowest threshold when examined 10 min (40.9 ng/ml) and 25 min (30.2 ng/ml) after. These results suggest that our analysis method is clinically applicable. In the present study, 0.5 ml of each plasma sample was subjected to extraction procedures. However, if double this amount of plasma were used, it might result in an improvement of the sensitivity.

Metocurine is not available in Japan. Therefore, we also used d-tubocurarine (d-Tc) as an IS. The retention time for d-Tc was ca. 3.6 min (a little shorter than that for metocurine), but the results with this substance were satisfactory in the other respects.

Thus, we confirmed that there was no appreciable difference between the use of metocurine or d-Tc as an IS.

Since atracurium is quickly decomposed by Hofmann elimination, the following procedures are necessary: (1) rapid separation of plasma after blood sampling, (2) acidification of plasma (to pH 4.5 or less), and (3) extraction under low temperature (0–4°C)[16].

As stated above, our method of extraction and determination of atracurium in human plasma which uses metocurine as an IS and a reversed phase C_{18} column, is a highly reproducible, extremely accurate, simple and quick analysis; we believe it to be appropriate for studying the pharmacokinetics of atracurium.

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