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Simple and rapid high-performance liquid chromatography method for the determination of alcuronium in human plasma and urine

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Abstract

A simple and quick HPLC assay for alcuronium is presented. Its characteristics are: precipitation of plasma proteins by acetonitrile; Spherisorb 5-CN column; acetonitrile–water (46:54, v/v) as mobile phase; flow-rate 1 ml/min; laudanosine 0.06 mg/l as internal standard with plasma; external standard with urine; UV detection at 294 nm; retention time 5.4 min; detection limit 0.025 mg/l; documented linearity: 0.025–2.0 mg/l for plasma and 1.0–80 mg/l for urine; intra- and inter-assay variability below 4%. None of nine drugs used in perioperative pharmacotherapy interfered with the assay. Satisfactory performance was exemplified in a 12-h pharmacokinetic evaluation of two patients.

1. Introduction

More than twenty years ago, alcuronium (diallyl-NOR-toxiferine, Alloferin) was introduced into clinical anaesthesia as the first semisynthetic nondepolarizing neuromuscular blocker. Since the 1970s it has been gradually replaced by synthetic steroid and benzylisochinoline compounds with a more favorable clinical profile, such as pancuronium, vecuronium, and atracurium which, however, were more expensive. Recent restraints on hospital budgets may reawaken interest in alcuronium as still being the cheapest nondepolarizing muscle relaxant available. For historical reasons pharmacokinetic and pharmacodynamic studies on alcuronium are scarce in contrast to the extensive data which is available for its modern substitutes. Its renal elimination, for example, has only been investigated in three individuals [1]. Thus, the necessity for supplemental pharmacokinetic research, in particular on the long-term disposition of alcuronium, is obvious. The large series of blood and urine samples produced by such studies require an HPLC assay which should be both rapid and simple enough to be performed by non-specialized personnel in any standard HPLC laboratory. The techniques described hitherto

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rely on either too laborious solvent extraction [2–4] or solid-phase extraction [5], or are not fully satisfactory with respect to their sensitivity [2,6]. We are presenting an HPLC assay which is devoid of these disadvantages.

2. Experimental

2.1. Instrumentation

The method was developed using an isocratic Merck-Hitachi HPLC system (Merck, Darmstadt, Germany) consisting of an AS-4000 autosampler, a D-6000 interface, a L-6200 intelligent pump, and a T-6300 column-thermostat. Separations were done on a 250 mm × 4 mm I.D. Spherisorb 5-CN column (Chromatographie Service, Langerwehe, Germany) with a mobile phase consisting of 46% acetonitrile and 54% of an aqueous solution of 60 m*M* Na_2SO_4 and 5 m*M* H_2SO_4 [7]. The system was operated at room temperature with a flow-rate of 1 ml/min resulting in a column pressure of 100–130 bar. Alcuronium was detected by means of a L-4250 UV-Vis detector at 294 nm.

2.2. Reagents

The following chemicals were used for the analytical procedure: acetonitrile HPLC grade (LiChrosolv, acetonitrile gradient grade, Merck, Darmstadt, Germany), deionized water, AR grade sulphuric acid (Merck), analytical grade alcuronium hydrochloride (purity 99%, No. 798 175, Hoffmann-La Roche, Grenzach, Germany), laudanosine DL-standard (L-1389, Sigma, Deisenhofen, Germany), heparin disodium-sulfate (Liquemin, Merck). The drugs administered to patients were used as pharmaceutical preparations: alcuronium (Alloferin, Hoffmann-LaRoche), thiopental (Trapanal, BYK Gulden, Konstanz, Germany); midazolam (Dormicum, Hoffmann-LaRoche); flunitrazepam (Rohypnol, Hoffmann-LaRoche); propofol (Disoprivan, ICI-Pharma, Heidelberg, Germany); fentanyl (Fentanyl, Janssen, Neuss, Germany); droperidol (Dehydrobenzperidol, Janssen); ketamine (Ketanest, Parke-Davis, Berlin, Germany); metronidazol (Clont, Bayer, Leverkusen, Germany); and cefazolin (Gramaxin, Boehringer-Mannheim, Mannheim, Germany).

2.3. Analysis of plasma

Patient blood (2 ml per sample) was collected in cappable plastic vials containing 10 μl of heparin. The vials were then centrifuged for 3 min at 15 800 *g* (Eppendorf centrifuge 5145 C, Hamburg, Germany). The supernatant plasma (1 ml) was transferred into light-protected vials and frozen at −20°C. The blood cell pellet was discarded. For HPLC, the samples were defrosted, vortex-mixed (Vortex, Bender and Hobein Zürich, Switzerland) for 15 s and centrifuged for 2 min at 15 800 *g*. Out of each centrifuged plasma sample, 250 μl were pipetted (micropipette Eppendorf) into cappable plastic vials, and 400 μl of a solution of 3 mg laudanosine (I.S.) in 50 ml acetonitrile were added with acetonitrile to effect protein precipitation [8]. The vials were then vortex-mixed for 15 s, rotated for 2 min (rotator Heidolph, Kehlheim, Germany), and centrifuged at 15 800 *g* for 10 min. From the supernatant liquid, 250 μl were transferred into HPLC autosampling vials from which 75 μl were injected for chromatography.

2.4. Analysis of urine

Fresh urine samples were filled into cappable plastic tubes and deep frozen at −20°C for up to 24 h. The samples were then defrosted, vortex-mixed, centrifuged at 2400 *g* for 5 min (Minifuge CL, Heraeus-Christ, Osterode, Germany) and diluted 1:1 (v/v) with 50 m*M* phosphate buffer (pH 7.3). Aliquots of the diluted urine were frozen again at −20°C. For HPLC, the urine samples were defrosted, vortex-mixed and centrifuged at 2400 *g* for 5 min. Aliquots of the supernatant urine were transferred into HPLC autosampling vials, from where 40 μl were injected onto the HPLC system. Quantitation was performed by means of external standardization.

2.5. Calibration curves

For establishing calibration curves, 10 mg of analytical grade alcuronium hydrochloride were dissolved in 25 ml of deionized water. An aliquot of this solution was transferred into human plasma and urine for 2 mg/l and 100 mg/l stock solutions, respectively. Specimens of drug-free plasma (2 ml) and urine (10 ml) were spiked with aliquots of the respective stock solution to obtain concentrations of alcuronium which may be expected following its therapeutic administration: 0.025, 0.05, 0.1, 0.2, 0.4, 0.5, 0.6, 1.0, 1.3, and 2.0 mg/l plasma and 1.0, 4.0, 5.0, 20.0, 40.0, 50.0, and 80.0 mg/l urine. Five HPLC measurements were performed with each concentration. The results were evaluated for intra- and inter-assay variability, as well as for linearity.

2.6. Interference

Drugs which may be used as constituents of anesthesia or of perioperative therapy were tested for their interference with the alcuronium assay. These drugs were added to human plasma in the excess of concentrations occurring in the clinical setting: thiopental 25, flunitrazepam 0.3, midazolam 1, disoprivan 5, ketamine 3, fentanyl 50, droperidol 50, cefazoline 300, and metronidazol 50 mg/l. The HPLC analysis was performed as described above.

2.7. Clinical application

In two patients the neuromuscular blocking effect, the plasma concentration and the renal elimination of alcuronium were measured simultaneously. One patient, undergoing maxillary surgery, received a single intravenous dose of alcuronium (0.25 mg/kg), whereas the second patient, during open heart surgery with hypothermic cardiopulmonary bypass, required three doses (0.18, 0.11, 0.11 mg/kg). Central venous blood samples were drawn in at the times shown in Figs. 2 and 3, preceded by blank samples taken 5 min before the injection of alcuronium. Urine was collected 1, 3, 6, and 12 h after alcuronium administration. The specimens were processed as described above and analyzed in duplicate. The neuromuscular blocking effect of alcuronium was monitored by recording the evoked twitch tension of the adductor pollicis muscle.

3. Results

3.1. Chromatogram

Fig. 1 shows a sample chromatogram with the peaks of laudanosine (I.S.) and alcuronium at 3.2 and 5.4 min, respectively. Both peaks rose from an almost noise-free baseline and were more than 2 min apart. The internal standard

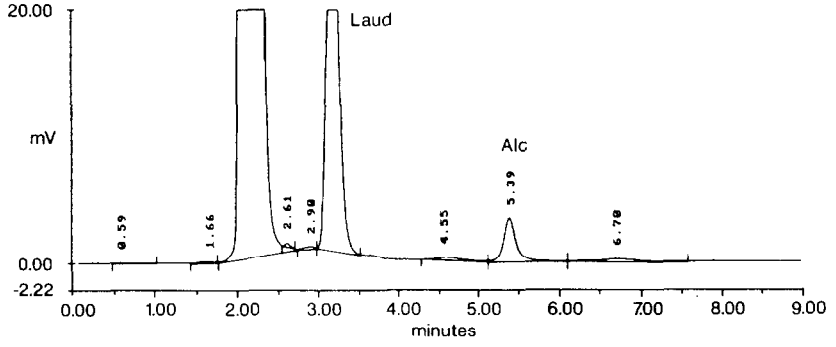

Fig. 1. High-performance liquid chromatogram of patient plasma containing alcuronium (Alc) 1.3 mg/l and laudanosine (Laud) 32 mg/l.

was also clearly separated from the plasma peak. None of the tested drugs interfered with the alcuronium assay.

3.2. Calibration

The linearity of the assay was determined by regression analysis of the values detected as a function of the spiked concentrations. With plasma, the correlation coefficient (r), the regression coefficient (B), and the intercept (A) were >0.999, 1.016, and -0.0255, respectively. The inter-assay variability was 2.1% on average. The intra-assay variability was 2.7%, 1.7%, and 1.2% for 0.25, 0.5, and 2.0 mg/l, respectively ($n=7$ each). With urine, the variables of the regression equation were $r>0.999$, $B=1.005$, and $A=-0.077$. The inter-assay variability was 3.6% average. The intra-assay variability was 1.13%, 1.61%, and 2.35% for 1.5, 5.0, and 20.0 mg/l, respectively.

3.3. Technical remarks

A single plasma sample was prepared for HPLC within 25 min. Processed in a series, 100 samples could be thawed and centrifuged at once and processed in an 8-h day shift. The average pre-HPLC working time per sample was then reduced to less than 5 min. The ensuing HPLC was performed automatically over night with a processing time of 8 min per sample. So far we have used the assay in more than a thousand samples where one column was good for at least 500 HPLC throughputs.

3.4. Clinical application

Figs. 2 and 3 illustrate the two patients' results, based on a total of 84 plasma and 18 urine samples. In patient 1 (Fig. 2), the plasma decay curve of alcuronium represented its typical exponential function with detectable concentrations persisting as long as 12 h. However, neuromuscular blocking had completely subsided at a plasma concentration of 0.25 mg/l. At this time

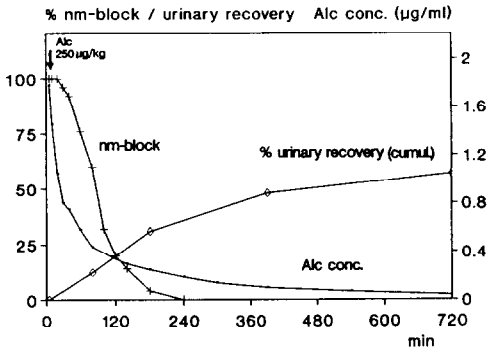

Fig. 2. Alcuronium (Alc) plasma concentration and renal elimination with simultaneous representation of neuromuscular block following a single intravenous dose of alcuronium 0.25 mg/kg in a patient undergoing maxillary surgery.

only 30% of the injected dose was recovered from the urine. In the second patient (Fig. 3) the peaks of the plasma concentration curve adequately reflected the repeated intravenous administration of alcuronium which was paralleled by an enhanced neuromuscular blocking. Both the disappearance of alcuronium from the plasma and the offset of neuromuscular blocking were slower during than before hypothermic cardiopulmonary bypass. Correspondingly, in

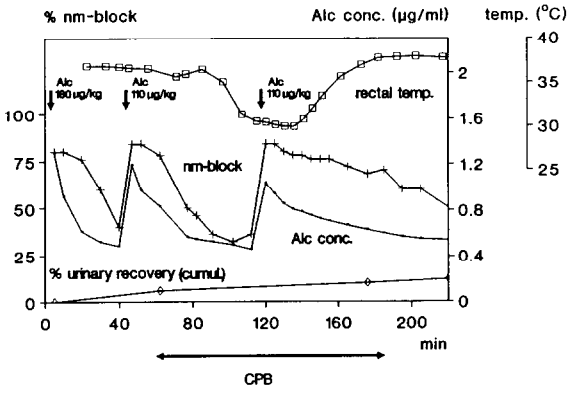

Fig. 3. Alcuronium (Alc) plasma concentration and renal elimination with simultaneous representation of rectal temperature, duration of cardiopulmonary bypass (CPB), and neuromuscular (nm) block following multiple intravenous doses of alcuronium in a patient undergoing open-heart surgery with hypothermia.

this patient, the 12-h renal elimination of alcuronium was only 9% of the injected dose.

4. Discussion

In a 12–24-h pharmacokinetic study as exemplified by our two patients, some 500 samples would accumulate for analysis. Since 100 samples can be thawed and centrifuged at once, their subsequent analytical procedure should be performed in the same session. In a set of 100 samples as little as five minutes extra processing time per sample would add up to more than a full day-shift, both in sample preparation and in occupation of the HPLC apparatus. Thus, an appropriate assay should ensure the simplest and quickest possible handling of the samples as well as the shortest possible chromatographic retention time.

Three approaches were employed for quantitative analysis of alcuronium in human body fluids: (1) administration of tritiated alcuronium [1], (2) coupling of alcuronium with an acid dye (rose bengal) followed by fluorometric detection [2,9,10], and (3) a variety of HPLC methods [3,4,6]. The tritium method is rejected for ethical reasons. The rose-bengal method, the only one used in three pharmacokinetic studies [2,9,10], requires plasma volumes as large as 1 ml per assay while leaving the method with a considerable 5 to 10% inter-assay variation coefficient [9]. The HPLC assays [3,4,6] which were much more sensitive and accurate than the rose-bengal method [2], were never used in a pharmacokinetic study. Technical difficulty may be one of several reasons: the assay of Parkin [3] depended on a 5-h extraction procedure while the use of Bond-Elut Phenyl cartridges [5] would also appear quite laborious and expensive. The method of Tovey *et al.* [6] avoided such difficulties by the use of acetonitrile for protein precipitation, however, its detection limit was only 0.1 mg/l.

Since Tovey *et al.* [6] have also shown that deproteinization by acetonitrile was not associated with any loss of alcuronium [6], we adopted this technique which allows the aqueous phase to be directly injected onto the HPLC system without the need of an internal standard. For the sake of accuracy, we abandoned the internal standard only for urine while maintaining it to assess the much lower concentrations of alcuronium in the plasma.

With the previous analytical techniques the alcuronium retention times were 12 min, [3,6], 7–10 min [5], and 4.5 min [4]. Thus, the method of Tovey *et al.* [6] was the simplest and quickest in pre-HPLC sample processing, while Bjorksten *et al.* [4] achieved the shortest retention time. Our method combines a short retention time with quick and easy handling of blood and urine samples. Despite such simplification and acceleration of the assay, our less than 3% inter- and intra-assay variability coefficients were at least as good as those of the previous techniques [3,4,6]. The improved performance of our technique, with respect to both retention time and detection limit was achieved by substituting the use of the reversed-phase technique (RP 8-column, particle size 10 μm) used by Tovey *et al.* [6] for ion chromatography (Spherisorb 5-CN, particle size 5 μm). This modification improved the efficiency of separation which allowed a higher elution strength with a reduced elution time to be used as well as an UV- rather than an electrochemical detector [6]. The specificity, as far as anaesthetic or perioperative drug therapy is concerned, was only tested by Tovey *et al.* [6]. We supplemented their selection of drugs by some modern intravenous anaesthetics, tranquilizers, and antibiotics.

Our two clinical examples demonstrate that both the sensitivity and linearity of our assay cover the range required for clinical pharmacokinetic studies, even beyond 12 h after alcuronium administration. The concentrations of alcuronium found in a total of 84 plasma and 18 urine samples are perfectly in line with those reported by previous workers [2,5,10]. Especially the changing plasma concentration of the patient with cardiopulmonary bypass clearly reflects the repeated administration of alcuronium while perfectly corresponding to the changing intensity of neuromuscular blocking. Equally well demonstrated is the reduced rate of renal alcuronium elimination following cardiopulmonary bypass

which is only one fifth of that found in the non-bypass patient. The good agreement of our data with both the clinical events and the results of previous workers is another validation of our assay.

In conclusion, we developed an HPLC assay for alcuronium which is sufficiently sensitive, specific, and simple to be performed in any standard HPLC laboratory by non-specialized personnel. The method is particularly useful for processing large series of blood and urine samples as obtained in extensive pharmacokinetic studies.

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6. References

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