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Stereoselective high-performance liquid chromatographic assay with fluorometric detection of the three isomers of mivacurium and their *cis*- and *trans*-alcohol and ester metabolites in human plasma

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Abstract

An improved high-performance liquid chromatography assay for the three stereoisomers of the muscle relaxant mivacurium and its metabolites in plasma is presented. The principal steps in the assay are precipitation of plasma proteins by acetonitrile, lyophilization of the supernatant and ion-exchange chromatography on Spherisorb 5-SCX column, with gradient elution (acetonitrile from 32 to 68% v/v and ionic gradient from 7 to 56 mmol l^{-1} Na_2SO_4), a flow-rate of 2.0 ml min^{-1}, D-tubocurarine as internal standard and fluorometric detection (excitation wavelength=280 nm, emission wavelength=320 nm). Quantitation limit of *cis–cis, cis–trans, trans–trans* isomers were 0.003, 0.002 and 0.005 μmol l^{-1}, respectively. Quantitation limits for the monoester$_{cis}$ metabolite were 0.011 μmol l^{-1}, for the monoester$_{trans}$ metabolite 0.017 μmol l^{-1}, for the amino-alcohol$_{trans}$ 0.020 μmol l^{-1} and for the amino-alcohol$_{cis}$ 0.021 μmol l^{-1}. None of eight drugs used during anaesthesia interfered with the assay in vitro. Satisfactory performance was demonstrated by the measurement of the isomers and their metabolites in plasma of two patients over a 6-h period after repeated injections of mivacurium.

Keywords: Enantiomer separation; Mivacurium

1. Introduction

Mivacurium dichloride (MIV) is a non-depolarizing neuromuscular blocking agent with a relatively short duration of action. It is rapidly hydrolyzed mainly by butyrylcholinesterases (EC 3.1.1.8) [1]. A quaternary monoester and a quaternary amino alcohol are the principal metabolites (Fig. 1). MIV consists of three stereoisomers: *trans–trans*, *cis–trans* and *cis–cis*. The percentage range specification for the isomers in clinical trials are 52.1–62.1, 34.1–39.6 and 3.8–8.2%, respectively [2]. Both metabolites are also present as *cis* and *trans* isomers. Collection of multiple blood samples is required during long-lasting surgical procedures for the definition of the pharmacokinetic variables of mivacurium. Hence, the volume of each blood sample should be as small as possible. Published procedures for determining the MIV isomers use solid-phase extraction and therefore require relatively large blood samples [2–4]. These methods are not suitable for measuring MIV and its metabolites in the same

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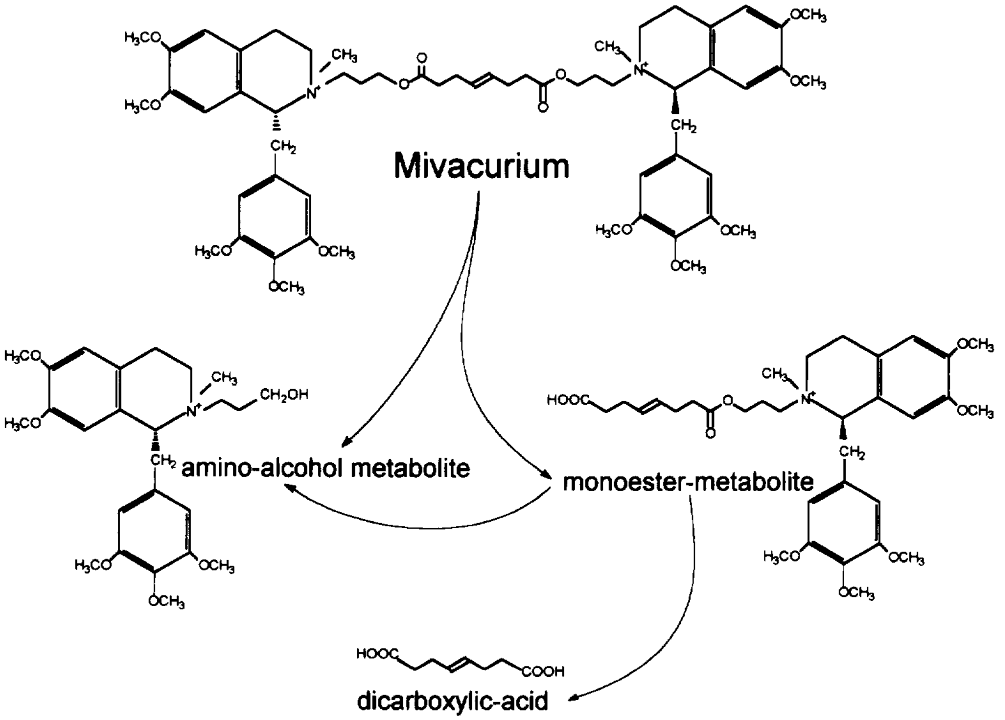

Fig. 1. Structure of mivacurium and the metabolic pathway (modified according to [8].

procedure. Furthermore, they were not tested for interference from therapeutic drugs. We developed a sensitive stereoselective assay for the determination of the three stereo isomers of MIV as well as the stereo isomers of the two metabolites in the same plasma sample without solid-phase extraction. Drugs commonly used in anaesthesia and perioperative medication were tested for interference with the procedure.

2. Experimental

2.1. Instrumentation

The method was developed using a Merck-Hitachi HPLC system (Merck, Darmstadt, Germany) consisting of an AS-4000 autosampler, a D-6000 interface, an L-6200 intelligent pump, and a T-6300 column thermostat. The storage rack for the vials in the auto-sampler was cooled to 5.0°C by a WK-5 cryothermostat (Colora Messtechnik, Lorch, Germany). The fluorometric detection was performed at 280 nm for excitation and 320 nm for emission with a F-1050 fluorescence spectrophotometer (Merck-Hitachi). Separations were done on a 250×4 mm I.D. Spherisorb 5-SCX column (Chromatographie Service, Langerwehe, Germany). The mobile phase was made up of acetonitrile and an aqueous solution of Na_2SO_4 in 9 mmol l^{-1} H_2SO_4 (pH 2). A gradient of 32 to 68% acetonitrile and 7 to 56 mmol l^{-1} Na_2SO_4 was used. The HPLC system was operated at room temperature with a flow-rate of 2 ml min^{-1}, resulting in a column pressure of 180–220 bar.

2.2. Chemicals

The following chemicals were used for the analytical procedure: HPLC grade acetonitrile (LiChrosolv, acetonitrile gradient grade, Merck, Darmstadt, Germany), deionized water, AR grade sulphuric acid (Merck), GR grade sodium sulphate anhydrous (Merck), analytical grade mivacurium dichloride (purity 99%, No. 86/0038-159-A), analytical grade monoester metabolite of MIV (purity 99%, No. 91/5440-098-1), analytical grade amino-alcohol metabolite of MIV (purity 99%, No. 91/1214-107, all mivacurium and metabolite standards from Burroughs Welcome, Research Triangle Park, USA), D-tubocurarine chloride (Lot 103H3283 Sigma, St. Louis, USA) and echothiopate iodide (Ayerst Laboratories, Philadelphia, USA). The drugs normally administered to patients during a surgical procedure were tested for interference with the analytical procedure in vitro: aprotinin (Trasylol Bayer, Leverkusen, Germany), fentanyl (Fentanyl, Jansen Jansen, Neuss, Germany), flunitrazepam (Rohypnol Hoffmann-La Roche, Grenzach, Germany), heparin sodium (Liquemin Hoffmann-La Roche), midazolam (Dormicum Hoffmann-La Roche), neostigmine bromide (Lot 359448 Prostigminbromid Hoffmann-La Roche), pyridostigmine bromide (Lot 0429031 Mestinon Hoffmann-La Roche), mivacurium chloride (Deutsche Welcome, Burgwedel, Germany), propofol (Disoprivan ICI-Pharma, Plankstadt, Germany), and lactated Ringer's solution (Fresenius, Bad Homburg, Germany).

2.3. Analysis of plasma

Blood samples (3 ml) from patients were transferred into prechilled vials containing heparin (10 $\mu l = 100$ IU) and echothiopate iodide (15 μl of an aqueous solution 0.2 mg l^{-1}) to inhibit further metabolisation of MIV by plasma esterases. The vials were gently shaken and centrifuged at 4°C for 2 min at 15 800 *g*. The supernatant was transferred to microvials and frozen at −20°C until analyzed. For HPLC analysis, the samples were thawed, stirred (vortex, Bender and Hobein, Zurich, Switzerland) for 10 s and centrifuged for 5 min at 15 800 *g* and 4°C (Eppendorf centrifuge 5402). Thereafter, 500 μl plasma was pipetted (Eppendorf micropipette) into plastic vials, and 800 μl of a solution containing D-tubocurarine in acetonitrile (2.5 mg D-tubocurarine in 1 l of acetonitrile) was added as an internal standard and for protein precipitation. The vials were then stirred for 15 s and centrifuged for 5 min at 16 000 *g* and 4°C (Eppendorf centrifuge 5402). The supernatant was transferred into plastic vials and lyophilized in a Hetovac VR-1 (Heto Lab Equipment, Allerod, Denmark; duration about 6 h). After lyophilization the pellet was reconstituted in 150 μl H_2SO_4 (5 mmol l^{-1}, pH 2) and transferred to HPLC autosampling vials from which 100 μl was injected for chromatography.

2.4. Assay validation

Drug-free plasma (2 ml) was spiked with aliquots from the stock solutions (100 μmol of MIV or its metabolites in 1 l of water) to obtain plasma concentrations of MIV and of its metabolites from 1 nmol l^{-1} up to 10 μmol l^{-1}. The lowest concentration with a coefficient of variation (C.V.) <20% was regarded as the lower limit of quantitation. Correlation between the spiked and the measured concentrations was tested by unweighted linear regression analysis. Variability was estimated as the standard deviation in percent of the mean value (C.V.) of the respective concentration. The intra-day (intra-assay) variability was determined by replicate measurements ($n=6$ on the same day) of seven different concentrations (concentrations shown in Table 2 and Table 3) processed in replicates on four days. The inter-day (inter-assay) variability was determined by assaying a specimen containing 1 μmol l^{-1} concentration of each isomer at ten different days during a period of one month. Recovery rates were determined by comparing the areas for each isomer obtained from plasma standards with those obtained from the injection of unextracted aqueous standards.

2.5. Interference

Drugs which may be used during anaesthesia or during the perioperative period (aprotinin, fentanyl, flunitrazepam, heparin sodium, midazolam, propofol, pyridostigmine, and neostigmine) were tested for interference with the MIV assay. These drugs were added to samples of human plasma to obtain concentrations in excess of concentrations occurring under clinical conditions. Additional interference testing included echothiopate, used to inhibit plasma esterases in vitro immediately after the collection of blood samples.

2.6. Clinical application

In patients undergoing open heart surgery, we measured the thumb twitch evoked by supra maximal stimulation of the ulnar nerve and the plasma concentrations of MIV and of its metabolites. MIV was administered intravenously at a dose of 150 µg kg^{-1} body weight prior to tracheal intubation. Additional doses of MIV (75 µg/kg) were administered whenever neuromuscular block decreased to 25%. Mixed venous blood samples were drawn at times shown in Fig. 3 and Fig. 4. An additional blood sample was collected 5 min before the first injection of MIV. Duplicate blood samples were processed as described above.

3. Results

3.1. Chromatogram

Fig. 2 shows a chromatogram of an extract of a plasma sample collected 2 min after i.v. injection of 10 mg MIV. It demonstrates the peaks of the three MIV isomers and of the four metabolites. This patient produced only a low concentration of amino-alcohol$_{cis}$. The MIV isomers were clearly separated as were those of the monoester and amino-alcohol metabolites. None of the tested drugs interfered with the MIV assay.

3.2. Calibration

3.2.1. Calibration

The correlation coefficient (r), the regression coefficient (B), the intercept (A), and the quantitation limits of the MIV isomers and their metabolites are shown in Table 1.

The average inter-assay variability was 5.5%, 7.6%, and 8.4% for MIV$_{trans-trans}$, MIV$_{cis-trans}$ and MIV$_{cis-cis}$, respectively. As documented in Table 2, the intra-assay variability of MIV$_{trans-trans}$ varied from 3.3% at 6.0 µmol l^{-1} to 15.1% at quantitation limit. MIV$_{cis-trans}$ had an intra-assay precision from 1.1% at 1.7 µmol l^{-1} to 12.6% at quantitation limit, and MIV$_{cis-cis}$ from 5.4% at 0.250 µmol l^{-1} to 19.5% at quantitation limit (n=6 each). Recovery for MIV$_{trans\ trans}$ was 96.3% (±2.2), for MIV$_{cis\ trans}$ 94.7% (±4.1) and for MIV$_{cis\ cis}$ 95.4% (±5.9).

The intra-assay precision (Table 3) of amino-alcohol metabolite$_{cis}$ varied from 3.3% at 5.4 µmol l^{-1} to 17.3% at quantitation limit. Amino-alcohol metabolite$_{trans}$ had an intra-assay variability from 1.4% at 1.6 µmol l^{-1} to 19.4% at quantitation limit. The monoester metabolite$_{trans}$ measurements varied from 1.5% at 1.56 µmol l^{-1} to 18.7% at quantitation limit and monoester metabolite$_{cis}$ had an intra-assay precision of 2.0% at 2.2 µmol l^{-1} to 19.2% at

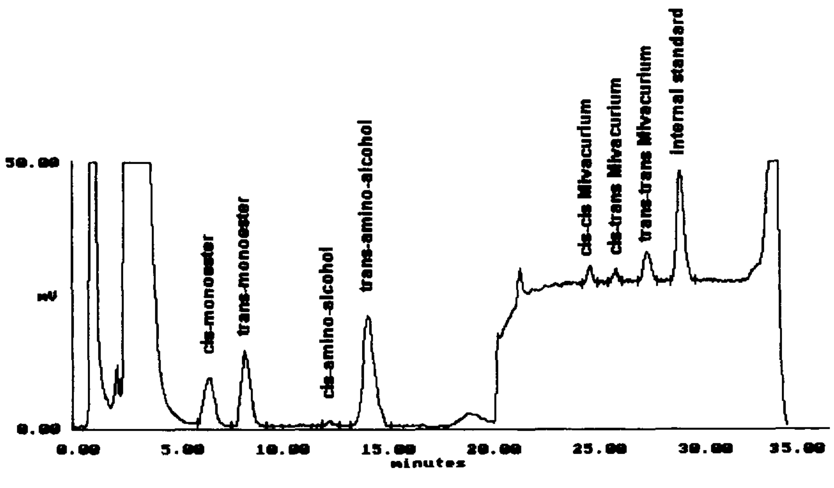

Fig. 2. Chromatogram of the mivacurium isomers and metabolites MIV$_{cis-cis}$ (t_R=24.6 min), MIV$_{cis-trans}$ (t_R=25.8 min), MIV$_{trans-trans}$ (t_R=27.3 min), monoester metabolite$_{cis}$ (t_R=6.7 min), monoester metabolite$_{trans}$ (t_R=8.5 min), amino-alcohol metabolite$_{cis}$ (t_R=12.6 min), amino-alcohol metabolite$_{trans}$ (t_R=14.4 min) and D-tubocurarine (IS, t_R=28.8 min). From 17.5 min to 22.5 min, the gradient procedure (higher acetonitrile and salt concentrations) resulted in an elevation of the baseline.

Table 1
Linearity and quantitation limit of the mivacurium isomers and its metabolites

	r	B	A (nmol l^{-1})	Concentration range tested (nmol l^{-1})		Recovery rate (%)
				Quantification limit	High	
MIV$_{trans-trans}$	>0.999	2.16	−110	5	10 200	96.3 (±2.2)
MIV$_{cis-trans}$	>0.999	2.11	−60	2	3400	94.7 (±4.1)
MIV$_{cis-cis}$	>0.999	1.63	−2	3	500	95.4 (±5.9)
Monoester metabolite$_{cis}$	>0.999	1.11	−4	11	5504	93.9 (±5.3)
Monoester metabolite$_{trans}$	>0.999	1.13	−3	17	7798	97.3 (±3.6)
Amino-alcohol metabolite$_{cis}$	>0.999	1.16	−12	21	5420	92.8 (±6.8)
Amino-alcohol metabolite$_{trans}$	>0.999	1.21	−44	20	7832	96.2 (±2.7)

r=correlation coefficient, B=regression coefficient, A=intercept.

Concentration range tested from the quantitation limit to the maximum concentration tested for linearity.

Table 2
Intra-assay variability of the mivacurium isomers

MIV$_{cis-cis}$		MIV$_{cis-trans}$		MIV$_{trans-trans}$	
Concentration (nmol l^{-1})	S.D. (%)	Concentration (nmol l^{-1})	S.D. (%)	Concentration (nmol l^{-1})	S.D. (%)
2.5	19.5	1.7	12.63	5	15.10
10	17.8	340	2.79	300	4.16
25	16.4	680	4.38	600	3.48
50	9.7	1020	4.85	1200	4.88
250	5.4	1700	1.14	3000	4.67
500	7.1	3400	3.82	6000	3.32
				10 200	3.56

S.D.=standard deviation in percent of the mean (n=6).

Table 3
Intra-assay variability of the mivacurium metabolites

Amino-alcohol metabolite$_{cis}$		Amino-alcohol metabolite$_{trans}$		Monoester metabolite$_{cis}$		Monoester metabolite$_{trans}$	
Concentration (nmol l^{-1})	S.D. (%)	Concentration (nmol l^{-1})	S.D. (%)	Concentration (nmol l^{-1})	S.D. (%)	Concentration (nmol l^{-1})	S.D. (%)
23	17.30	20	19.40	11	19.20	17	18.70
114	9.93	39	9.82	22	10.41	39	7.90
217	7.43	78	8.85	110	8.37	78	4.94
1084	4.97	392	2.47	220	6.40	390	5.72
2168	3.48	783	5.07	1108	2.83	780	2.03
5420	3.32	1566	1.42	2202	2.01	1560	1.48
		3916	2.55	5504	3.29	3899	1.60
		7832	4.36			7798	2.18

SD=standard deviation in percent of the mean (n=6).

quantitation limit ($n=6$ each). Recovery rates for the metabolites varied from 92.8% to 97.3% (Table 1).

3.2.2. Technical remarks

A single plasma sample required 0.5 man-hour for preparation for HPLC. Processed in a series, 100 samples could be thawed and centrifuged at once and processed in an eight-hour shift. The average pre-HPLC working time per sample was thus less than 5 min. The subsequent HPLC analysis was performed automatically overnight with a processing time of 34 min per sample. So far, we have used the assay in more than 1500 samples. One column was good for at least 300 analyses.

3.2.3. Clinical application

Fig. 3 illustrates the time course of the MIV concentrations in plasma over 3.5 h in a patient undergoing open heart surgery with hypothermic cardiopulmonary bypass (CPB). During this period 50 blood samples were collected. The intensity of the thumb twitch and the rectal temperature were recorded as well. The peak concentrations of $\mathrm{MIV}_{trans-trans}$ and $\mathrm{MIV}_{cis-trans}$ increased during the CPB, but the peak concentration of $\mathrm{MIV}_{cis-cis}$ did not change. Fig. 4 represents the plasma concentration curves of the metabolites of MIV in the same patient. During the CPB and hypothermia, the product of the concentration and time (area under the curve, AUC) of the metabolites of MIV in plasma increased. The plasma concentrations of $\mathrm{MIV}_{cis-trans}$ and $\mathrm{MIV}_{trans-trans}$ paralleled the intensity of neuromuscular block (Fig. 3).

4. Discussion

Our assay is the first method that allows the determination of the three MIV isomers as well as of the four metabolites of MIV from the same 500 μl plasma sample in a single HPLC run. Compared to the method of Cook et al. [1] who used UV detection and reported a detection limit of 0.1 μmol l^{-1}, the quantitation limit in our method was 20 to 45 times lower by using fluorometric detection. In addition, stereoselective analysis could not be done. To de-

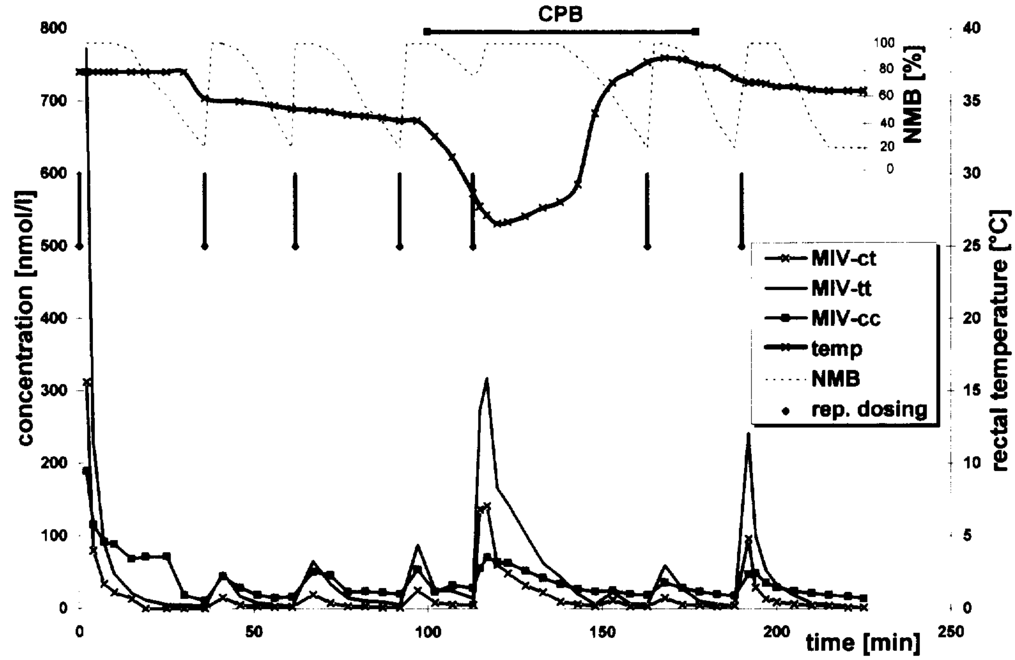

Fig. 3. Time course of the mivacurium isomers and neuromuscular block in a patient during open heart surgery with cardiopulmonary bypass: MIV-ct: $\mathrm{mivacurium}_{cis-trans}$; MIV-tt: $\mathrm{mivacurium}_{trans-trans}$; MIV-cc: $\mathrm{mivacurium}_{cis-cis}$; temp: rectal temperature; NMB: intensity of neuromuscular block in percentages; rep. dosing: repeat dosing; CPB: duration of cardio-pulmonary bypass.

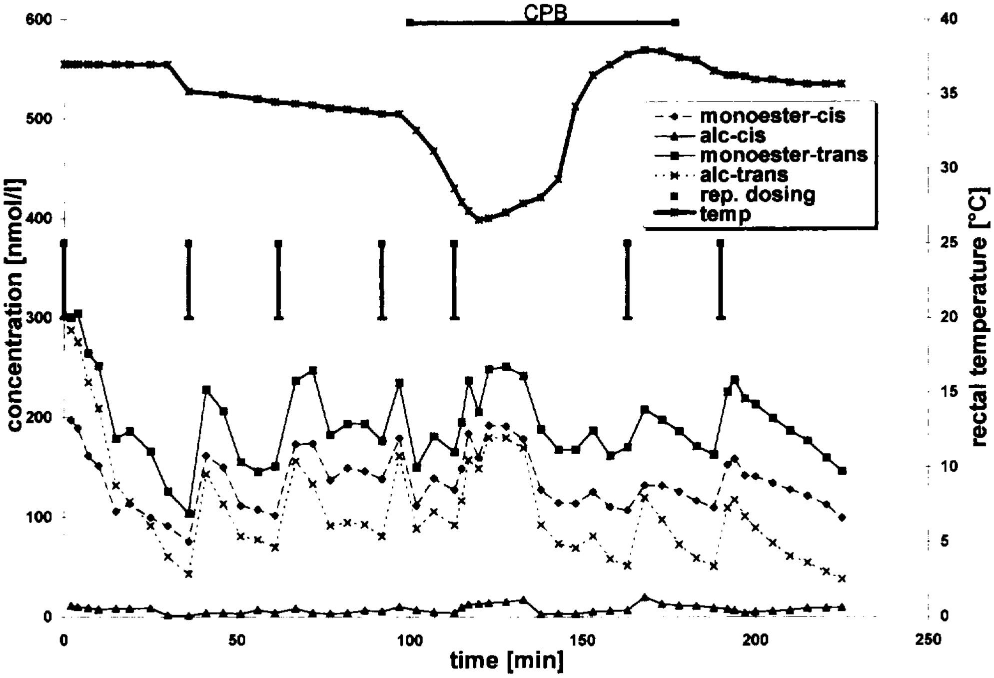

Fig. 4. Time course of the mivacurium metabolites in the same patient: monoester-*cis*: monoester metabolite$_{cis}$; alc-*cis*: amino-alcohol metabolite$_{cis}$; monoester-*trans*: monoester metabolite$_{trans}$; alc-*trans*: amino-alcohol metabolite$_{trans}$; rep.dosing: repeat dosing; temp: rectal temperature; CPB: duration of cardio-pulmonary bypass.

termine the terminal elimination half life of MIV isomers in pharmacokinetic studies, a highly sensitive assay is required. The assay we developed meets this requirement. No interfering peaks appear in chromatograms of blank plasma and none of eight drugs used during anaesthesia interfered with the method.

The potency of MIV$_{cis-trans}$ and MIV$_{trans-trans}$ is 10 times greater than that of MIV$_{cis-cis}$ [5]. MIV$_{cis-cis}$ has a much longer elimination half-life. The half-life was reported as 22 min [6], 50.3 min [3], or 52.9 min [7]. When the diastereomers differ in such relevant pharmacokinetic and pharmacodynamic properties, it is most important to have a stereoselective assay for analysis. Pharmacokinetic analysis of mixtures of diastereomers as a single entity may be misleading. The isomers of mivacurium are hydrolyzed at different rates in plasma. The half-lives are 1.8 min for MIV$_{trans-trans}$, 1.9 min for MIV$_{cis-trans}$, and 52.9 for MIV$_{cis-cis}$ [7]. Therefore, blood samples should be taken in prechilled vials and an inhibitor of butyrylcholinesterases must be added as soon as possible to minimize the metabolization of MIV in the collected samples.

All published stereoselective assays for the determination of MIV use solid-phase extraction [2–4]. The procedures are time-consuming and expensive. Additionally, relatively large plasma samples are required. The method of Brown et al. [2] requires 1000 μl to measure the mivacurium isomers only and that of Lacroix et al. [4] requires 1250 μl to measure the mivacurium isomers and the metabolites. The total volume of plasma required can become a limiting factor in long term pharmacokinetic studies. The method we developed is suitable for measurement of MIV and its metabolites in as little as 500 μl plasma. Protein precipitation by acetonitrile is a quick and cheap way to create a sample that is sufficiently clean for HPLC analysis. Using this method, the incomplete recovery of the monoester metabolite isomers reported by Lacroix et al. [4] and the loss of mivacurium during sample preparation in the assay of Brown et al. [2] are avoided.

Reproducibility data demonstrated that the sensitivity of the presented method meets the requirements of a clinical pharmacokinetic study.

5. Conclusion

A sensitive stereoselective assay with fluorescence detection has been developed for the measurement of the MIV isomers and their metabolites. Protein precipitation with acetonitrile is a simple, rapid and cheap method that requires no additional extraction procedures. The results have confirmed that the method is precise, specific, and sensitive for the mivacurium isomers and their metabolites.

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