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Short Communication

Stereoselective high-performance liquid chromatographic assay with fluorometric detection for the isomers of mivacurium in human plasma

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ABSTRACT

A high-performance liquid chromatographic assay with fluorometric detection was developed for the analysis of the stereoisomers of mivacurium, a new short-acting neuromuscular blocker, in plasma. The isomers were isolated from plasma by solid-phase extraction with C_{18} and anion-exchange cartridges. The extracts were chromatographed on a LiChrosphere 60 RP Select B column (125 mm × 4.6 mm I.D.) using a mobile phase of acetonitrile–water (4:6, v/v) containing 0.005 *M* octanesulfonic acid. The fluorescence excitation and emission wavelengths were 202 and 320 nm, respectively. The accuracy and precision of the assay, expressed as the percentage deviation of measured values from true values and the percentage coefficient of variation, respectively, were $\leq 10\%$ at all concentrations except for the percentage coefficient of variation at the lower limit of quantitation (5 ng/ml). The assay has been successfully used for the analysis of plasma samples from a pharmacokinetic study in human volunteers.

INTRODUCTION

Mivacurium, (*E*)-(1*R*,1′*R*)-2,2′-[4-octenedioylbis(oxytrimethylene)]bis[1,2,3,4-tetrahydro-6,7-dimethoxy-2-methyl-1-(3,4,5-trimethoxybenzyl)-isoquinolium] dichloride (Fig. 1), is a short-acting non-depolarizing neuromuscular blocking agent which is rapidly hydrolyzed to inactive metabolites by plasma esterases [1,2]. Mivacurium consists of a mixture of three stereoisomers; the *cis–trans* and *trans–trans* isomers are equipotent while the *cis–cis* isomer is approximately one tenth as active as the other isomers. The percentage range specification for the *trans–trans*, *cis–trans* and *cis*–cis isomers in clinical trial material are 52.1–62.1, 34.1–39.6 and 3.8–8.2%, respectively. In initial clinical studies, plasma concentrations of total mivacurium (without separation of the isomers) were measured by a reversed-phase high-performance liquid chromatographic (HPLC) method with UV detection similar to that described for the long-acting neuromuscular blocker doxacurium [3]. The plasma concentration–time profiles of patients in these studies were multi-exponential and the pharmacodynamics of

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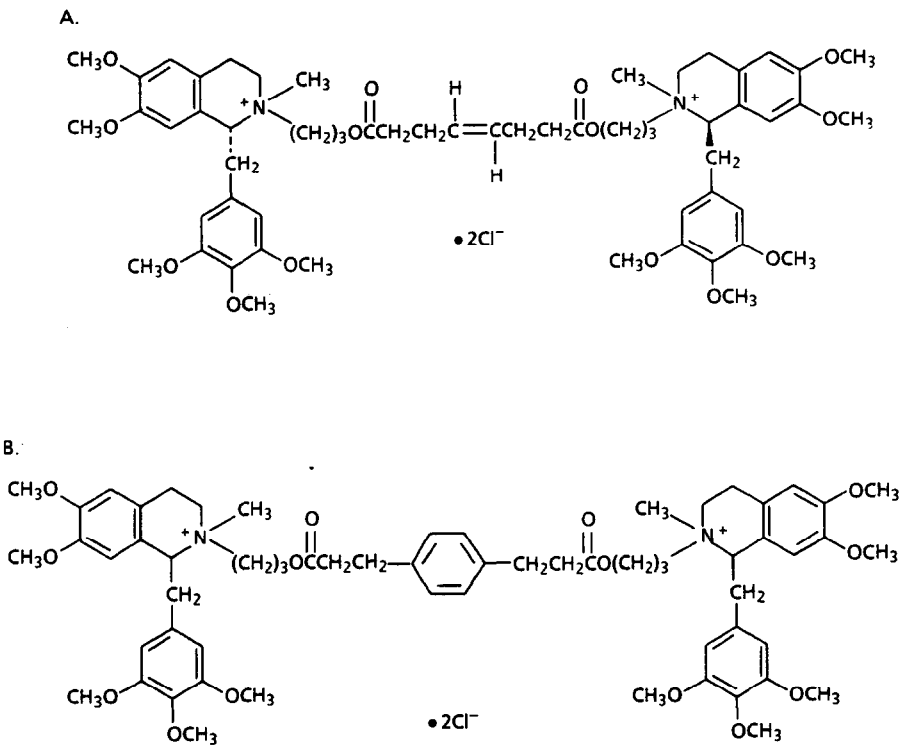

Fig. 1. Structures of mivacurium (A) and the internal standard (B). For mivacurium, the stereochemistry of the 2-positions of the isoquinolinium rings is mixed. The compound is a mixture of three stereoisomers: (1*R*, 1′*R*, 2*S* and 2′*S*), the *trans–trans* isomer; (1*R*, 1′*R*, 2*R* and 2′*R*), the *cis–trans* isomer; and (1*R*, 1′*R*, 2*R* and 2′*R*), the *cis–cis* isomer. The structure shown represents the *cis–trans* isomer.

mivacurium correlated most closely with the initial short elimination phase rather than the much slower terminal elimination phase [4]. Further studies have demonstrated that the *in vitro* hydrolysis rate of the *cis–cis* isomer in human plasma is significantly slower than the *cis–trans* or *trans–trans* isomers [5], suggesting that the terminal phase represents primarily the *cis–cis* isomer.

To elucidate the pharmacokinetics of the individual isomers in humans, we have developed a sensitive stereoselective assay incorporating solid-phase extraction and fluorometric detection. This method has been used to quantitate plasma concentrations of the three isomers in plasma after intravenous administration of mivacurium.

EXPERIMENTAL

Materials

Mivacurium (BW 1090U81), which consists of a mixture of the *cis–trans*, *trans–trans* and *cis–cis* isomers, was synthesized at Burroughs Wellcome (Research Triangle Park, NC, USA). The stereoisomers were prepared by chromatographic separation on Waters preparative silica gel cartridges (Waters Assoc., Milford, MA, USA) by Chemical Development Laboratories, Burroughs Wellcome RTP, USA. The internal standard, bis-{3-[*trans*-1,2,3,4-tetrahydro-6,7-dimethoxy-N-methyl-1-(3,4,5-trimethoxybenzyl)isoquinolinium]propyl}-1,3-phenylenedipropionate dichloride (BW 785U77), was also synthesized at Burroughs Wellcome.

Octanesulfonic acid (PIC B-8, low UV) was purchased from Waters Assoc. High-purity hydrochloric acid was purchased from GFS Chemicals (Columbus, OH, USA). Phospholine iodide, used as an inhibitor of plasma cholinesterase, was obtained from Ayerst Labs. (New York, NY, USA). All solvents used were HPLC grade (Omnisolv, EM Science, Cherry Hill, NJ, USA).

Instrumentation

Sep-Pak solid-phase extraction cartridges (Waters Assoc.) were used for sample preparation in conjunction with a Waters Millilab automated sample preparation unit. A Waters 600 multi-solvent delivery system was used with a 125 mm × 4.6 mm I.D. LiChrosphere 60 RP Select B, 5-μm column (EM Science, Gibbstown, NJ, USA) for the separation of the three isomers. A precolumn cartridge containing the same bonded phase was coupled directly to the analytical column with a Merck cartridge system (EM Science). The analytical column was maintained at 35°C with a Systek thin-foil heating system (Systek, Minneapolis, MN, USA). Samples were injected onto the column with a Waters 712B autosampler and the analytes quantitated with a HP 1046A fluorescence detector (Hewlett Packard, Avondale, PA, USA). The excitation and emission wavelengths were 202 and 320 nm, respectively. The HPLC mobile phase was acetonitrile–water (4:6, v/v) containing 0.005 *M* octanesulfonic acid. With a flow-rate of 1 ml/min, the retention times of the *trans–trans*, *cis–trans* and *cis–cis* isomers and the internal standard were 8.4, 9.3, 10.2 and 13.1 min, respectively. Chromatographic data acquisition, peak-area analysis and data reduction were performed by VG Multichrom software (VG Data Systems, Altrincham, UK) on a VAX 6000-320 computer (Digital Equipment, Maynard, MA, USA).

Extraction procedure

Plasma samples (1.0 ml) and method standards (5–250 ng/ml) containing the esterase inhibitor phospholine iodide (0.33 mg/ml of plasma) were transfered to 75 mm × 10 mm disposable glass tubes and placed on a Waters Millilab workstation for automated sample extraction. Internal standard (1 ml of a 100 ng/ml solution in saline) was added to each tube. The samples were then loaded onto Sep-Pak C_{18} cartridges preconditioned with methanol (5 ml) and distilled water (8 ml). The cartridges were washed with water (4 ml), methanol (4 ml) and acetonitrile (4 ml). The isomers and internal standard were then eluted with acetonitrile acidified with 0.33% 6 *M* hydrochloric acid (2.8 ml). The eluates were loaded onto QMA anion-exchange cartridges preconditioned with methanol (5 ml) and water (8 ml). The isomers and internal standard were eluted with acetonitrile acidified with 0.33% 6 *M* hydrochloric acid (0.9 ml). The eluates were evaporated to dryness at 55°C in a Savant Speed Vac centrifugal vacuum evaporation system (Savant, Farmingdale, NY, USA) and the residues reconstituted in 200 μl of mobile phase. A 50-μl aliquot was injected onto the HPLC system.

Assay validation

To determine the intra-day accuracy and precision of the assay, replicate ($n = 5$) plasma samples at seven different concentrations of the individual isomers (5–250 ng/ml) were analyzed. To determine the inter-day accuracy and precision of the assay, replicate plasma samples were analyzed on eight different days. The peak-area ratios of each compound and the internal standard were calculated, and a least-squares regression analysis was performed on the logarithmically transformed data. Precision was estimated from the standard deviation expressed as a percentage of the mean (percentage coefficient of variation, C.V.). Accuracy was calculated as the percentage difference between the mean calculated concentration and the amount added (percentage relative error, R.E.).

The stability of the isomers in human plasma stored at −20°C was determined. Plasma containing phospholine iodide was spiked with 25 or 200 ng/ml of each isomer. Replicate samples ($n = 2$) were analyzed immediately and at various times after storage at −20°C. The stability of the isomers was also determined in standard solutions stored at 4°C, in method standards (25 and 200 ng/ml) stored at room temperature and in plasma samples (25 and 200 ng/ml) after five freeze–thaw cycles.

The extraction efficiency of the assay was determined by comparing the peak heights for each isomer in extracted standards with those obtained by the injection of unextracted standards.

Biomedical application

A clinical study was designed to investigate the pharmacokinetics of the isomers of mivacurium in human volunteers. Informed written consent was obtained from healthy male volunteers undergoing elective surgery. During the surgical procedure, a 2-h infusion of mivacurium was initiated at 5 μg/kg per min for 60 min followed by 10 μg/kg per min for 60 min. Venous blood samples were taken at various times during the in-

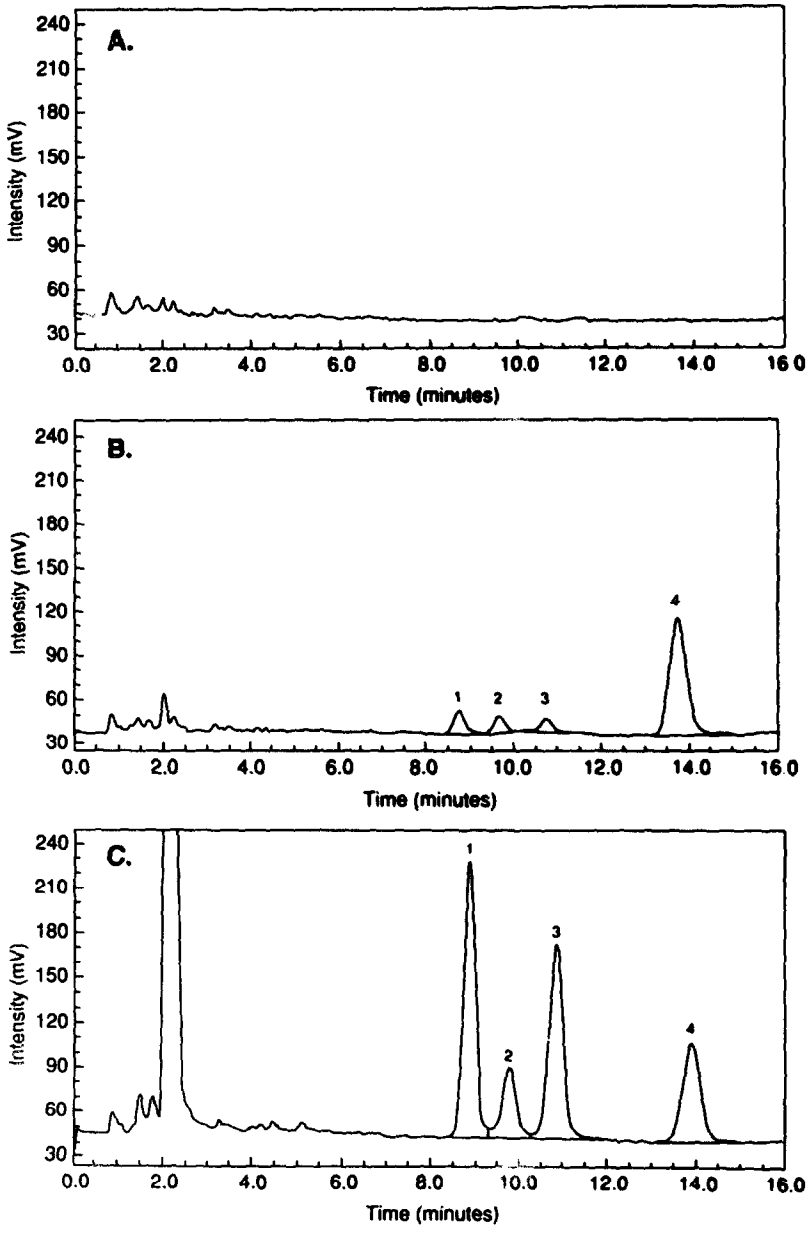

Fig. 2. HPLC of (A) extracted blank human plasma, (B) a 10 ng/ml method standard, and (C) a plasma extract taken from a human volunteer at 1 min after the end of a 10 μg/kg/min infusion of mivacurium for 60 min. Peaks 1, 2, 3 and 4 represent the *trans–trans*, *cis–trans* and *cis–cis* isomers and the internal standard, respectively. The concentrations of the *trans–trans*, *cis–trans* and *cis–cis* isomers in this sample were 165, 64 and 168 ng/ml, respectively.

fusion and up to 2 h post-infusion. Blood samples were added immediately to tubes containing the inhibitor, phospholine iodide. Plasma was prepared and frozen at −20°C until analyzed.

RESULTS AND DISCUSSION

A sensitive and selective assay has been developed for the quantitation of the isomers of mivacurium in human plasma. The assay is specific for the isomers and no interfering peaks appear in chromatograms of blank plasma (Fig. 2). Authentic samples of the quaternary and mono-ester metabolites eluted from the HPLC column in the void volume indicating these compounds do not interfere in the assay. Fig. 2 also shows a plasma standard of each isomer (10 ng/ml) and a chromatogram from a human volunteer taken at 1 min following intravenous infusion of mivacurium. The lower limit of quantitation for each isomer was 5 ng/ml of plasma. The separation of the isomers was relatively insensitive to changes in chromatographic conditions and the isomer peaks did not coalesce with small changes in mobile phase composition. Deterioration of column performance resulted in incomplete resolution between the isomers and excessively wide peaks.

The intra-day and inter-day accuracy and precision data for the assay of the isomers of mivacurium in human plasma are shown in Table I. The intra-day and inter-day precision, as indicated by the C.V., were 10% or less for all three isomers except at the lower limit of quantitation (16% or less at 5 ng/ml). The intra-day and inter-day accuracy, as indicated by the R.E., ranged from −6.0 to 6.7% for all three isomers.

The extraction efficiencies for the three isomers at three different concentrations are shown in Ta-

TABLE I

PRECISION AND ACCURACY FOR THE *TRANS–TRANS*, *CIS–TRANS* AND *CIS–CIS* ISOMERS IN HUMAN PLASMA

Compound	Concentration added (ng/ml)	Mean concentration found (ng/ml)	C.V.[a] (%)	R.E.[b] (%)
Intra-day (n = 5)				
Trans–trans	5	5.0	0.0	0.0
	50	47.0	2.6	−6.0
	250	255.8	1.2	2.3
Cis–trans	5	5.2	16.1	4.0
	50	47.2	4.8	−5.6
	250	250.2	3.4	0.1
Cis–cis	5	5.0	14.1	0.0
	50	48.4	5.6	−3.2
	250	240.2	4.7	−3.9
Inter-day (n = 14–16)				
Trans–trans	5	5.3	9.2	6.7
	50	47.9	6.2	−4.2
	250	257.0	5.7	2.8
Cis–trans	5	5.0	12.7	0.3
	50	49.9	6.8	−0.1
	250	245.3	5.6	−1.9
Cis–cis	5	5.1	16.2	2.8
	50	50.5	7.1	0.9
	250	241.5	6.8	−3.4

[a] Precision expressed as coefficient of variation.

[b] Accuracy expressed as relative error.

TABLE II

EXTRACTION EFFICIENCIES OF THE *TRANS–TRANS*, *CIS–TRANS* AND *CIS–CIS* ISOMERS IN HUMAN PLASMA

n = 3 for each concentration.

Compound	Concentration (ng/ml)	Recovery (mean ± S.D.) (%)
Trans–trans	10	56.7 ± 5.8
	50	54.7 ± 5.0
	250	54.4 ± 1.2
Cis–trans	10	53.3 ± 5.8
	50	55.3 ± 5.0
	250	55.7 ± 1.0
Cis–cis	10	53.3 ± 5.8
	50	54.0 ± 4.0
	250	53.3 ± 1.3

ble II. The mean extraction efficiencies were 55, 55 and 54% for the *trans–trans*, *cis–trans* and *cis–cis* isomers, respectively. The extraction efficiency results were confirmed by extracting plasma spiked with [^{14}C]mivacurium, a mixture of the three stereoisomers. Results using the radiolabel also indicated that a significant proportion of the spiked compound was being lost from the C_{18} cartridge in the methanol wash. However, without this wash, endogenous peaks appeared in chromatograms which interfered with the quantitation of the stereoisomers. The extraction method was not modified because the extraction efficiencies were reproducible, and the lower limit of quantitation for the assay was acceptable (5 ng/ml) for the application. We have not seen variations in isomer recovery with different batches of Sep-Pak cartridges; however, an internal standard with similar physico-chemical properties to mivacurium was specifically chosen to compensate for any changes in column packing.

The isomers of mivacurium are rapidly hydrolyzed in plasma to relatively inactive metabolites, the quaternary alcohol and mono-ester. The *in vitro* half-lives of the active isomers in human plasma are approximately 2 min [6]. Therefore, an inhibitor of plasma esterases must be added within 20 s after taking blood samples to minimize the loss of mivacurium (< 10%) during the sampling procedure. The inhibitor must also be added to method standards. Studies with the non-specific esterase inhibitor, phospholine iodide, demonstrated that a concentration of 0.33 mg of inhibitor per milliliter of plasma fully inhibited the hydrolysis of the isomers. With this inhibitor present, the stability of the isomers in plasma stored at −20°C was studied. No degradation of either the *trans–trans*, *cis–trans* or *cis–cis* isomers was detected in plasma samples stored for at least 86 days at this temperature. In addition, no degradation of the isomers was detected in standard solutions stored at 4°C for three months, in method standards stored at room temperature for three days or in plasma samples after five freeze–thaw cycles.

The assay has been used to quantitate plasma concentrations of the *trans–trans*, *cis–trans* and *cis–cis* isomers in volunteers after intravenous administration of mivacurium. As an example of

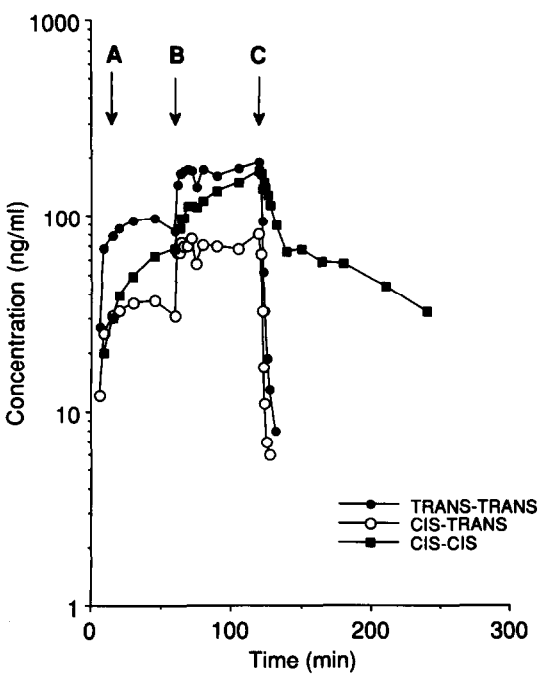

Fig. 3. Plasma concentrations of the *trans–trans* (●), *cis–trans* (○) and *cis–cis* isomers (■) during a 60-min intravenous infusion of 5 μg/kg mivacurium per min (A), during a 60-min intravenous infusion of 10 μg/kg mivacurium per min (B) and post-infusion (C).

the utility of the assay, a plasma concentration *versus* time curve for each isomer following intravenous infusion of mivacurium to one human volunteer is shown in Fig. 3.

In this clinical study, the *cis–cis* isomer represents 6.1% of the clinical trial material compared to 57.2 and 35.8% for the *trans–trans* and *cis–trans* isomers, respectively. Since plasma concentrations of the *cis–cis* isomer are low, a high-sensitivity assay is required to define the terminal elimination phase of this compound. As shown in Fig. 3, by using the stereoselective assay with fluorometric detection, the pharmacokinetics of all three isomers have been defined. The results have confirmed that the more active *trans–trans* and *cis–trans* isomers have a shorter plasma elimination half-life (approximately 2 min) than the *cis–cis* isomer (approximately 51 min) which is consistent with the short duration of block associated with mivacurium [1]. Although the *cis–cis* isomer has a longer plasma half-life, it does not appear to contribute to neuromuscular blockade because of its relatively low potency.

In conclusion, a new sensitive stereoselective assay has been developed for the analysis of the isomers of mivacurium. Over 600 clinical samples have been analyzed with this assay. Results have confirmed that the assay is both accurate and highly reproducible.

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