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ASSAY OF VECURONIUM IN PLASMA USING SOLID-PHASE EXTRACTION, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND POST-COLUMN ION-PAIR EXTRACTION WITH FLUORIMETRIC DETECTION

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SUMMARY

An assay has been developed and validated for the routine monitoring of vecuronium in plasma. It consists of solid-phase extraction using C_{18} disposables as sample pre-treatment, high-performance liquid chromatography and post-column ion-pair extraction with fluorimetric detection. The fluorescent anion 9,10-dimethoxyanthracene-2-sulphonate (DAS) is used as the counter ion. The detection limit is ca. 5 ng/ml in plasma with a signal-to-noise ratio of 10. The assay is also applicable for monitoring vecuronium and its potential metabolites in other biological media, e.g., urine, bile and tissue (liver, kidney) homogenates.

INTRODUCTION

Vecuronium bromide (Norcuron®), 1-[(2β,3α,5α,16β,17β)-3,17-bis(acetyloxy)-2-(1-piperidinyl)androstan-16-yl]-1-methylpiperidinium bromide (Fig. 1), is a clinically effective [1] non-depolarizing neuromuscular blocking agent with a wide margin of cardiovascular safety [2,3]. A bioanalysis for vecuronium using normal-phase high-performance liquid chromatography (HPLC) and UV detection has been described [4]. The assay method is selective enough to determine vecuronium and its metabolites (hydrolysis products), but it is not sensitive enough [200 ng/ml in plasma, signal-to-noise ratio (S/N) = 10] to monitor vecuronium in all clinical situations. When applied for vecuronium, a fluorimetric assay [5] described for the structurally related pancuronium has a detection limit of approximately 10 ng/ml in plasma, but it lacks selectivity. Recently, the determination of vecuronium by direct insertion probe chemical ionization mass spectrometry was reported by Castagnoli et al. [6]. The assay is

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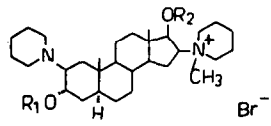

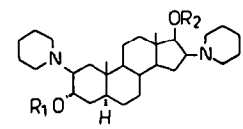

R_1=Ac ; R_2=Ac	vecuronium bromide	R_1=Ac ; R_2=Ac internal standard
R_1=H ; R_2=Ac	3-OH-vecuronium bromide (I)	
R_1=H ; R_2=H	3,17-diOH-vecuronium bromide (II)	
R_1=Ac ; R_2=H	17-OH-vecuronium bromide (III)	

Fig. 1. Structures of vecuronium, its hydroxy derivatives and the internal standard.

laborious, time-consuming and dependent on the availability of a mass spectrometer. The detection limit is 5 ng/ml in plasma at S/N = 2.

Recently, the liquid chromatographic determination of a quaternary ammonium steroidal type of drug in plasma was reported using post-column ion-pair extraction with the fluorescent anion 9,10-dimethoxyanthracene-2-sulphonate (DAS) and fluorimetric detection [7]. This method combines the selectivity of reversed-phase HPLC and the low-nanogram range sensitivity of fluorimetric detection. This paper describes the adaptation of the latter assay method for routine monitoring of vecuronium in plasma and reports applications of the assay to other biological media such as bile and urine.

EXPERIMENTAL

Chemicals

Vecuronium bromide and other reference standards (I, II and III) and the internal standard (Fig. 1) were supplied by Organon Labs. (Newhouse, U.K.).

[N-Methyl-^{3}H]vecuronium was synthesized by reaction of the internal standard with tritiated methyl iodide in dichloromethane. The final product was purified over alumina with 2-propanol–water. The radiochemical purity exceeded 95% as determined by thin-layer chromatography in three systems. The specific activity was 85 Ci (3.1 TBq)/mmol.

Methanol, acetonitrile, 1,4-dioxane and 1,2 dichloroethane were of Uvasol quality from Merck (Darmstadt, F.R.G.). Water was of Millipore Q-quality (Millipore, Bedford, MA, U.S.A.). DAS (purum quality), obtained from Fluka (Buchs, Switzerland), was subjected to Soxhlet extraction with dichloroethane before use. NaH_2PO_4 and orthophosphoric acid (35%) were of pro analysi quality from Merck.

The solid-phase extraction procedure was carried out on 1-ml C_{18} Bond-Elut disposable solid-phase columns (Baker, Phillipsburg, NJ, U.S.A.) for off-line preparation or 0.25-ml C_{18} disposable columns in cartridges of ten (Analytichem International, Harbor City, CA, U.S.A.) using the AASP (Analytichem automated sample processor) on-line. The HPLC Nova-Pak C_{18} column (15 cm × 3.9 mm I.D.; particle size 4 μm) was obtained from Millipore Waters (Milford, MA, U.S.A.).

Instrumental

A Waters Model M-6000 HPLC pump, an Eldex single-piston pump with pulse damper as post-column pump and a Model 710 B WISP autosampler were used (Millipore). The AASP module and the Vac-Elut module were purchased from Analytichem International and the solid-phase extraction manifold (10 SPE) from Baker. The micro control needle valve BMVC was obtained from SGE (Ringwood, Australia). The phase separator was custom-made and constructed according to De Ruiter et al. [8]. The phase separator consists of two stainless-steel blocks, one with a groove and one without a groove, and a PTFE disc with a groove, sandwiched together. As a result, a groove is obtained, the upper half being of stainless steel and the lower half of PTFE. The aqueous outlet is connected to a micro needle valve to allow the application of back-pressure to adjust the organic flow through the fluorimetric detector. The total internal volume of the sandwich phase separator is approximately 30 μl. This phase separator is very efficient in terms of minimization of external band broadening [8]. In addition, it does not, contrary to membrane-type phase separators, show lifetime problems when it is used for the analysis of plasma samples [7]. A Model LS-3 fluorimetric detector was obtained from Perkin-Elmer (Beaconsfield, U.K.). The integrator used was obtained from Spectra-Physics (San Jose, CA, U.S.A.).

Nuclear magnetic resonance (NMR)

To investigate how many equivalents of DAS were associated by ion-pairing with vecuronium upon post-column ion-pair extraction, a bench extraction was performed with dichloroethane for vecuronium and DAS dissolved in the HPLC eluent. The dichloroethane extract was evaporated to dryness, the residue dissolved in C^2HCl_3 and the solution subjected to NMR analysis (Bruker AM 360 spectrometer).

Stock and working solutions

Stock solutions of all reference compounds (0.1 mg/ml*) were prepared in 0.1 *M* NaH_2PO_4 adjusted to pH 3 with orthophosphoric acid. Working solutions for spiking to plasma were prepared weekly by dilution with 0.1 *M* NaH_2PO_4 (pH 3) to a final concentration of 10 or 1 ng/μl.

Acidification and storage of plasma samples

Vecuronium is liable to hydrolyse in plasma at physiological pH. Therefore, plasma samples were acidified by addition of ca. 150 μl of 1 *M* NaH_2PO_4 per ml of plasma. This prevented the hydrolysis of vecuronium. Plasma samples were stored at $-20°C$ until required for analysis.

Extraction and calibration

The design of the clinical study, the administered dose and time of blood sampling were used to predict the vecuronium plasma level. Variable volumes of internal standard from the working solution were added to the clinical plasma sample

*All concentrations refer to cationic entities without bromide.

at a concentration equal to that of the anticipated vecuronium concentration. With this assay, plasma samples of varying volumes (from a few μl to 1 ml) can be processed. Plasma samples with volumes less than 1 ml were diluted with 0.1 *M* NaH_2PO_4 (pH 3) to 1 ml.

For the extraction procedure the C_{18} columns were pre-conditioned with 1 ml of methanol–acetonitrile (2:1, v/v) and 1 ml of water. The 1-ml samples were applied to the columns and washed with 1 ml of water and 1 ml of 0.1 *M* NaH_2PO_4 (pH 3). The 0.25-ml C_{18} columns in a cartridge were then ready for HPLC analysis using the AASP. For off-line elution using the 1-ml C_{18} disposable columns, the columns were eluted with 400 μl of HPLC eluent, discarding the first 100 μl. An aliquot of 200 μl was introduced on to the HPLC column using the WISP autosampler.

To test the overall assay recovery, 1-ml plasma samples were spiked in replicate ($n=5$) with [^{3}H]vecuronium equivalent to 5, 10, 50 and 100 ng/ml. Off-line elution using Baker columns and the WISP and the on-line elution using the AASP were compared. The plasma samples were processed and the recovery was assessed by sampling the dichloroethane leaving the fluorimetric detector over the time interval in which the [^{3}H]vecuronium eluted from the HPLC system (corrected for the hold-up time in the outlet tubing).

For calibration purposes 1-ml plasma samples were spiked with vecuronium in concentrations of 10, 20, 50 and 100 ng/ml and 20 ng/ml internal standard. For quality control purposes, the accuracy (relative difference between mean measured and spiked analyte plasma concentrations) and the precision (coefficient of variation, C.V.) were calculated, using plasma samples spiked with vecuronium and internal standard in a 1:1 ratio at concentration levels of 10, 20, 50 and 100 ng/ml. The peak areas of the analytical signals of interest were taken as quantitative measures. Calibration graphs were constructed by calculating the peak-area ratio of vecuronium to the internal standard and applying linear regression between peak-area ratio and concentration ratio.

HPLC system

The HPLC system is shown schematically in Fig. 2. The injector was either the AASP module or the WISP autosampler. The eluent consisted of 0.1 *M* NaH_2PO_4 and 0.44 m*M* DAS adjusted to pH 3 with phosphoric acid in water–dioxane (4:1, v/v). A Nova-Pak C_{18} column (15 cm $\times$ 3.9 mm I.D.) was used. The eluent was permanently degassed with helium to avoid the occurrence of air in the system, which is detrimental for the post-column phase separation and fluorimetric detection. The eluent flow-rate was 1 ml/min and the dichloroethane flow-rate was 1.6 ml/min. The low-dead-volume T-piece was made of stainless steel. The capillary coil was made of stainless steel (1 m $\times$ 0.25 mm I.D.) with a coil diameter of ca. 9 mm. The splitting ratio after phase separation was $0.6/1.6=37.5\%$, which is in the optimal range between 30 and 40% [8]. The fluorimetric detector was operated at 380 nm (excitation wavelength) and 452 nm (emission wavelength).

After each series of analyses the whole HPLC system was flushed with methanol (ca. 200 ml). Chromatographic performance was restored after pumping ca. 120 ml of eluent through the system.

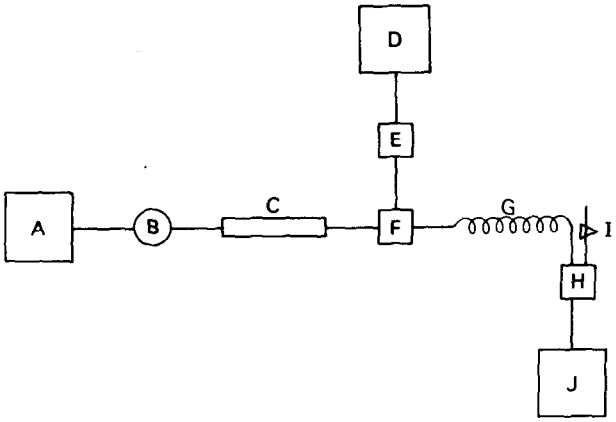

Fig. 2. Schematic diagram of HPLC configuration. A = pump; B = injector (AASP or WISP); C = HPLC column; D = pump; E = pulse damper; F = T-piece; G = extraction coil; H = phase separator; I = needle valve; J = fluorimetric detector.

RESULTS AND DISCUSSION

Representative chromatograms of extracts of blank (free of drug), spiked and clinical human plasma samples are given in Fig. 3. The chromatogram for blank plasma shows that there is no interference from endogenous plasma components.

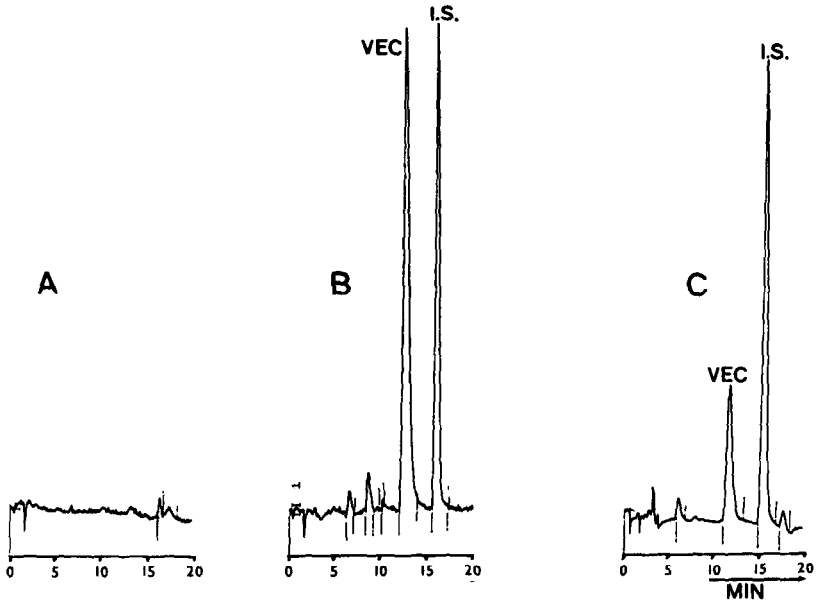

Fig. 3. Chromatograms of processed human plasma samples. (A) Drug-free plasma. (B) Drug-free plasma, spiked with 200 ng of vecuronium (VEC) and 200 ng of internal standard (I.S.); 500 μl of plasma were processed. (C) Clinical plasma, spiked with 200 ng of internal standard (I.S.); 50 μl of plasma were processed. Intravenous bolus dose, 19 mg of vecuronium bromide; time after administration, 10 min.

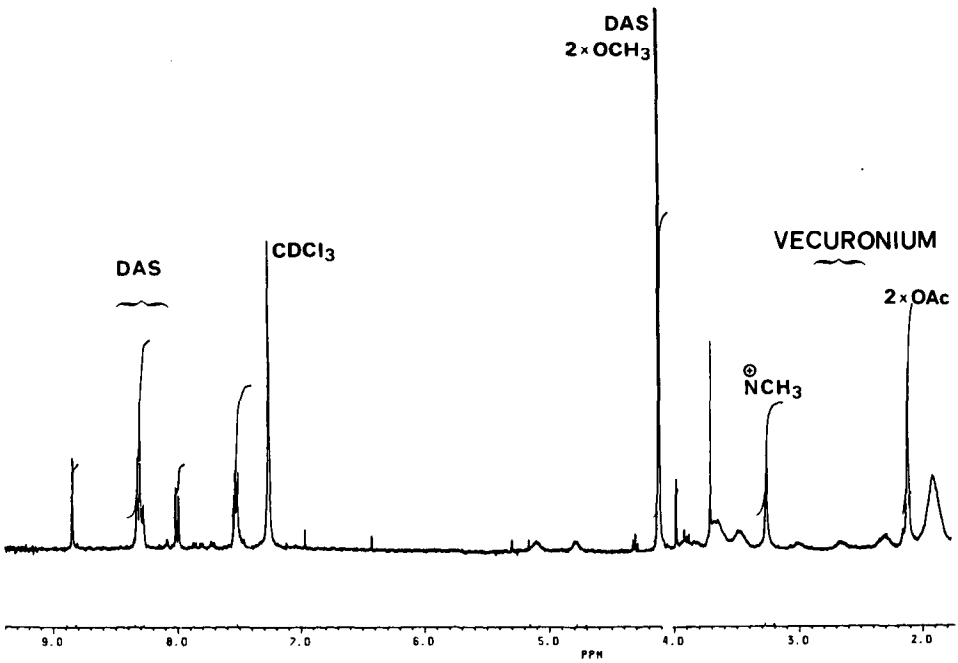

Fig. 4. NMR spectrum in C^2HCl_3 of the dichloroethane extract of a mixture of vecuronium and DAS.

The high selectivity is due to the solid-phase extraction of the compounds of interest from plasma, the separation by reversed-phase chromatography and the additional post-column ion-pair extraction into dichloroethane and fluorimetric detection. The sensitivity of the assay is determined by the amount of DAS (as an ion pair) extracted into the dichloroethane.

On systematically lowering the DAS concentration in the mobile phase to 0.05 and 0.005 m*M*, the retention of the cationic analytes of interest decreased. This indicates that the retention is governed by ion-exchange and ion-pair formation mechanisms. In addition, on lowering the DAS concentration in the mobile phase to 0.05 and 0.005 m*M*, the sensitivity decreased substantially: a factor 2 at 0.05 m*M* and a factor of 15 at 0.005 m*M*. In principle, the DAS in the mobile phase can be replaced by another anionic ion-pair former such as heptanesulphonic acid or octanesulphonic acid. On adding DAS dissolved in water post-column with an extra pump, comparable HPLC selectivities were obtained; however, the sensitivity decreased by a factor of 2–5. Obviously ion-pair formation with DAS is less readily achieved in the latter situation. This is due to the competition between, e.g., octanesulphonic acid and DAS for ion-pair formation with the cation of interest. Therefore, it was finally decided to add DAS to the mobile phase both for the retention and the enhancement of detection sensitivity. As illustrated in Fig. 4 for vecuronium, the ion-pair formation between vecuronium and DAS as extracted into dichloroethane occurs in an approximately 1:1.3 ratio.

Data on the recovery of vecuronium using [^{3}H]vecuronium as a tracer at different plasma concentrations ranging from 5 to 100 ng/ml, taken through the

TABLE I

ASSAY RECOVERY DATA OF [^{3}H]VECURONIUM ($n=5$)

Concentration (ng/ml)	On-line (AASP)		Off-line (Bond-elut)	
	Recovery (%)	S.D. (%)	Recovery (%)	S.D. (%)
100	93.9	3.2	68.8	14.4
50	86.4	9.1	92.0	3.6
10	81.6	13.3	96.0	3.6
5	72.0	3.2	86.0	4.8

TABLE II

ASSAY QUALITY CONTROL ($n=5$)

Vecuronium added (ng/ml)	Found (mean ± S.D.) (ng/ml)	Accuracy (%)	Precision (C.V., %)
10	8.8 ± 1.4	−12.0	13.8
20	20.4 ± 2.8	+ 2.0	13.7
50	51.2 ± 1.0	+ 2.4	2.0
100	104.3 ± 4.0	+ 4.3	3.8

assay procedure, are given in Table I. The average overall assay recoveries using on-line solid-phase extraction (Vac-Elut and AASP) or Baker SPE manifold and WISP were 83.5 and 85.7%, respectively. The data were corrected for the splitting ratio after phase separation. These recovery data indicate that solid-phase

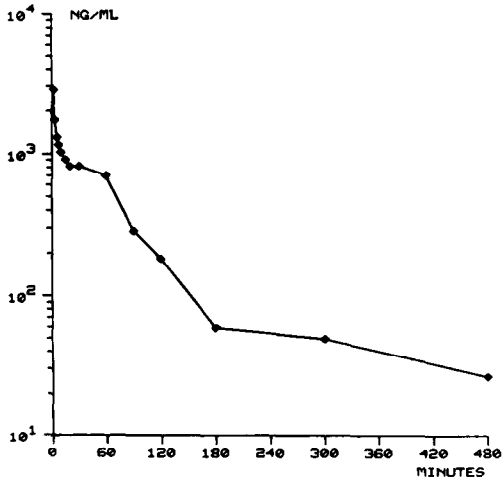

Fig. 5. Time course of vecuronium plasma levels after intravenous administration of 19 mg of vecuronium bromide.

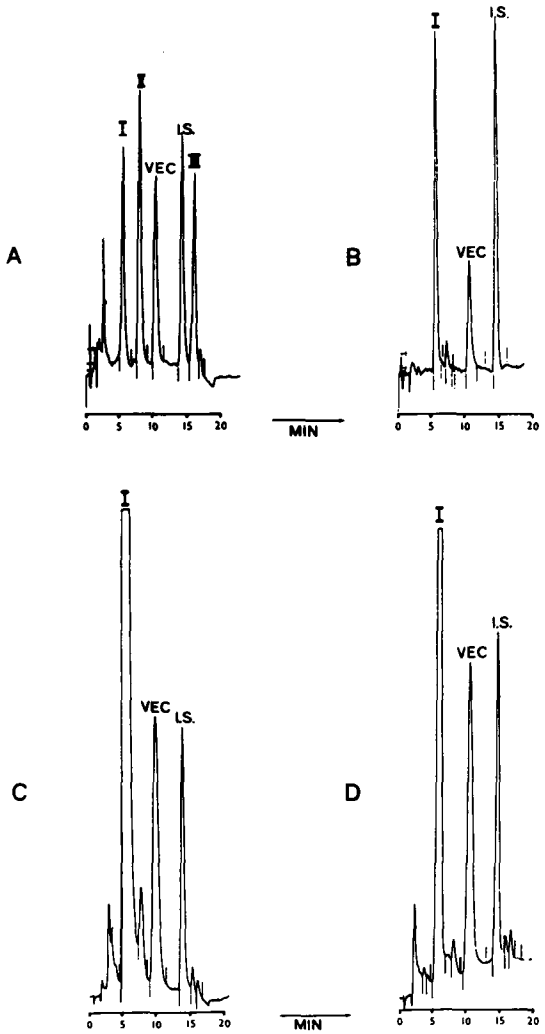

Fig. 6. Chromatograms of processed biological samples. (A) Drug-free plasma, spiked with 100 ng of I, II, vecuronium (VEC), internal standard (I.S.) and III; 1 ml of plasma was processed. (B) Rat liver perfusate. Dose, 1 mg of vecuronium bromide; time after addition, 80 min; 200 μl of perfusate, spiked with 200 ng of internal standard, were processed. (C) Dog bile, spiked with 200 ng of internal standard; 1 μl was processed. (D) Dog urine, spiked with 200 ng of internal standard; 1 μl was processed.

extractions using the AASP on-line or the Baker SPE manifold off-line are equally efficient. Owing to automation possibilities and an enhancement in sensitivity, it was better to use the AASP on-line, whereby the sample is quantitatively transferred on-line from the AASP to the HPLC column.

On calibration for vecuronium the following representative linear regression equation was obtained using peak-area ratios (y) versus concentration ratios (x): $y=0.93x-0.05$ ($r^2=0.9927$).

The accuracy and precision data for vecuronium at the various concentration

levels added to human plasma are given in Table II. The detection limit for vecuronium is ca. 5 ng/ml in plasma (S/N = 10).

Applications of the assay

An example of the time course of vecuronium plasma levels after intravenous bolus administration of 19 mg of vecuronium bromide to a subject (75 kg) undergoing elective surgery is given in Fig. 5. Vecuronium plasma levels were monitored up to 8 h after administration of vecuronium bromide. In addition, the assay allows the selective determination of vecuronium and its potential metabolites in various biological media.

Fig. 6A shows a chromatogram of a human plasma sample spiked with vecuronium, I, II, III and internal standard. Fig. 6B shows a chromatogram of perfusate (processed in the same way as described for plasma) used in an isolated rat liver perfusion experiment with vecuronium bromide. Fig. 6C and D show chromatograms of 1 μl of bile and urine from a dog dosed with vecuronium bromide. The samples were diluted to 1 ml with 0.1 *M* NaH_2PO_4 (pH 3) and processed in the same way as described for plasma. The detection limits obtained for the potential metabolites are comparable to that for vecuronium. Obviously, ion-pair formation and extraction are achieved equally readily and to the same extent as for vecuronium.

The assay has also been applied for the determination of vecuronium and its potential metabolites in the liver of rats, cats and dogs. To that end, the liver (or parts of it) was meticulously homogenized and centrifuged and the supernatants were processed in the same way as described for plasma.

The assay is also applicable to other amino-steroidal compounds such as pancuronium bromide. The HPLC retention can be controlled by adapting the DAS and/or the dioxane moderator concentration in the eluent within certain limits.

CONCLUSION

An assay has been developed for the routine monitoring of vecuronium in plasma. The assay is also applicable to the monitoring of vecuronium and its potential metabolites in other biological media.

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