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High-performance liquid chromatography–electrochemical detection of vecuronium and its metabolites in human plasma

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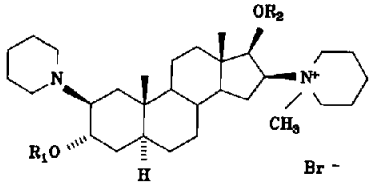
ABSTRACT

A high-performance liquid chromatographic assay coupled with electrochemical detection has been developed for the determination of vecuronium and its three putative deacetylated metabolites in human plasma. A novel solid-phase extraction procedure allowed good recovery of both vecuronium and its metabolites, together with ease and speed of execution. This method was sensitive, reproducible and accurate over the therapeutic range of concentrations of vecuronium and its metabolites, and was applied successfully to a study of the pharmacokinetics of vecuronium in anaesthetized patients.

INTRODUCTION

Vecuronium bromide, 1-[(2β,3α,5α,16β,17β)-3,17-bis(acetyloxy-2-(1-piperidinyl)androstan-16-yl]-1-methylpiperidinium bromide, is a non-depolarizing neuromuscular blocker of intermediate duration of action with minimal haemodynamic effects and an apparent lack of histamine-releasing properties [1]. In humans, its high liver uptake and metabolism may lead to deacetylation into 3α-hydroxyvecuronium, 3α,17β-dihydroxyvecuronium and 17β-hydroxyvecuronium [2] (Fig. 1). However, in most pharmacokinetic studies, no metabolites of vecuronium were detected in human plasma following the administration of a single dose of vecuronium [3–5]. In contrast, Lebrault and co-workers [6,7] estimated



	R_1	R_2
Vecuronium	CH_3CO	CH_3CO
3α-OH-vecuronium	H	CH_3CO
17β-OH-vecuronium	CH_3CO	H
3α,17β-OH-vecuronium	H	H
Org 7465	CH_3CH_2CO	CH_3CH_2CO

Fig. 1. Structures of vecuronium, its three putative metabolites and the I.S. (Org. 7465).

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that the 3α-hydroxy metabolite accounted for 5–10% of the total plasma concentrations. This metabolite has also been found in negligible amounts in urine (3%) and bile (1.5%) [3,4]. The prolonged neuromuscular blockade observed in critically ill patients following repeated bolus injections of vecuronium over several days has been attributed to the accumulation of 3α-hydroxyvecuronium [8]. This could be important as dose–response experiments in animals predict that 3α-hydroxyvecuronium should be almost equipotent to vecuronium, whereas 3α,17β-dihydroxyvecuronium, which is thought to be the terminal metabolite, would be 60 times less potent [9,10].

A sensitive and specific assay for the determination of vecuronium and its metabolites which is readily applicable in a clinical setting is still not available. In most pharmacokinetic studies, a fluorimetric assay similar to that described for pancuronium [11] is combined with thin-layer chromatography to determine, in a semi-quantitative manner, the relative proportions of vecuronium and its metabolites in plasma [3–7,12]. High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection led to the development of a selective method which lacked sensitivity [13]. More recently, an HPLC assay using post-column ion-pair extraction and fluorimetric detection has been reported [14]. This method was unsuccessful in this laboratory because of baseline fluctuations and erratic post-column extraction. In addition, it required a lengthy equilibration period with expensive organic solvents. The determination of quaternary steroid neuromuscular blockers by capillary gas chromatography was sensitive and specific but necessitated the derivatization of the 3-hydroxy metabolites [15]. Other assays based on chemical ionization selected-ion mass spectrometry [16] or mass spectrometry with a moving-belt liquid chromatography–mass spectrometry interface [17,18] require the use of sophisticated equipment which is not readily available in clinical laboratories. Furthermore, these methods require laborious ion-pair liquid–liquid extraction procedures, regardless of their detection system.

A reversed-phase HPLC method based on solid-phase extraction and electrochemical detection is proposed for the quantitative determination of vecuronium and its deacetylated metabolites in human plasma. It is anticipated that this assay will provide the means to further characterize the pharmacokinetic–pharmacodynamic relationship of vecuronium.

EXPERIMENTAL

Chemicals and reagents

Vecuronium bromide, its three metabolites and an internal standard (I.S.; Org 7465) were kindly supplied by Organon Labs. (West Orange, NJ, USA). Organic solvents and ammonium phosphate were of HPLC grade and all other chemicals were of analytical-reagent grade (Fisher Scientific, Montreal, Canada). High-quality water was obtained using five distillation columns connected in series (Oy Santasalo-Sohlberg, Helsinki, Finland). The water was stored refrigerated in Pyrex bottles and was passed through a Sep-Pak C_{18} cartridge (Millipore, Waters Assoc., Milford, MA, USA) immediately before use. All solutions were filtered through 0.2-μm membranes (Type HVLP, Millipore).

Standard solutions

Pure standard stock solutions of vecuronium and its metabolites (1 mg/ml) were prepared in 0.1 *M* monobasic ammonium phosphate (pH 3.0) and stored at 4°C. Working solutions (10 ng/μl) were made by further dilution in the same buffer and adjusted to pH 5.0.

A standard stock solution of the internal standard (Org 7465, 1 mg/ml) was prepared in phosphate buffer (pH 5.0) and further diluted in the same medium to yield the working solution (15 ng/μl). All solutions were tested weekly for degradation of the drug.

Chromatographic apparatus and conditions

The chromatographic system consisted of a Constametric III pump (LDC Milton Roy, Riviera Beach, FL, USA) an LP-21 pulse damper (Scientific System (SSI), State College, PA, USA), a 7125 injector fitted with a 50-μl injection loop (Rheodyne, Cotati, CA, USA) and a Coulochem 5100A electrochemical detector linked to a 5010 analytical cell (Environmental Science As-

soc., Bedford, MA, USA). The potentials of the screen electrode (detector 1) and the sample electrode (detector 2) were varied from 0 to +1.2 V to find the optimal detection conditions. The response time was 2 s. The 1000-mV output from detector 2 was connected to a CR3-A integrator (Shimadzu, Kyoto, Japan). A prepacked Spherisorb CN (5-μm particle size) column, 12.5 cm × 4.6 mm I.D. (Hichrom, Reading, UK), was used for the chromatographic separation of the analytes. SSI in-line filters of 0.45 and 0.2 μm were placed before the column and the analytical cell, respectively.

The mobile phase was prepared daily from 0.033 *M* phosphoric acid (60% v/v) and acetonitrile (40% v/v) and adjusted to a final pH of 5.55 with ammonium hydroxide. The solution was degassed under vacuum and pumped at a flow-rate of 2 ml/min. The mobile phase was allowed to recirculate during the equilibration period of the coulometer, but not thereafter. The chromatographic system was operated at room temperature.

Sample preparation

Bond Elut C_1 solid-phase extraction cartridges (Analytichem International, Harbor City, CA, USA) were conditioned with methanol (2 × 3 ml) and water (2 × 3 ml). Plasma (1 ml), water (1 ml) and internal standard working solution (100 μl) were added to the cartridges and then aspirated through the sorbent. The cartridges were sequentially washed with water (1 × 3 ml), acetonitrile (1 × 3 ml) and methanol (1 × 3 ml) under vacuum. Analytes were eluted into silanized glass tubes with 2 × 500 μl of 0.01 *M* sodium perchlorate solution in methanol. Elution was carried out under gravity for 3 min, then a small vacuum (less than 20 kPa) was applied to the cartridges. The eluents were evaporated using a Speed-Vac concentrator (Model SVC100H, Savant Instruments, Farmingdale, NY, USA). The dry residues were dissolved in 100 μl of mobile phase and a volume of *ca.* 30 μl was injected onto the analytical column.

Calibration graphs

Plasma samples were acidified to prevent the degradation of analytes [15]. Drug-free plasma used for the preparation of standards and spiked samples was acidified with 12 μl of 2 *M* sulphuric acid per ml prior to the addition of the analytes. A pool of plasma containing 4000 ng/ml vecuronium was serially diluted with drug-free plasma to give standard plasma at concentrations ranging from 3.9 to 4000 ng/ml. A similar procedure was used for each of the metabolites. Calibration graphs were generated by least-squares regression of the analyte/I.S. peak-height ratio against the concentration of the analyte.

Recovery

The recovery of vecuronium and its metabolites from human plasma was determined in triplicate at concentrations within the range of the calibration graphs (20, 50, 250, 500 and 4000 ng/ml). Blank plasma spiked with known amounts of analytes and 100 μl of internal standard were extracted and compared with blank plasma extracts subsequently spiked with the same amounts of analytes and I.S. The two sets of extracts were evaporated to dryness using the Speed-Vac system. Residues were reconstituted in mobile phase. The recovery was assessed by comparing the peak-height ratios of analyte/I.S. in the two sets of extracts. The recovery of the I.S. was previously assessed by comparing the peak heights of ten extracted samples to ten spiked samples at the same concentration used for the extraction of vecuronium.

Precision and accuracy

Intra-assay precision was assessed using triplicate spiked samples at four different concentrations ranging from 20 to 4000 ng/ml. Inter-assay precision was studied using spiked plasma samples (from the calibration graph) that were independently analysed at least four times within a 30-day period.

Drug-free plasma was spiked with vecuronium and its three metabolites to give twelve concentrations ranging from 20 to 3200 ng/ml. The samples were assayed blindly and concentrations derived from the calibration graphs. Accuracy was evaluated by comparing the estimated concentration with the known concentration of the analyte.

Limit of quantitation

Diluted solutions of vecuronium and its metabolites were injected directly into the HPLC system to estimate the minimum amount of analyte detectable electrochemically with a signal-to-noise ratio of 3. For each analyte, the lowest plasma concentration of the standard graph is defined as the lower limit of quantitation.

Stability

The stability of vecuronium and its deacetylated metabolites was studied with spiked plasma samples (400 and 4000 ng/ml) stored at −14°C. Samples were assayed in triplicate on the day of preparation (day 0) and one month later. Plasma samples spiked with vecuronium only were also assayed after three months of storage.

Clinical study

This method of determination of vecuronium and its metabolites in plasma was used in a pharmacokinetic study involving consenting patients undergoing elective surgery. The results obtained with one patient are presented. Heparinized blood samples were kept on ice until acidification within 15 min of collection. Samples were centrifuged and plasma was acidified as previously described. All samples were stored at −20°C until analysis.

RESULTS AND DISCUSSION

The optimum oxidative potentials at electrodes 1 and 2 were determined through repeated on-column injections of stock solutions of vecuronium, its deacetylated metabolites and the I.S. diluted in the mobile phase. The resulting hydrodynamic voltammograms at detector 2 are shown in Fig. 2. At potentials higher than +0.9 V, the current attained a plateau phase. However, increases in the voltage to greater than 0.8 V were associated with technical difficulties such as a decreased signal-to-noise ratio and a lengthy period of equilibration. Therefore 0.8 V was chosen as the selective oxidation potential for the second electrode. The first electrode was set at 0.4 V to screen for possible oxidizable impurities in the mobile phase or the biological sample without any significant loss of analytes.

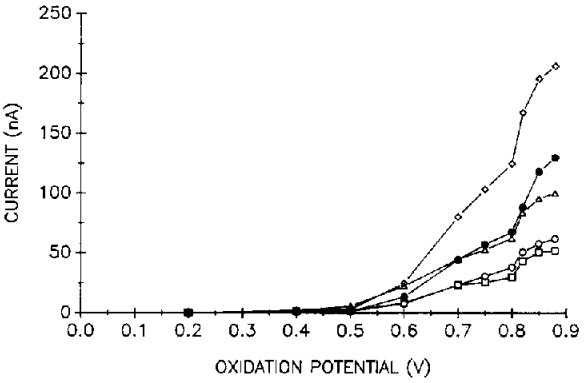

Fig. 2. Hydrodynamic voltammograms of vecuronium (●), 3α-hydroxyvecuronium (△), 3α,17β-dihydroxyvecuronium (□), 17β-hydroxyvecuronium (◇) and the I.S. (○).

Earlier studies showed that the oxidation peak potential of N-substituted piperidine molecules was dependent on the inductive or field effect of the substituent on the oxidation centre (*e.g.* N-methylpiperidine, 0.80 V; N-(2-aminoethyl)piperidine, 0.89 V) [19]. Thus the electro-activity of vecuronium and its metabolites is thought to be based on the oxidation of the tertiary piperidinic nitrogen in their structure. Similarly, the presence of a tertiary amine on neuromuscular blockers such as pipecuronium (N-substituted morpholine) or Org 9426 (N-substituted piperazine) may be responsible for their oxidative properties, which are similar to those of vecuronium. Pancuronium, a steroidal neuromuscular blocker with quaternary amines at positions 2 and 16, was not amenable to oxidation at the potentials used in this study.

The performance of the analytical cell was regularly monitored by repeated injections of diluted solutions of the analytes and comparison of the peak heights. The analytical cell was reconditioned whenever a decrease of more than 10% of the original response was observed. Sequential rinsing with water, methanol, water, acidified methanol and water was found to be effective in restoring the optimum sensitivity. Working with high-quality water, buffers and organic solvents, carefully degassed and filtered through 0.2-μm filters before use, allowed reproducible analyses.

The retention time of all compounds, including the I.S., was highly dependent on the ionic strength and pH of the mobile phase. A faster

TABLE I

RECOVERY OF VECURONIUM AND ITS METABOLITES FROM HUMAN PLASMA SAMPLES AND INTRA-ASSAY VARIABILITY

Concentration added (ng/ml)	Recovery (mean ± S.D., n = 3) (%)	Coefficient of variation (%)
Vecuronium		
20	87 ± 9	9.9
50	107 ± 1	1.0
250	106 ± 1	0.9
500	104 ± 1	0.6
4000	96 ± 5	4.9
3-Hydroxyvecuronium		
20	68 ± 1	1.5
250	73 ± 1	0.7
500	110 ± 4	3.4
4000	96 ± 5	5.2
3,17-Dihydroxyvecuronium		
50	71 ± 11	14.4
250	110 ± 2	2.2
500	101 ± 6	6.2
4000	100 ± 5	4.6
17-Hydroxyvecuronium		
20	107 ± 4	3.9
250	103 ± 1	0.8
500	93 ± 1	0.6
4000	90 ± 7	7.3

elution was achieved by increasing the ionic strength or lowering the pH. However, the resolution of chromatographic peaks was at an optimum at low ionic strength and high pH. Increases in sensitivity of the analytes have been associated with increments in pH or, to a lesser extent, in ionic strength. Alterations in the percentage of acetonitrile had a minimal effect on either the retention times or resolution. A mobile phase based on 0.02 *M* phosphoric acid precisely adjusted to pH 5.55 with ammonium hydroxide allowed a rapid separation of the analytes, and their detection at concentrations below 10 ng/ml of plasma.

Although C_{18} columns have been used by other workers for the solid-phase extraction of quaternary ammonium products from biological fluids [20,21], clean plasma samples and adequate recoveries for vecuronium and its metabolites could not be obtained in this work. This led to a study of the potential use of columns with more polar functional groups (*e.g.* C_1, CBA, SCX). A C_1 reversed-phase matrix provided the best compromise between retention and elution parameters. Using these columns, clean plasma extracts were obtained, allowing half of the reconstituted sample to be injected onto the column. The recovery of 1500 ng of I.S. was 104 ± 6% (mean ± S.D.). The mean recoveries of vecuronium obtained from five plasma concentrations were greater than 85% (Table I). The overall recovery

TABLE II

CALIBRATION GRAPHS FOR VECURONIUM AND ITS METABOLITES IN PLASMA AND INTER-ASSAY VARIABILITY

Concentration (ng/ml)	Peak-height ratio (mean ± S.D., n = 4)			
	Vecuronium	3-Hydroxyvecuronium	3,17-Dihydroxyvecuronium	17-Hydroxyvecuronium
3.9	0.008 ± 0.001	NQ[a]	NQ	NQ
7.8	0.014 ± 0.001	0.013 ± 0.002	NQ	NQ
15.6	0.027 ± 0.003	0.025 ± 0.002	NQ	0.030 ± 0.004
31.2	0.039 ± 0.004	0.039 ± 0.002	0.018 ± 0.002	0.049 ± 0.004
62.5	0.078 ± 0.005	0.077 ± 0.003	0.033 ± 0.002	0.096 ± 0.004
125	0.146 ± 0.005	0.155 ± 0.002	0.063 ± 0.002	0.195 ± 0.011
250	0.294 ± 0.018	0.294 ± 0.020	0.127 ± 0.004	0.379 ± 0.020
500	0.545 ± 0.012	0.526 ± 0.015	0.254 ± 0.014	0.762 ± 0.018
1000	1.093 ± 0.017	0.977 ± 0.076	0.522 ± 0.017	1.573 ± 0.029
2000	2.069 ± 0.064	2.053 ± 0.091	0.988 ± 0.072	3.068 ± 0.261
4000	3.760 ± 0.103	3.666 ± 0.343	1.937 ± 0.067	5.660 ± 0.633

[a] NQ = Not quantifiable.

was also good for the metabolites, although lower recoveries were obtained at low concentrations.

On the column, the limit of detection was 1 ng for vecuronium and its 3- and 17-hydroxy metabolites. The dihydroxy metabolite was slightly less reactive, with an on-column detection limit of 5 ng. The lower limits of quantitation in plasma were 3.9 ng/ml for vecuronium, 7.9 ng/ml for 3α-hydroxyvecuronium, 15.6 ng/ml for 3α,17β-dihydroxyvecuronium and 31.2 ng/ml for 17β-hydroxyvecuronium (Table II).

To achieve linearity over such a wide range of concentrations, two standard graphs were established for each analyte. The lower range covered plasma concentrations up to 250 ng/ml and the higher range those of 250 ng/ml and above. The regression equations for vecuronium at low and high concentrations were $y = 0.0012x + 0.0050$ ($r^2 = 0.993$) and $y = 0.0009x + 0.0813$ ($r^2 = 0.994$), respectively. Similarly, for 3α-hydroxyvecuronium, the regression equations were $y = 0.0012x + 0.0032$ ($r^2 = 0.995$) and $y = 0.0009x + 0.1237$ ($r^2 = 0.997$). For 3α,17β-dihydroxyvecuronium, the equations were $y = 0.0005x + 0.0012$ ($r^2 = 0.995$) and $y = 0.0005x + 0.0220$ ($r^2 = 0.995$). Finally, for 17β-hydroxyvecuronium, the equations were $y = 0.0015x + 0.0042$ ($r^2 = 0.993$) and $y = 0.0014x + 0.1009$ ($r^2 = 0.992$).

The intra-assay precision for samples containing 20–4000 ng/ml of either vecuronium or its metabolites were in all instances but one less than 10% (Table I). Because its physicochemical and electrochemical characteristics were similar to those of the analytes, the I.S., Org 7465, improved the intra-assay reproducibility. The inter-assay reproducibility gave a mean coefficient of variation of 5.8% for vecuronium, and 6.3, 5.5 and 6.5% for the three metabolites (Table II).

Blindly assayed spiked samples of vecuronium at concentrations covering the standard graphs showed an accuracy of 98.9 ± 5.5% (mean ± S.D., $n = 12$). The mean accuracies for the 3α-, 3α,17β- and 17β-hydroxy metabolites were 108.3 ± 9.1, 108.3 ± 3.5 and 98.7 ± 5.4%, respectively.

Vecuronium was stable in plasma for at least three months when stored at −14°C. The deacetylated metabolites showed no significant decay in their concentrations when stored for one month under the same conditions (Table III). Dry residues of extracted samples could be stored overnight in a freezer without any loss of analyte.

TABLE III

STABILITY OF VECURONIUM AND ITS METABOLITES IN PLASMA SAMPLES STORED AT −14°C

Concentration added (ng/ml)	Concentration found (mean ± S.D., $n = 3$) (ng/ml)		
	Day 0	One month	Three months
Vecuronium			
400	354 ± 1	387 ± 16	351 ± 5
4000	3965 ± 107	3936 ± 153	3919 ± 73
3-Hydroxyvecuronium			
400	385 ± 6	402 ± 12	ND[a]
4000	3979 ± 283	3535 ± 196	ND
3,17-Dihydroxyvecuronium			
400	425 ± 2	381 ± 9	ND
4000	3912 ± 19	3496 ± 81	ND
17-Hydroxyvecuronium			
400	388 ± 1	419 ± 43	ND
4000	3959 ± 275	3732 ± 148	ND

[a] ND = Not determined.

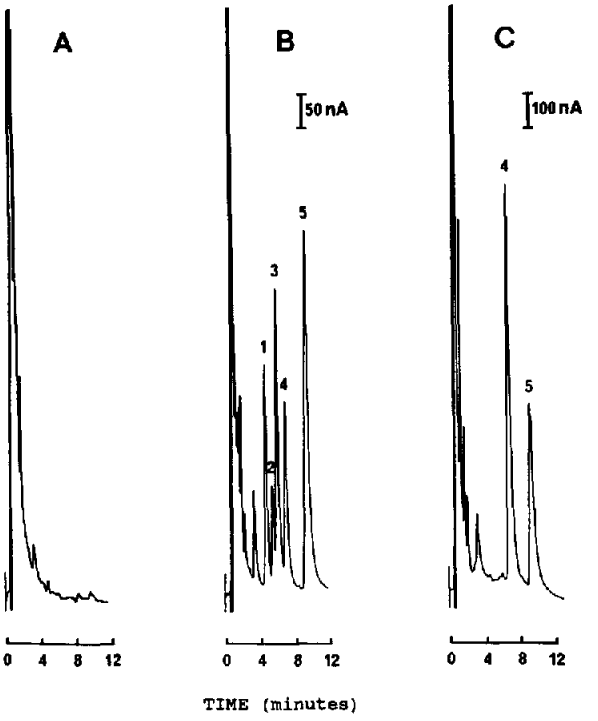

Fig. 3. HPLC results for: (A) plasma sample collected from a patient before the injection of vecuronium; (B) drug-free plasma spiked with 500 ng/ml vecuronium and its three metabolites and 1500 ng/ml I.S.; and (C) plasma sample collected from a patient 2 min after the injection of an intravenous bolus of 0.1 mg/kg vecuronium bromide. Peaks: 1 = 3α-hydroxyvecuronium; 2 = 3α,17β-dihydroxyvecuronium; 3 = 17β-hydroxyvecuronium; 4 = vecuronium; 5 = I.S.

Fig. 3 shows representative chromatograms of extracts of drug-free plasma spiked or unspiked with vecuronium and its metabolites. The retention times of 3α-hydroxyvecuronium, 3α,17β-dihydroxyvecuronium, 17β-hydroxyvecuronium, vecuronium and the I.S. were 5.0, 5.8, 6.5, 7.3 and 9.8 min, respectively (Fig. 3B). The total time for a chromatographic run, including the time needed for the baseline to return to its pre-injection value, was less than 12 min.

This method was applied to a study of the pharmacokinetics of vecuronium given as an intravenous bolus of 0.1 mg/kg to patients undergoing elective surgery. A plasma sample collected from a patient before the injection of vecuronium was free from interferences, either endogenous or from other drugs currently given during anaesthesia (Fig. 3A). A chromatogram obtained from a plasma sample taken 2 min after injection of vecuronium in the same patient is shown in Fig. 3C. In some samples, concentrations of the 3-hydroxy metabolite not exceeding 40 ng/ml could be determined. None of the other metabolites could be detected. The plasma concentration–time profile for this patient is shown in Fig. 4. Plasma concentrations declined from approximately 4000 to 5 ng/ml 5 h after injection of the drug and were well within the limit of quantitation of our assay. Full results of this study will be presented elsewhere.

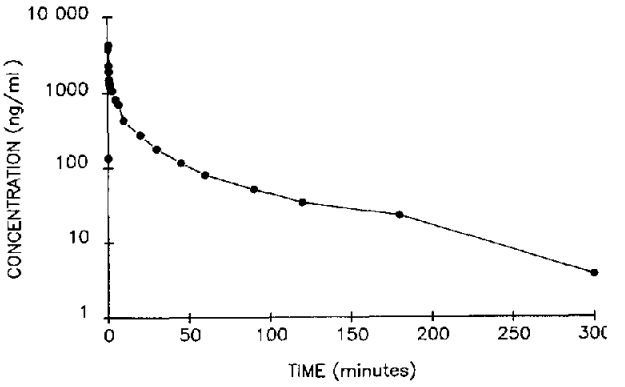

Fig. 4. Concentration–time curve for vecuronium in human plasma following the intravenous injection of 0.1 mg/kg vecuronium bromide in one patient.

CONCLUSIONS

A simple and highly selective HPLC assay with electrochemical detection has been developed for the simultaneous determination of vecuronium and its deacetylated metabolites in plasma samples. It provides a less expensive and time-saving alternative to HPLC with fluorimetric detection and post-column ion-pair extraction or gas chromatography coupled with mass spectrometry. The method is also sensitive and reproducible, and was successfully used in pharmacokinetic studies in humans. This method could also be applied to the determination of therapeutic concentrations of pipecuronium and Org 9426 in human plasma samples.

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