Journal of Chromatography, 533 (1990) 241–247
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO 5497

Note

Simple high-performance liquid chromatographic method for the analysis of the non-depolarizing neuromuscular blocking drugs in clinical anaesthesia

ANDREW R BJORKSTEN*

University Department of Surgery, Royal Melbourne Hospital, Melbourne, Victoria 3050 (Australia)

GEOFF H BEEMER

Department of Anaesthesia, Royal Melbourne Hospital, Melbourne, Victoria 3050 (Australia)

and

DAVID P. CRANKSHAW

University Department of Surgery, Royal Melbourne Hospital, Melbourne, Victoria 3050 (Australia)

(First received April 9th, 1990; revised manuscript received July 24th, 1990)

Lipophilic quaternary amines are often considered to be difficult to chromatograph with standard high-performance liquid chromatographic (HPLC) techniques [1]. The non-depolarizing neuromuscular blocking drugs used in clinical anaesthesia are typical in this regard due to the conflicting chemical properties confered by their large organic structures which contain one or two positively charged quaternary ammonium groups. Various means have been used to overcome this conflict with ion-paired normal-phase [2], ion-suppressed reversed-phase [3] and ion-exchange [4,5] methods appearing in the literature.

This report describes a simple and relatively inexpensive HPLC system, using a silica column with an acidic aqueous mobile phase, which may be used to determine plasma concentrations of all the commonly used drugs of this group. Laudanosine, a major metabolite of atracurium [6] with central nervous system (CNS) stimulant properties [7], may also be measured with a modified mobile phase.

EXPERIMENTAL

The methods were developed with an isocratic HPLC system consisting of a Model 510 pump, a Model 481 variable-wavelength UV absorbance detector or Model 431 conductivity detector, and a Model 710B WISP autosampler (Waters

0378-4347/90/$03.50

Assoc., Milford, MA, U.S.A.). The analytical column was a 15 cm × 3.9 mm I.D. μPorasil (Waters Assoc.) with an extra 0.002-mm pre-column frit to minimize particulate contamination of the column. Detector output was recorded on an AR25 chart recorder (Pye-Unicam, Cambridge, U.K.) with quantitation by peak-height ratio.

Reagents

Acetonitrile, isopropanol and dichloromethane were all HPLC grade (Millipore, Bedford, MA, U.S.A.) and sulphuric acid was AR grade (Ajax, Sydney, Australia). Picric acid was a 1:10 or 1:50 (v/v) dilution of saturated picric acid solution (Kock-Light, Haverhill, U.K.). Muscle relaxant standards were provided by the manufacturers: vecuronium from Organon Teknika (Sydney, Australia), atracurium and *d*-tubocurarine from Wellcome (Sydney, Australia) and alcuronium from Roche Products (Sydney, Australia). Pancuronium was diluted from the Pavulon commercial formulation (Organon Teknika), metocurine from the Metocurine Iodide USP formulation (Quad Pharmaceuticals, Indianapolis, IN, U.S.A.) and tacrine from THA (H W. Woods, Melbourne, Austrialia). Laudanosine was purchased from Sigma (St. Louis, MO, U.S A.).

Collection of blood samples

After giving informed consent in accordance with approved institutional protocol five patients were administered a single dose of one each of the following: 8 mg of vecuronium, 5 mg of pancuronium, 25 mg of atracurium, 20 mg of alcuronium, or 40 mg of *d*-tubocurarine as part of normal anaesthetic practice. Metocurine could not be administered as it is not approved for use in Australia. Blood samples (5 ml) were taken via a radial artery cannula placed for blood pressure monitoring. The blood was collected into heparinized plastic tubes (10 ml, Johns Professional Products, Melbourne, Australia), immediateley transferred to a second tube containing 0.05 ml of 2 *M* sulphuric acid and stored on ice. In addition to preventing the spontaneous hydrolysis of either vecuronium [8] or atracurium [9], acidification of the blood improves the efficiency of the extraction. All blood was centrifuged within 2 h of collection and the plasma stored frozen (−20°C) until analysis.

Chromatographic methods

The assays used similar methods for extraction and chromatography, with only minor changes where necessary. The general method for extraction from plasma was: 0.25 ml of dilute picric acid (1:50, v/v, dilution of a saturated solution), 0.25 ml of internal standard (I.S.) solution, 0.25 ml of water or drug standard, 0.25 ml of sample or blank plasma and 2.5 ml of isopropanol–dichloromethane (15:85, v/v) were added to a 10-ml polypropylene centrifuge tube. The mixture was vortex-mixed for 15 s and centrifuged at 1500 *g* in a Sigma 3E-1 for 10 min. The organic (lower) phase was transfered to a second 10-ml polypropylene tube and

evaporated to dryness in a waterbath at 40°C under a stream of nitrogen gas. The residue was reconstituted in 0.15–0.25 ml of acetonitrile–water (40:60, v/v) and centrifuged at 1500 *g* for 4 min before 0.02–0.10 ml was injected into the HPLC system.

The atracurium (alcuronium I.S.), alcuronium (atracurium I.S.), *d*-tubocurarine (metocurine I.S.), metocurine (*d*-tubocurarine I.S.) methods all used the general extraction method as detailed above, with a mobile phase of acetonitrile–0 002 *M* sulphuric acid (50:50, v/v) at 2 ml min^{-1}. Detection was by UV absorbance in each case, at 210 nm for atracurium, metocurine and *d*-tubocurarine, and 292 nm for alcuronium.

For vecuronium (pancuronium I.S.) and pancuronium (vecuronium I.S.), 2 ml of plasma rather than 0.25 ml, 1:10 instead of 1:50 dilution of saturated picric acid and 5 ml of isopropanol–dichloromethane (15:85, v/v) instead of 2.5 ml were used. The mobile phase was as above. Detection was by ionic conductivity in both cases

The analysis of laudanosine used the general extraction method with a mobile phase of methanol–0.0001 *M* sulphuric acid (50:50, v/v) at 2 ml min^{-1}, detection by UV absorbance at 210 nm and tacrine as the internal standard.

RESULTS

A representative chromatogram for vecuronium and pancuronium is shown in the left-hand panel of Fig. 1, with retention times of 4.7 and 5.7 min, respectively. The standard curves were linear from 50 to 2000 ng ml^{-1} ($r > 0.999$) with a within-day coefficient of variation of 5.9% for vecuronium at 300 ng ml^{-1} and

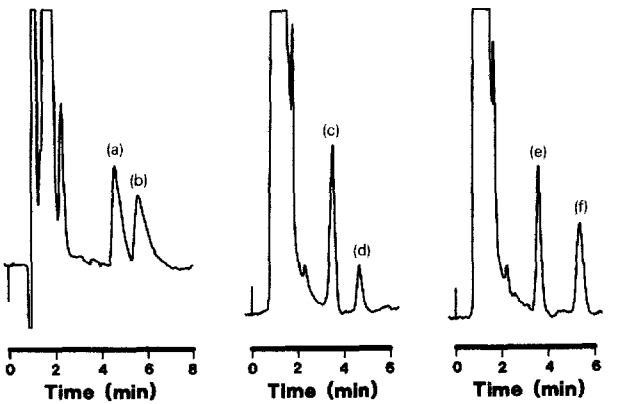

Fig 1 Representative chromatograms of extracts from plasma spiked with (a) vecuronium, (b) pancuronium (both 500 ng ml^{-1}, injection volume 0 1 ml, detector setting 2500 nS full scale), (c) atracurium, (d) alcuronium, (e) *d*-tubocurarine and (f) metocurine (all 1000 ng ml^{-1}; injection volume 0 05 ml, detector setting 0 02 a u f.s at 210 nm) Note for analysis of alcuronium 292 nm is used

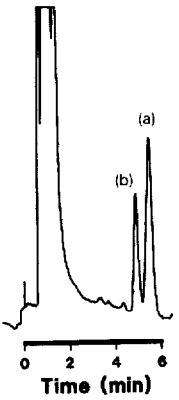

Fig 2 Representative chromatogram of an extract from plasma spiked with 1000 ng ml^{-1} laudanosine (a) The internal standard was 1000 ng ml^{-1} tacrine (b) (injection volume 0 025 ml and detector setting 0 02 a u f s at 210 nm)

pancuronium at 350 ng ml^{-1} (n = 6). The limits of detection were 10 and 20 ng ml^{-1} respectively.

The center panel of Fig. 1 shows a representative chromatogram of atracurium and alcuronium with retention times of 3.5 and 4.5 min, respectively The standard curves were linear from 50 to 10 000 ng ml^{-1} (r > 0.999) with a within-day coefficient of variation of 4.2% at 1000 ng ml^{-1} (n = 6) for the two drugs. The limits of detection were 25 and 15 ng ml^{-1}, respectively.

The right-hand panel of Fig. 1 shows a representative chromatogram of d-tubocurarine and metocurine with retention times of 3.7 and 5.3 min. The standard curves were linear form 50 to 10 000 ng ml^{-1} (r > 0.999) with a within-day coefficient of variation (n = 6) of 2.3% for d-tubocurarine at 1250 ng ml^{-1} and

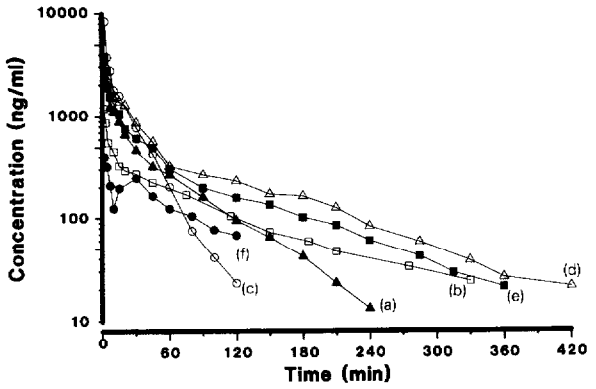

Fig 3 Representative plasma concentrations in surgical patients following 8 mg of vecuronium (a), 5 mg of pancuronium (b), 25 mg of atracurium (c), 20 mg of alcuronium (d) or 40 mg of d-tubocurarine (e) Laudanosine concentrations (f) were measured from the patient who received atracurium

metocurine at 600 ng ml^{-1}. The limits of detection were 20 and 25 ng ml^{-1}, respectively.

For laudanosine, the standard curve was linear from 50 to 5000 ng ml^{-1} ($r > 0.999$) with a coefficient of variation of 3.9% at 1000 ng ml^{-1} ($n = 6$) and a detection limit of 20 ng ml^{-1}. Fig. 2 shows a representative chromatogram for laudanosine and tacrine, with retentions of 5.7 and 5 min, respectively.

Extraction recoveries, determined by comparison of extracted standards with direct injection of the standards, were greater than 90% in all cases.

Fig. 3. shows representative concentration profiles for the administration of a single dose of vecuronium, pancuronium, atracurium, alcuronium, *d*-tubocurarine and laudanosine produced from the administration of atracurium in surgical patients.

DISCUSSION

The measurement of ionic conductivity in the aqueous mobile phase used in this report proved to be a useful and sensitive means of quantitating the otherwise inert and difficult to detect drugs vecuronium and pancuronium As all non-depolarizing muscle relaxants contain at least one quaternary ammonium group in their chemical structures, it seems likely that this mode of detection may be useful in this class of drugs, particularly those with little UV absorption.

The vecuronium and pancuronium methods presented here compare well with published alternatives. They are of comparable sensitivity to spectrofluorimetric methods [10], while retaining the property of differentiating the parent compounds from their metabolites The two published HPLC methods offering greater sensitivity, post-column ion-pair extraction (5 ng ml^{-1}) [11] and mass spectrometry (2 ng ml^{-1}) [12] are complex and costly exotic techniques with questionable reliability and robustness in general use. These difficulties, which have prevented their widespread use, are not applicable to the conductivity method described here. Conductivity detection proved unnecessary for the other drugs of the group as they possess sufficient UV absorbance for comparable or greater sensitivity to published methods for alcuronium (100 ng ml^{-1}) [13,14], metocurine (25 ng ml^{-1}) [15] and *d*-tubocurarine (25–50 ng ml^{-1}) [15–17]. The published methods for the analysis of atracurium and laudanosine in plasma rely on fluorimetric detection to achieve marginally greater sensitivity (10 and 5 ng ml^{-1}, respectively) [3–5]. However, this small difference is more than offset by the much greater availability of UV absorbance detection, particularly in small laboratories.

The chromatography is relatively simple and inexpensive to run, particularly when compared with methods requiring expensive strong cation-exchange columns [4,5], while the short retention times allow a rapid sample throughput. Column life is quite acceptable in routine usage, even at the low pH conditions of the sulphuric acid mobile phase, allowing in excess of one thousand extracted

plasma samples without a noticeable decrease in retention. Even then, the gradual reduction in retention can be counteracted by gradually decreasing the sulphuric acid concentration in the aqueous part of the mobile phase to enhance the useful life of the silica column.

The development of a single general method for the group of drugs confers the benefits of standardization of usage, storage and preparation of reagents and equipment. The modifications reguired for individual drugs are minor, allowing rapid changeover between drugs of interest, with one column sufficing for all methods. In addition, this general method should be applicable to the next generation of non-depolarizing muscle relaxants currently under development, including pipecuronium, doxacurium and mivacurium.

The mode of separation appears to be mixed: a combination of ion exchange and normal phase, representing the novel chemistry of silica under acidic aqueous conditions. Increasing the sulphuric acid concentration or decreasing the organic solvent of the mobile phase reduces retention times. The ion-exchange effect predominates as retention is more dependent on acid concentration than on organic solvent content.

The ion-exchange character is particularly useful as sensitive reversed-phase methods are difficult to develop for drugs which exhibit only low-wavelength UV absorbance. The retention and detection of many components of plasma produces a baseline with many interfering small peaks. This is generally less of a problem with ion-exchange methods as most of these interfering substances are eluted near the void volume, producing an effective increase in assay selectivity over reversed-phase methods. The use of an efficient extraction method employing a different mode of chemistry to the chromatography further enhances this selectivity. The enhancement of sensitivity was sufficient in the case of atracurium to allow UV absorbance detection to produce comparable sensitivity to methods using fluorescence detection [3–5].

In summary, we have developed a selective, inexpensive and simple general HPLC assay method for non-depolarizing muscle relaxants and the major atracurium metabolite laudanosine. The method is particularly applicable to small laboratories with access to isocratic HPLC equipment.

ACKNOWLEDGEMENTS

This work was supported by grants from Organon Teknika (Australia) and Wellcome (Australia). The support of Tim Bowser and Gill Whitney of Millipore (Melbourne, Australia) in allowing us the use of their laboratory and equipment, in particular the Model 431 conductivity detector, is much appreciated.

REFERENCES

1 B A Bidlingmeyer, *J Korpl, Anal Chem* , 54 (1982) 442–447
2 J E Paanakker and G L M van de Laar, *J Chromatogr* , 183 (1980) 459–466
3 R L Stiller, B W Brandon and D R. Cook, *Anesth. Analg* , 64 (1985) 58–62.
4 R J Simmonds, *J Chromatogr* , 343 (1985) 431–436
5 E. A M Neill and C. R Jones, *J. Chromatogr* , 274 (1983) 409–412
6 W Ward and B. C Weatherley, *Br J Anaesth* , 58 (1986) 6S–10S
7 G H Beemer, A. R. Bjorksten, P J. Dawson and D P Crankshaw, *Br J Anaesth* , 63 (1989) 76–80
8 C Lebrault, J L Berger, A A D'Hollander, R Gomeni, D Henzel and P Duvaldestin, *Anesthesiology,* 62 (1985) 601–605
9 R L Stiller, D R Cook and S. Chakravorti, *Br J Anaesth* , 57 (1985) 1085–1088.
10 U W. Kersten, D K F. Meijer and S Agoston, *Clin Chim Acta,* 44 (1973) 59-66
11 J E Paanakker, J. M S L Thio, H. M van den Wildenberg and F M Kaspersen, *J Chromatogr.,* 421 (1987) 327–335
12 R Cronnelly, D. M Fisher, R D Miller, P Gencarelli, L Ngugen-Gruenke and N Castagnoli, *Anesthesiology,* 58 (1983) 405–408.
13 J E Parkin, *J Chromatogr* , 225 (1981) 240–244
14 C. Tovey, D W A. Bourne and J Schneider, *J Chromatogr* , 278 (1983) 216–219
15 M J Avram and C A Shanks, *J. Chromatogr.,* 306 (1984) 398–404.
16 F de Bros and A J Gissen, *Anesthesiology,* 51 (1979) S265
17 A Meulemans, J Mohler, D Henzel and P Duvaldestin, *J Chromatogr* , 226 (1981) 255–258