Journal of Chromatography, 343 (1985) 431—436
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2702

Note

Determination of atracurium, laudanosine and related compounds in plasma by high-performance liquid chromatography

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(First received February 8th, 1985; revised manuscript received May 6th, 1985)

Atracurium besylate, 2,2′-(3,11-dioxo-4,10-dioxatridecylene)-bis[6,7-dimethoxy-1-(3,4-dimethoxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinolium], is a potent neuromuscular blocking agent marketed as Tracrium. At physiological pH atracurium undergoes rapid Hofmann elimination [1] to yield laudanosine which may be further metabolised to tetrahydropapaverine (THP). Ester hydrolysis is also possible to give quaternary alcohol and other derivatives (Fig. 1). Interest in plasma laudanosine concentrations following atracurium administration [2—4] necessitated the development of a method of analysis for atracurium and its derivatives in samples from patients undergoing surgery. The published method for atracurium [5] did not resolve laudanosine, THP or other atracurium derivatives from each other or from plasma peaks.

Solid-phase extraction of plasma followed by gradient ion-exchange high-performance liquid chromatography (HPLC) was the method of choice, since this enabled sample preparation and analysis without further breakdown of atracurium.

EXPERIMENTAL

Chemicals and reagents

Atracurium besylate, THP, and the monoquaternary alcohol derivative of atracurium were supplied by the Wellcome Foundation (Dartford, U.K.). Laudanosine was supplied by Aldrich (U.K.). N-Methyl laudanosine iodide and *d*-tubocurarine, used as internal standards, were supplied by the Wellcome Foundation. Acetonitrile and methanol (HPLC grade 'S') were supplied by

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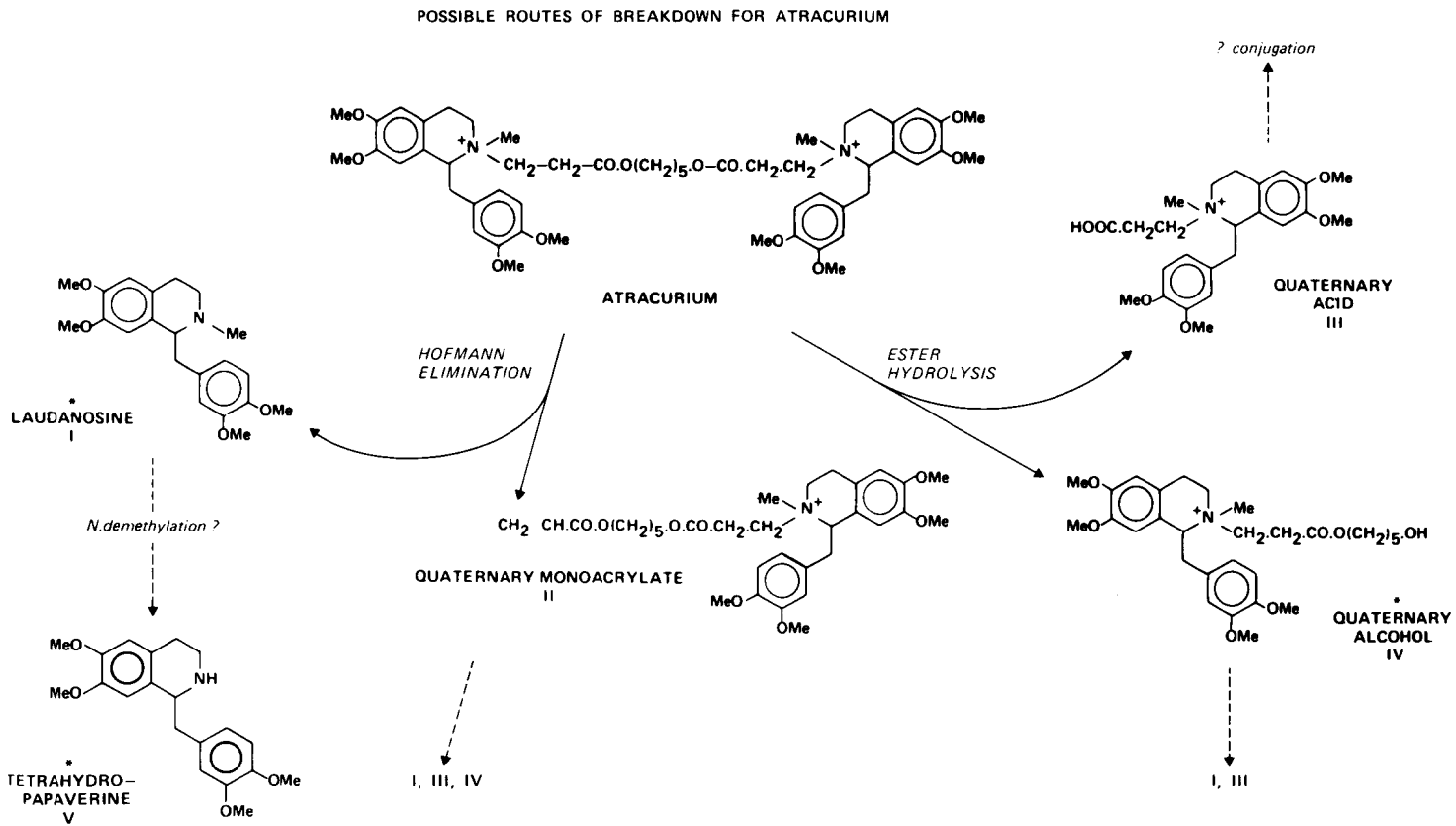

Fig. 1. Breakdown of atracurium in the body.

Rathburn Chemicals (Walkerburn, U.K.). All other chemicals were supplied by BDH (Poole, U.K.) and were of Analar or Aristar grade. All water was deionised and glass-distilled.

Equipment

Analytichem Bond-Elut phenyl cartridges, 100 mg, and Vac-Elut ten-place vacuum manifolds were obtained from Jones Chromatography (Llanbradach, U.K.). An Eppendorf centrifuge Model 5514 and plastic Eppendorf vials were used for rapid separation of plasma from whole blood.

Preparation of standards for calibration curve

Atracurium and the monoquaternary alcohol derivative were dissolved in 0.0005 *M* sulphuric acid (pH 3.0) at 1 mg/ml. These stock solutions were stored at −20°C and diluted with 0.0005 *M* sulphuric acid before spiking control plasma. Laudanosine, N-methyl laudanosine and THP were dissolved in 0.005 *M* sulphuric acid at 1 mg/ml. Further dilutions were made in water before use.

Stabilisation of plasma samples

Immediately after collection 1 ml of whole blood was centrifuged at 8000 *g* for 2 min, 0.2 ml of plasma was removed and mixed with 0.8 ml of 0.015 *M* sulphuric acid. These samples could be stored for up to 24 h at room temperature, or at least a week if frozen, without loss of atracurium.

Extraction

Samples were spiked with 20 μl of N-methyl laudanosine solution (5 μg/ml) and passed through Bond-Elut cartridges primed with 3 ml acetonitrile and 3 ml of 0.005 *M* sulphuric acid. Cartridges were then washed with 3 ml of 0.005 *M* sulphuric acid and 2 ml methanol—water (50:50). Atracurium and its derivatives and internal standard were eluted with 0.6 ml acetonitrile—0.06 *M* sodium sulphate (60:40) in 0.005 *M* sulphuric acid. After elution the pressure in the Vac-Elut manifold was further reduced in order to evaporate the eluates in situ to 0.2—0.3 ml.

HPLC

HPLC was carried out using a Hewlett-Packard HP1084 chromatograph linked to a Hitachi 650-10LC fluorescence detector, set at 280 nm (excitation) and 320 nm (emission). The column was a Partisil 10 SCX (strong cation exchange, 10 μm particle size), 250 × 4.6 mm (Hichrom, Woodley, U.K.) with an efficiency of 12 000 theoretical plates/m, as measured with laudanosine. This was maintained at 60°C. Mobile phases were (A) acetonitrile—0.01 *M* sodium sulphate (60:40) in 0.005 *M* sulphuric acid; (B) acetonitrile—0.06 *M* sodium sulphate (60:40) in 0.005 *M* sulphuric acid. After injection of 50—100 μl of eluate, a stepped gradient at a flow-rate of 2 ml/min was applied, consisting of 0—7 min 100% A, 7—10 min 0—100% B, 10—17 min 100% B, 17—20 min 100—0% B. Peaks were integrated automatically, and concentrations were calculated by the peak area ratio of analyte to internal standard by using the internal standard option of the HP1084.

RESULTS

Extracts of pre-dose (control) human plasma showed no interfering peaks from endogenous compounds or from drugs given concomitantly with atracurium, even at highest sensitivities (Fig. 2a, b). Laudanosine was resolved from the monoquaternary alcohol (IV) and THP (V) (Fig. 1). THP (V) was less well resolved from the quaternary alcohol (IV). Peaks corresponding to the quaternary monoacrylate (II) and the quaternary acid (III) were not seen in extracts of plasma from patients given a single dose of atracurium. Atracurium was eluted in the second part of the gradient, which produced little shift in baseline (Fig. 2b). The recovery of analytes from plasma, estimated by comparing peak areas given by unextracted standards and standards extracted from control plasma, was about 95% in all cases.

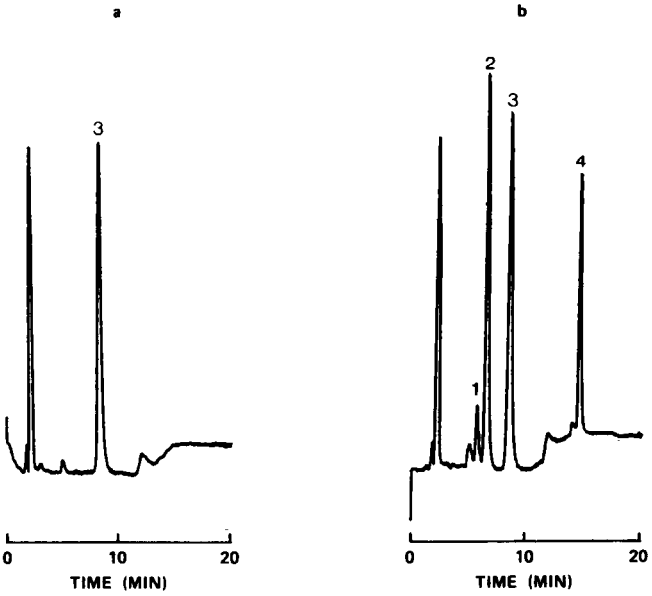

Fig. 2. Gradient HPLC of (a) extract of pre-dose (control) plasma; (b) extract of post-dose plasma. Conditions: 250 × 4.6 mm Partisil 10 SCX column at 60° C; mobile phases: (A) acetonitrile—0.01 *M* sodium sulphate (60:40) in 0.005 *M* sulphuric acid; (B) acetonitrile—0.06 *M* sodium sulphate (60:40) in 0.005 *M* sulphuric acid; flow-rate 2 ml/min. Gradient is 0—100% B starting from 7 to 10 min into run. Fluorescence detection 280 nm (ex), 320 nm (em). Peaks: 1 = monoquaternary alcohol derivative, 0.1 μg/ml; 2 = laudanosine, 0.2 μg/ml; 3 = N-methyl laudanosine (internal standard), 0.5 μg/ml; 4 = atracurium, 0.5 μg/ml.

Isocratic methods

Since gradient elution is not always convenient, and necessary only when either atracurium or laudanosine is required in the presence of the other, the method was adapted to give isocratic analysis of laudanosine and THP in dog plasma, and atracurium, alone, in human plasma.

Laudanosine and THP in dog plasma

For this assay the sample preparation was unchanged though it was found useful to extract only 0.1 ml of plasma. Analytes were eluted from the

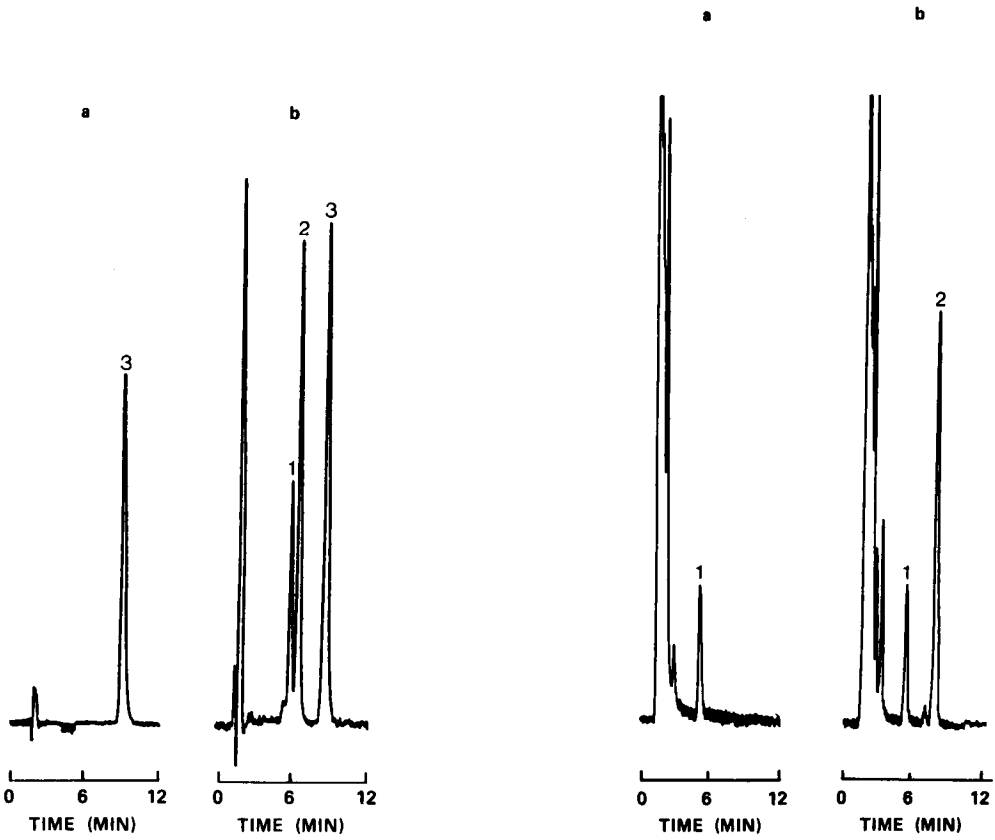

Fig. 3. Isocratic HPLC of laudanosine and THP in dog plasma: (a) extract of pre-dose plasma; (b) extract of post-dose plasma. Conditions: 250 × 4.6 mm Partisil 10 SCX column kept at 50°C; mobile phase: acetonitrile—0.012 *M* sodium sulphate (60:40) in 0.005 *M* sulphuric acid; flow-rate 2 ml/min. Fluorescence detection 280 nm (ex), 320 nm (em). Peaks: 1 = THP, 0.1 μg/ml; 2 = laudanosine, 0.2 μg/ml; 3 = internal standard (N-methyl laudanosine), 1.0 μg/ml.

Fig. 4. Isocratic HPLC of atracurium in human plasma: (a) extract of pre-dose plasma; (b) extract of post-dose plasma. Conditions: Spherisorb CN 250 × 4.6 mm column kept at 50°C; mobile phase: acetonitrile—0.02 *M* sodium sulphate (60:40) in 0.005 *M* sulphuric acid; flow-rate 2 ml/min. Fluorescence detection 280 nm (ex), 320 nm (em). Peaks: 1 = *d*-tubocurarine, 5.0 μg/ml; 2 = atracurium, 1.0 μg/ml.

cartridge with 0.6 ml acetonitrile- 0.012 *M* sodium sulphate (60:40) in 0.005 *M* sulphuric acid. The HPLC mobile phase was acetonitrile—0.012 *M* sodium sulphate (60:40) in 0.005 *M* sulphuric acid, at a flow-rate of 2 ml/min. Extracts of control dog plasma showed no interfering peaks and THP and laudanosine were resolved (Fig. 3a and b).

Atracurium alone in human plasma

For atracurium alone, in human (or dog) plasma 0.2 ml plasma was spiked with 1 μg of *d*-tubocurarine in place of N-methyl laudanosine. Extraction was otherwise unchanged. HPLC analysis was accomplished using a Spherisorb CN (nitrile phase, 5 μm) column, 250 × 4.6 mm (Hichrom) maintained at 50°C and a mobile phase of acetonitrile—0.02 *M* sodium sulphate (60:40) in 0.005 *M*

sulphuric acid at a flow-rate of 2 ml/min. This stationary phase gave a better efficiency for atracurium (N = 20 000 theoretical plates/m, measured with atracurium), than did ion-exchange phases, and made an increase in sensitivity possible. No interfering peaks in plasma were seen (Fig. 4a and b). Alternatively, the HPLC conditions of Neill and Jones [5] can be used.

DISCUSSION

These analytical techniques have several advantages over the previously published method [5]. The carrier compound is not now required, and its replacement with N-methyl laudanosine as a true internal standard has given an increase in robustness and reproducibility. Atracurium and its derivatives are analysed in a single chromatographic run. Because the gradient consists of a simple increase in salt concentration no lengthy re-equilibration is required and column life and performance are not affected. If Aristar reagents are used in the mobile phases UV detection can be employed without large changes in baseline.

The methods are suitable for the analysis of multiple plasma samples from the same individual, since only 0.2 ml or less is required for a sensitivity of about 0.01 μg/ml for atracurium and 0.005 μg/ml for laudanosine or THP. Miniaturisation of the method is partly a consequence of using Bond-Elut 100-mg cartridges; these proved robust in use and no batch-to-batch variation was seen. The acidification step ensured that atracurium in plasma was kept at its most stable pH, so that serial samples could be stored and up to fifty extractions could be made at a time (by the linking of Vac-Elut manifolds).

The extract was very clean and large volumes (up to the whole of the sample) could be injected without HPLC column deterioration. The extraction method was also used with plasma from anephric and hepatic failure patients, and with 0.2 ml urine without problems of recovery or interfering peaks in chromatograms. Extracts were stable for at least three days at room temperature and were thus amenable to automatic analysis and the reinjection of suspect samples.

In conclusion the method has shown itself reproducible and of more than sufficient sensitivity in the analysis of serial samples from human volunteer clinical experiments and animal studies.

ACKNOWLEDGEMENTS

The enthusiasm and technical expertise of Miss T.A. Dopson is gratefully acknowledged in the development and application of this analysis.

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