Journal of Chromatography, 274 (1983) 409—412
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1601

Note

Determination of atracurium besylate in human plasma

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(First received October 11th, 1982; revised manuscript received December 7th, 1982)

Atracurium besylate, 2,2′-(3,11 dioxo-4,10-dioxatridecylene)-bis-[6,7 dimethoxy-1(3,4 dimethoxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinolinium] dibenzene sulphonate (Fig. 1) has been developed as a neuromuscular blocking agent which undergoes non-enzymic decomposition under physiological conditions, thus having a predictable duration of action [1]. Earlier studies had shown that the compound does undergo a facile decomposition at pH 7.4 by Hofmann elimination [2]. Analysis of atracurium therefore necessitated the

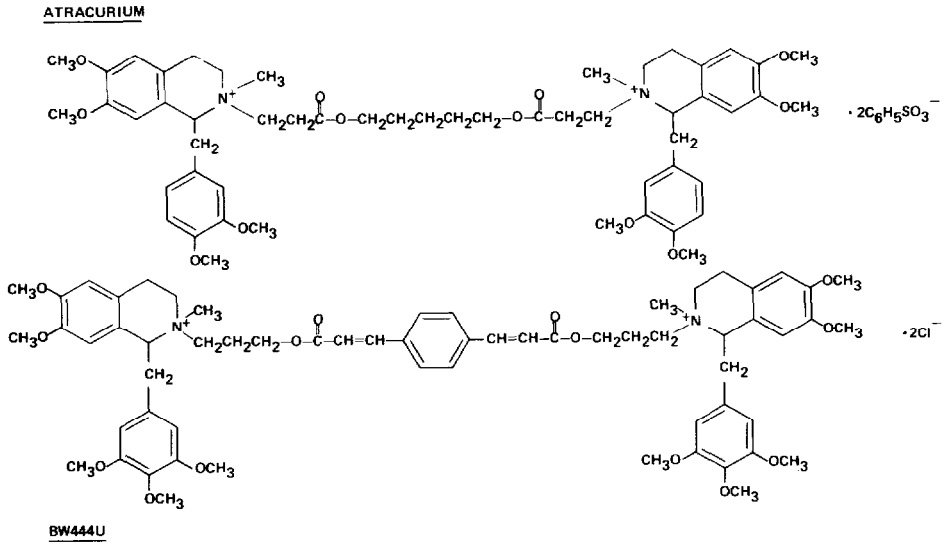

Fig. 1. Chemical structures of atracurium and BW444U.

0378-4347/83/$03.00

use of rapid sample preparation and relatively mild chromatographic conditions if the integrity of the molecule were to be preserved.

High-performance liquid chromatography (HPLC) was the method of choice, since this was readily applicable for fairly high molecular weight ionized compounds. Reversed-phase chromatography with a variety of mobile phases including ion-pairing reagents was investigated, but efficiency and capacity were low so a method was developed using cation-exchange chromatography.

EXPERIMENTAL

Chemicals

Atracurium besylate and compound BW444U were supplied by the Wellcome Foundation (Dartford, Great Britain). Acetonitrile, HPLC grade, was obtained from Rathburn Chemicals (Walkerburn, Great Britain); sodium sulphate and sulphuric acid, both AnalaR grade, were obtained from BDH Chemicals (Poole, Great Britain).

Equipment

Sep-Pak C_{18} cartridges (Waters Assoc., Northwich, Great Britain) were prewetted before use by successive passage of 2 ml acetonitrile, 2 ml acetonitrile—sulphuric acid, pH 2 (80:20) and 5 ml distilled water.

An Eppendorf centrifuge, Model 5414 was used for the rapid separation of blood samples.

HPLC equipment consisted of a Hewlett-Packard 1084B chromatograph linked to a Hitachi 650-LC fluorescence detector. The column used was 250 × 4.9 mm Partisil 10 SCX, particle size 10 μm, packed by Hichrom (Woodley, Great Britain).

Preparation of standards for calibration curve

Samples of atracurium were prepared at 0.01, 0.1 and 1 mg/ml in dilute sulphuric acid (pH 3.2) containing BW444U as a carrier at 5 μg/ml and were used to spike plasma for calibration purposes. An additional quantity of 2.5 μg BW444U was added to each ml of plasma to suppress adsorption.

Replicate 2-ml portions of the spiked plasma were dispensed into glass vials containing 200 μl of sulphuric acid (pH 1) and immediately frozen in solid carbon dioxide. These standards were prepared on the same day as blood samples were obtained.

Preparation of samples

Fresh heparinised blood samples (10 ml) were centrifuged for approximately 30 sec in 1.5-ml micro-test tubes. Plasma was dispensed as two 2-ml portions into glass vials containing 200 μl of sulphuric acid (pH 1) and 5 μg of BW444U and frozen as above.

Extraction of samples and standards

Plasma samples were extracted within 24 h of freezing. They were gently thawed and immediately centrifuged in the Eppendorf for approximately 30 sec to remove any precipitated protein. The supernatant (2 ml) was applied

through a Luer-tip syringe to a Sep-Pak cartridge and then washed with 1 ml sulphuric acid (pH 1) followed by 0.4 ml acetonitrile—sulphuric acid, pH 2 (80:20). These effluents were discarded. The retained atracurium was eluted with 1 ml acetonitrile—sulphuric acid, pH 2 (80:20) into a micro test tube. All samples were centrifuged for approximately 1 min to remove any precipitate which might have formed, 0.8 ml of the supernatant was transferred to a glass vial for HPLC and tightly capped. Extracted samples were found to be stable for overnight runs and could be kept in a refrigerator for several days.

HPLC conditions

The mobile phase was acetonitrile—sulphuric acid, pH 2 (50:50) containing 0.03 *M* sodium sulphate. At a flow-rate of 3.5 ml/min atracurium eluted in approximately 4 min with an oven temperature of 60°C. Normally 20 μl were injected.

Fluorimeter settings were 280 nm and 320 nm for excitation and emission, respectively. Under these conditions BW444U does not fluoresce and does not appear on the chromatogram.

RESULTS AND DISCUSSION

Atracurium is, by design, difficult to handle under physiological conditions, so the assay was developed to minimise possible degradation during sample processing. The small amount of acid added to plasma samples was sufficient to take the pH to approximately 6 without causing excessive protein precipitation. BW444U was added because it is sufficiently similar in structure to atracurium to reduce the extent of adsorption of atracurium on glass vials used for storage. These should preferably be of neutral glass, since problems of breakdown with solutions inadvertently stored in soda glass vials have occurred. Even at −20°C, Hofmann elimination can occur under mild basic conditions, so the 24-h limit on sample storage before extraction was established as a working practice.

No suitable internal standard was available for this assay. This caused no problems in practice but to allow for any extraction variability, duplicate samples were used. The coefficient of variation of the extraction and assay of atracurium from spiked control plasma was typically 10% over the range 0.05—10 μg/ml (3% at 3 μg/ml, 16% at 0.3 μg/ml and 19% at 0.05 μg/ml; $n=6$).

Fig. 2 shows a typical HPLC trace for extracted standards. Linearity was observed over the range 0.05—10 μg/ml; $r=0.9983$ for extracted standards; $r=0.9999$ for unextracted standards. Extraction efficiency was found to be approximately 70% by comparison with unextracted standards. Peak heights were measured as this was found to be more reliable than peak area integration, especially at low concentrations. About 1 ng is the limit of detection on column.

With several samples to extract, we found it more convenient to set up a succession of Sep-Pak cartridges inserted through pierced "Suba-seal" stoppers into tubes under vacuum suction. In this way a steady negative pressure could be applied.

In general this method was easy, convenient and reliable over many months of use. Occasional topping up of the column with, if necessary, a slight adjust-

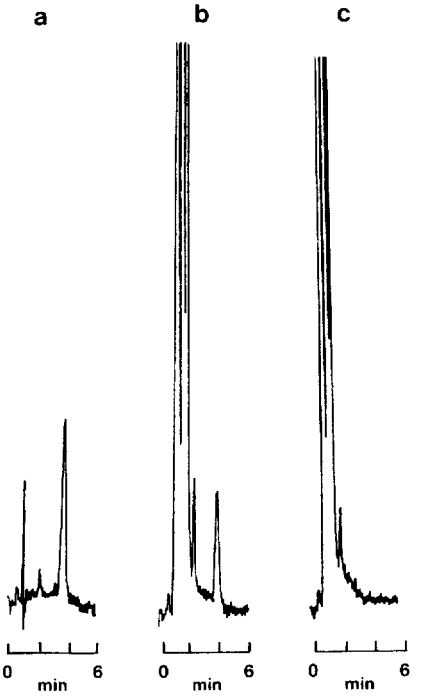

Fig. 2. Sample traces from HPLC. (a) Unextracted standard equivalent to 300 ng/ml atracurium; (b) extracted plasma containing 300 ng/ml atracurium; (c) extracted plasma blank.

ment of the inorganic salt content was all that was required. With small injection volumes we were able to recycle the mobile phase during a run, since this did not cause a significant rise in the baseline and ensured steady run conditions. This method allowed separation of atracurium from all its known breakdown products except for the monoquaternary compound which was present only as a very minor impurity.

The wide range of standards was necessary to measure plasma concentrations of atracurium in man following the intravenous administration of a paralysing dose [3]. The assay has been found to be adequate for all the clinical studies so far undertaken, which at present exceeds 500 samples.

ACKNOWLEDGEMENTS

Our thanks are due to Ian Pratt and Paul Johnson for their expert technical assistance.

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