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Determination of atracurium and laudanosine in human plasma by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method coupled with fluorometric detection has been developed for the determination of atracurium and its major end-product laudanosine in human plasma. The method enables good separation of atracurium from its metabolites after direct precipitation of plasma proteins. The assay is sensitive, reproducible and linear for atracurium concentrations ranging from 31.25 to 8000 ng/ml. In a clinical setting, drugs commonly administered during anesthesia did not interfere with the assay. This method provides a simple and time-saving alternative to existing methods.

INTRODUCTION

Atracurium besylate, 2,2′-[1,5-pentanediylbis[oxy-(3-oxo-3,1-propanediyl)]]bis[1-[(3,4-dimethoyxyphenyl)methyl]-1,2,3,4-tetrahydro-6,7-dimethoxy-2-methylisoquinolinium] dibenzenesulphonate (Fig. 1) is an intermediate-acting non-depolarizing neuromuscular blocker. Atracurium has a unique feature in that its elimination is independent of both hepatic [1] and renal [2]

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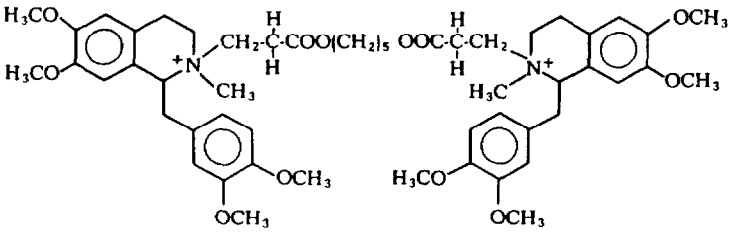
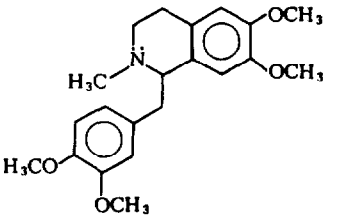


ATRACURIUM



LAUDANOSINE

Fig. 1. Structures of atracurium and laudanosine, its major end-product.

functions. Following intravenous administration to humans and laboratory animals atracurium is predominantly degraded, under physiological temperature and pH, by a Hofmann process to laudanosine (Fig. 1) and pentamethylene-1,5 diacrylate [3,4]. A less important route of metabolism involves the enzymatic hydrolysis of atracurium by plasma esterases leading to the formation of a monoquaternary alcohol and acid, which are eventually degraded to laudanosine by a Hofmann reaction [5,6]. In view of the low potency of atracurium metabolites, it is unlikely that their contribution to the neuromuscular blocking effect is of pharmacological importance [7].

Several high-performance liquid chromatographic (HPLC) methods have been reported for the determination of atracurium alone or in combination with its metabolites in human plasma [8–11]. However, these methods often lack sensitivity or require solid-phase extraction, silanization or chemicals not widely available. Recently, Nehmer [12] examined, on a reversed-phase column, the influence of mobile phase composition (acetonitrile proportion, buffer concentration) and pH on the separation of atracurium from its metabolites. His method allowed a good resolution of the parent compound from six possible metabolites; however, concentrations greatly exceeding those expected in a clinical setting were used.

This report described a new HPLC–fluorometric detection assay for the rapid determination of atracurium and its main end-product laudanosine. The method is specific and sensitive enough to enable pharmacokinetic–pharmacodynamic modeling. Furthermore, it requires no extraction from body fluids

and uses conventional instruments and chemicals making this method suitable for drug monitoring.

EXPERIMENTAL

Chemicals and reagents

Solvents of HPLC grade and all other chemicals of analytical grade were purchased from Fisher Scientific (Montreal, Canada). Atracurium besylate was kindly supplied by Wellcome Research Labs. (Beckenham, U.K.). Laudanosine and verapamil hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.).

Instrumentation

The HPLC system consisted of two Constametric III pumps (LDC Milton Roy, Riviera Beach, FL, U.S.A.), a Model 1601 gradient controller (LDC Milton Roy), a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) and a Model RF 535 fluorescence detector (Shimadzu, Kyoto, Japan) set at excitation and emission wavelengths of 240 and 320 nm, respectively. The column (10 cm × 4.9 mm I.D.) was packed with 5-μm Spherisorb C_8 (Hichrom, Reading , U.K.). Mobile phase A (acetonitrile–methanol–0.03 *M* dibasic potassium phosphate buffer, 37.5:5:57.5) and B (acetonitrile–methanol–0.1 *M* dibasic potassium phosphate buffer, 37.5:15:47.5) were prepared daily and the final pH was adjusted to 5. Compounds were separated using a linear gradient (from 0 to 100% of mobile phase B over 8 min.). The solvent flow-rate was 1.7 ml/min and the chromatographic system was operated at room temperature.

Standard solutions

Stock solutions of atracurium and laudanosine (1 mg/ml in 0.005 *M* sulphuric acid; pH 2.5) were appropriately diluted in the same acidic medium to obtain working standards. A stock solution of verapamil (internal standard) was prepared at 0.1 mg/ml in distilled water. All solutions were stored at 4 °C. Standard solutions were tested weekly for stability and replaced every month.

Calibration curves

Drug-free plasma for the preparation of spiked samples and standards was always adjusted to pH 4 with 2 *M* sulphuric acid before the addition of atracurium (and laudanosine) to prevent its degradation. A pool of plasma containing 8000 ng/ml atracurium was further diluted with drug-free plasma to give final plasma standards at concentrations ranging from 31.25 to 8000 ng/ml. Similar dilutions were carried out in plasma with laudanosine (from 4000 ng/ml down to 31.25 ng/ml). Calibration curves for atracurium and laudanosine were generated by least-squares regression of the analyte/internal standard peak-height ratio versus standard concentration.

Plasma sample preparation

In a glass tube, 0.25 ml of acidified plasma sample, 10 μl of 0.5 *M* sulphuric acid and 50 μl (250 ng) of internal standard were added sequentially and mixed. Then, 0.6 ml of acetonitrile was added, the sample was vortex-mixed for 1 min and centrifuged for 10 min at 2400 *g*. An aliquot of 50 μl of the supernatant was injected onto the HPLC column.

Recovery

Recovery of atracurium from plasma was assessed with spiked samples at a concentration of 4000 ng/ml ($n=4$). Atracurium and the internal standard were added to plasma before protein precipitation and to the supernatant, after protein precipitation. Recovery estimates were based on a comparison of the atracurium/internal standard peak-height ratios.

Sensitivity

The limit of detection was defined as the sample concentration of an analyte that provides a detector response with a signal-to-noise ratio of at least 3:1. The limit of detection was 20 ng/ml for atracurium and 10 ng/ml for laudanosine using 0.25 ml of plasma.

Accuracy and precision

Drug-free plasma was spiked with atracurium to achieve eight different concentrations ranging from 50 to 2000 ng/ml, and samples were blindly assayed. Intra-assay variability was assessed by replicate measures ($n=4$) on the day of preparation of plasma spiked with the analyte at 250 and 2000 ng/ml. Inter-assay variability was determined by analyzing several samples on different days. The same standards were used to derive the calibration curves.

Stability

The stability of the analyte (atracurium or laudanosine) at concentrations of 250 and 2000 ng/ml in acidified plasma was determined ($n=4$). Plasma samples were assayed on the day of preparation (day 0) and after being stored at $-14\,^{\circ}C$ for 7, 14 and 21 days.

Patient study

Following anesthesia, nine patients scheduled for elective surgery were given atracurium besylate (0.2 mg/kg) as an intravenous bolus. An aliquot of the injected solution was collected for further HPLC analysis. Blood samples (2 ml) were withdrawn at frequent intervals for up to 1 h with heparinized syringes, immediately placed in an ice-water bath for 5 min and then centrifuged for 2 min. The plasma was transferred, acidified to pH 4 with 2 *M* sulphuric acid and frozen in dry ice. Samples were kept at $-14\,^{\circ}C$ and assayed within one week. Informed consent was obtained from all patients before the study.

RESULTS AND DISCUSSION

Representative chromatograms of extracts obtained from drug-free and spiked plasma are presented in Fig. 2. Our method provides chromatograms devoid of interfering endogenous substances. The analysis of a plasma sample taken 3 min after the administration of atracurium to a patient shows good resolution of atracurium from its metabolites. The retention times were 6.2 min for atracurium, 1.4 min for laudanosine and 4 min for verapamil. The peak which occurs shortly after laudanosine (peak 4) is believed to represent the quaternary alcohol derivative (if we extrapolate the retention times of the various metabolites from Nehmer's study [12]). The total HPLC run time for atracurium and its metabolites including reequilibration was less than 10 min.

All calibration curves for the analytes were linear over a wide concentration range. The regression equations for atracurium at low (31.25–500 ng/ml) and high (500–8000 ng/ml) concentrations were $y=0.00116x+0.0123$ ($r^2=0.996$) and $y=0.00104x+0.0749$ ($r^2=0.993$), respectively. For laudanosine concentrations between 31.25 and 4000 ng/ml, the regression equation was $y=0.00441x-0.0702$ ($r^2=0.994$).

Since there is no extraction involved in our method, the loss of analytes could result from either degradation or trapping within the protein precipitate. When atracurium was added before acetonitrile a mean peak-height ratio of

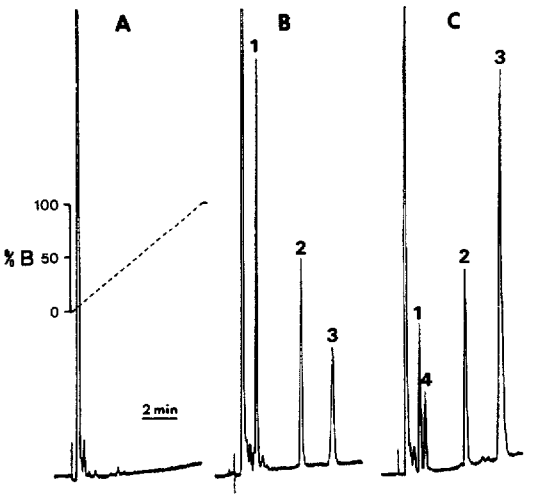


Fig. 2. Chromatographic profiles of (A) drug-free plasma, (B) drug-free plasma spiked with 500 ng/ml laudanosine and atracurium and 250 ng/ml verapamil and (C) patient plasma sample collected 3 min after the administration of 0.2 mg/kg atracurium besylate as an intravenous bolus. The percentage of increase of mobile phase B versus time is shown by the dotted line on the first chromatogram. Peaks: 1 = laudanosine; 2 = verapamil; 3 = atracurium; 4 = monoquaternary alcohol derivative.

3.919 ± 0.127 was obtained compared to 4.084 ± 0.081 when atracurium was added in the supernatant after protein precipitation. The possibilities mentioned above were ruled out since a difference of less than 5% in the peak-height ratio was observed. In addition, there was no adsorption of the analytes on the glass surface.

An accuracy of 103.2 ± 7.7% (mean ± S.D.) was achieved for all investigated atracurium concentrations. The intra-assay variability of the method for atracurium and laudanosine was less than 4% (Table I). The inter-day mean variability was 4.9 and 4.4% for atracurium (Table II) and laudanosine (Table III), respectively.

Concentrations of atracurium in plasma samples stored at −14 °C were stable for up to two weeks (Table IV). However, under similar conditions, there was a reduction of 10% in the concentration of laudanosine in the samples. After three weeks, atracurium and laudanosine concentrations were reduced by approximately 10 and 20%, respectively, in the two samples tested. In the

TABLE I

INTRA-ASSAY VARIABILITY OF THE METHOD IN PLASMA

Analyte	Concentration (ng/ml)	*n*	Peak-height ratio (mean ± S.D.)	Coefficient of variation (%)
Atracurium	250	4	0.293 ± 0.008	2.6
	2000	4	2.226 ± 0.075	3.4
Laudanosine	250	4	1.128 ± 0.023	2.1
	2000	4	8.933 ± 0.152	1.7

TABLE II

ATRACURIUM CALIBRATION CURVES IN PLASMA AND INTER-ASSAY VARIABILITY

Concentration (ng/ml)	*n*	Peak-height ratio (mean ± S.D.)	Coefficient of variation (%)
31.25	5	0.045 ± 0.002	4.3
62.5	6	0.091 ± 0.008	8.6
125	6	0.158 ± 0.013	8.1
250	5	0.294 ± 0.012	4.2
500	4	0.597 ± 0.019	3.1
1000	4	1.132 ± 0.056	4.9
2000	5	2.336 ± 0.095	4.1
4000	4	3.885 ± 0.136	3.5
8000	4	8.533 ± 0.298	3.5

TABLE III

LAUDANOSINE CALIBRATION CURVE IN PLASMA AND INTER-ASSAY VARIABILITY

Concentration (ng/ml)	n	Peak-height ratio (mean ± S.D.)	Coefficient of variation (%)
31.25	4	0.125 ± 0.001	0.8
62.5	8	0.275 ± 0.013	4.7
125	7	0.558 ± 0.028	5.1
250	7	1.101 ± 0.027	2.5
500	7	2.156 ± 0.121	5.6
1000	8	4.365 ± 0.215	4.9
2000	8	8.872 ± 0.573	6.5
4000	8	17.615 ± 0.889	5.0

TABLE IV

STABILITY OF ATRACURIUM AND LAUDANOSINE IN PLASMA SAMPLES STORED AT −14°C

Concentration added (ng/ml)	Time (days)	n	Concentration found (mean ± S.D.) (ng/ml)	
			Atracurium	Laudanosine
250	0	4	241.7 ± 11.4	255.1 ± 5.3
	7	4	265.3 ± 11.9	235.1 ± 11.1
	14	4	242.5 ± 6.5	229.2 ± 8.6
	21	2	218.9 ± 31.7	213.6 ± 24.1
2000	0	4	2174.1 ± 44.1	2148.7 ± 147.9
	7	4	2165.6 ± 118.7	2013.2 ± 34.2
	14	4	2064.7 ± 72.4	1958.6 ± 74.3
	21	2	1881.4 ± 272.3	1614.2 ± 79.2

clinical study, acidified plasma samples were always assayed within one week after blood withdrawal.

This assay was directly applied to the pharmacokinetic study of atracurium in patients undergoing surgery. There was no interference from drugs commonly co-administered during anesthesia. Special attention was paid to sample handling. Merrett et al. [13] showed that the half-life of atracurium in plasma decreased from 15.5 h at 0°C to 18 min at 37°C. Acidification of plasma samples considerably reduced the degradation of atracurium, but at a pH of about 3 or less ester hydrolysis could take place. Therefore, blood samples should be immediately chilled (5 min in an ice water bath), then centrifugation can be carried out at 4°C without noticeable degradation of atracurium. If the first

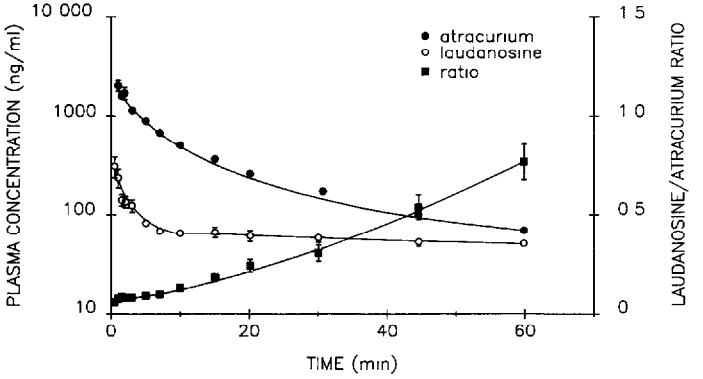


Fig. 3. Plasma concentration versus time curves for atracurium and laudanosine following intravenous administration of 0.2 mg/kg atracurium besylate in nine patients (mean ± standard error of the mean). For each time point, the mean concentration ratio of laudanosine/atracurium was calculated.

step is omitted, the temperature of the samples remains elevated and degradation occurs.

The mean plasma concentration versus time curves for atracurium and laudanosine are shown in Fig. 3. In the first 10-min blood samples, the laudanosine/atracurium concentration ratio is constant, corresponding to approximately 5% degradation of atracurium. Up to 2% of this degradation is attributable to the laudanosine already present in the injectable solution. The remaining degradation is accounted for by the ex vivo procedure (from blood sampling to plasma acidification). A significant increase in the laudanosine/atracurium concentration ratio is noticeable only 10 min after the administration of atracurium and would correspond to the in vivo formation of laudanosine.

CONCLUSION

In summary, the advantage of this method is that it requires no extraction, offers a major improvement in ease and speed of execution without sacrifice of precision, sensitivity or selectivity. Finally, atracurium and laudanosine could be quantified as efficiently in much smaller blood samples than those used in our experiments.

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