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Note

Measurement of D-tubocurarine chloride in human urine using solid-phase extraction and reversed-phase high-performance liquid chromatography with ultraviolet detection

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D-Tubocurarine chloride (DTC) is a neuromuscular blocking drug which is a benzylisoquinoline quaternary ammonium compound [1]. It is used in the operating room and intensive care unit to produce skeletal muscle relaxation. Drugs such as DTC were once thought to pass through the body unmetabolized [1,2]. Recent clinical studies, however, indicate that in certain pathological states, and in the presence of drugs such as phenytoin and phenobarbital, which induce liver metabolizing enzymes, the requirement for neuromuscular blockers is increased [3,4]. Whether this increased dose requirement is due to increased metabolic clearance and elimination is unclear.

The presence of two hydroxyl groups on the DTC molecule lends itself to conjugation, resulting in non-polar metabolites which can be excreted in the urine. The potential for DTC to be metabolized is confirmed by the fact that not all of the injected parent compound can be accounted for in the bile and urine at 24–48 h after dose [5–7]. The main elimination route of the parent drug and possibly the metabolites is in the urine [2].

Previous assays for DTC in urine have utilized radiolabelled compounds [6,7], radioimmunoassay (RIA) [8], fluorimetry [6] and paper chromatography followed by densitometric detection [7]. Fluorescence and RIA meth-

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ods, though sensitive, lack specificity in that the assay detects the parent compound as well as other structurally similar compounds [8]. The use of radioactive compounds is also fraught with the problem that simple radioactive counting will measure the radioactivity of the parent and any radioactive metabolites. Furthermore, the technique is not practical for human experimentation. Cohen et al. [5], using [^{3}H]DTC and ion-pair thin-layer and paper chromatography, found no evidence of DTC metabolites in the urine.

In order to examine the role of enzyme induction following the onset of pathological states and during the use of drugs such as phenytoin, and in order to accurately quantitate the increased drug metabolism, a sensitive and specific assay is required for the detection of the parent compound in urine. Recently, several high-performance liquid chromatographic (HPLC) assays for the measurement of plasma DTC levels have been reported [9–11]. One of these [11] uses simple solid-phase extraction methods for sample clean-up followed by reversed-phase HPLC using an isocratic ion-paired mobile phase. Detection of 25 ng/ml has been reported with this method. The success of the HPLC method for plasma DTC encouraged us to try and develop a similar system for the determination of DTC in human urine.

This paper describes an assay for the detection of DTC in urine using a solid-phase extraction (SPE) silica gel procedure followed by reversed-phase HPLC, and provides preliminary evidence that DTC may be conjugated to glucuronic acid, but not to sulfate.

EXPERIMENTAL

Chemicals and reagents

DTC and the internal standard (I.S.), D-chondocurarine, were purchased from Sigma (St. Louis, MO, U.S.A.) and E.R. Squibb and Sons (Nutley, NJ, U.S.A.), respectively, and used as received. HPLC-grade methanol, reagent-grade orthophosphoric acid, dibutylamine, ethyl acetate and sodium hydroxide were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). HPLC-grade water was produced in an Organopure water system (Millipore, Milford, MA, U.S.A.).

Preparation of standards and samples

Stock solutions of DTC (10 μg/ml) and the I.S. (2.8 μg/ml) were prepared in water. These solutions were stable for at least four months. Lower concentrations were prepared by serial dilution from these stock solutions. Standards were prepared by adding known amounts of DTC to 2 ml of drug-free urine.

Seven patients, having no systemic pathology and not receiving any enzyme-inducing drugs, each received a single dose of 0.5 mg/kg DTC intravenously as a bolus during general anesthesia for surgical procedures. Other drugs administered as supplements to general anesthesia included nitrous oxide with oxygen and intravenous narcotics (fentanyl and/or morphine). The urine that

was excreted over the following 24-h period was collected by an indwelling catheter and urine bag. The urine was refrigerated for later analysis.

Since it was likely that any metabolites present would be in small concentrations, a 40-ml aliquot of each patient urine sample was concentrated by lyophilization. The dried samples were reconstituted in 10 ml of 0.1 *M* acetate buffer (pH 5.0) and centrifuged at 5000 *g* for 15 min. The supernatant was divided into three 3-ml portions. The first, serving as a control, was incubated in 10 μl of the acetate buffer. The second was incubated with 10 μl of the enzymes glucuronidase (500 U/μl) and sulfatase (26.4 U/μl), respectively (Sigma). The third sample was incubated with the same enzyme solution in the presence of 10 m*M* G saccharo-1,4-lactone (Sigma), an inhibitor of β-glucuronidase. Each sample was prepared in triplicate.

The treatment of the urine samples with β-glucuronidase and sulfatase served to convert any glucuronidated or sulfated metabolites back to the parent compound. Treatment of the third aliquot with the same enzymes, but in the presence of the β-glucuronidase inhibitor, meant that only the sulfatase was active. All samples were incubated overnight at 37°C. The incubation was halted by freezing the samples. The frozen samples were then lyophilized and stored at −20°C. Prior to HPLC analysis each of the samples was reconstituted in 2 ml of water. A paired *t*-test was used to evaluate changes in post-enzyme-treated DTC concentrations with control sample concentrations.

Extraction

SPE of DTC and I.S. was carried out using 100 mg silica gel SPE cartridges and a vacuum manifold (Analytichem International, Harbor City, CA, U.S.A.). After the addition of 28 μg of I.S. (10 μl of the stock solution) to the reconstituted urine samples, 200 μl of each were applied to an SPE cartridge that had been prewashed successively with 2 ml each of ethyl acetate, methanol and water. The cartridges were then rinsed with 2 ml of water followed by 500 μl of methanol. The compounds were eluted with two successive 1-ml volumes of the HPLC mobile phase.

For the assay of free DTC in urine at clinical levels, 600 ng of the I.S. (10 μl of a 60 ng/μl solution) were added to 1 ml of urine and the entire sample was extracted as above.

The recovery of DTC and the I.S. was determined by injecting a known amount of a standard solution and comparing the resulting peak area to the peak area from an injection of an extracted sample.

Chromatography

The HPLC system consisted of Beckman Model 110B pumps with a Model 420 gradient controller (Beckman Instruments, San Ramon, CA, U.S.A.). Samples were introduced through a Rheodyne (Cotati, CA, U.S.A.) 7125 injector with a 200-μl loop and detected with a Waters Model 481 variable-wave-

length ultraviolet (UV) detector (Milford, MA, U.S.A.) operated at 204 nm. Reversed-phase ion-pair chromatography was carried out using a Beckman ODS-IP column, 150 mm × 4.6 mm I.D. (5 μm particle size). A guard column (20 mm × 4.6 mm I.D.) packed with 10 μm particle size C_{18} (Rainin, Woburn, MA, U.S.A.) was used prior to the analytical column. Isocratic elution was employed using water–methanol–dibutylamine phosphate (79.1:19.8:0.1, v/v) as a mobile phase. The dibutylamine phosphate stock solution was prepared by adding 32 g of dibutylamine to 250 ml of concentrated orthophosphoric acid. The mobile phase was adjusted to pH 2.5 with 1 *M* sodium hydroxide solution. A flow-rate of 1.5 ml/min was used for all analysis.

RESULTS AND DISCUSSION

C_{18} reversed-phase SPE columns have been used for the extraction of DTC and other related quaternary ammonium anesthetics from human plasma [11]. Unfortunately they proved to be of little use for the clean-up of urine samples. Determination of DTC levels from the HPLC profiles following extraction, with the HPLC mobile phase, from C_{18} SPE cartridges, was impossible because of the high levels of endogenous materials remaining in the extracts (see Fig. 1A). Work on the separation of organic amines on silica gel HPLC columns with reversed-phase solvents [12] led us to investigate the potential utility of silica cartridges for the SPE of DTC and the I.S. from urine. Fig. 1A and B show chromatograms resulting from the extraction of 1 ml of drug-free urine on both a C_{18} and a silica SPE cartridge using the HPLC mobile phase as the eluent.

The recovery of DTC from the silica SPE cartridges at concentrations of 0.20, 0.60 and 6.0 μg/ml was 97.6, 93.7 and 97.0%, respectively ($n=6$). The recovery of the I.S. was dependent on the volume of methanol used as a wash solvent. While 2 ml of methanol resulted in an almost total loss of the compound, a 1-ml wash yielded a 92.7% ± 15% recovery (mean ± S.D., $n=11$) for 2.8 μg/ml. Further experimentation showed that a 500-μl wash, while not improving the recovery any, gave better intra-assay reproducibility (±4.5%, $n=6$).

Fig. 1B and C show the chromatograms of a drug-free urine and a 50 ng/ml urine standard, respectively, assayed for DTC after silica gel SPE. The limit of detection for this method was 15 ng/ml (signal-to-noise ratio of 4). The removal of endogenous materials from the extracts is the limiting factor in improving the sensitivity of the assay.

Intra-assay accuracy and precision data over three orders of magnitude are presented in Table I. Linear regression analysis of standard curves over the two operating ranges shows a correlation (r^2) of 0.9997 for the clinical levels (25–1000 ng/ml) and a correlation of 0.9990 for levels used in the metabolism (clinical) study (5–50 μg/ml).

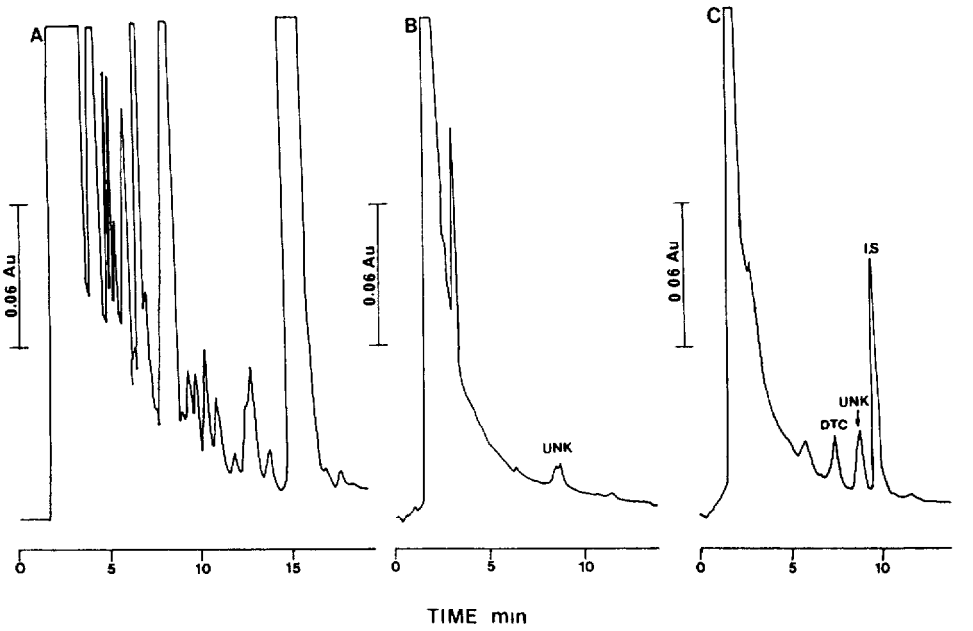

Fig. 1. Chromatograms of (A) drug-free urine after C_{18} SPE extraction, (B) drug-free urine after silica gel SPE extraction and (C) urine standard containing 50 ng/ml DTC and 300 ng/ml D-chondocurarine (I.S.) after silica gel SPE extraction. Chromatographic conditions as described in the text.

TABLE I

INTRA-ASSAY ACCURACY AND PRECISION FOR URINE D-TUBOCURARINE CHLORIDE ASSAY

DTC added (μg)	DTC measured (mean ± S.D.) (μg)	n	Accuracy[a] (%)	Precision[b] (%)
0.40	0.36 ± 0.02	6	90	5 7
1.20	1.33 ± 0.12	6	111	8.9
12.00	12.00 ± 0.20	4	100	1 6
50.00	49 50 ± 1.60	5	99	2.8

[a]Accuracy = (found/true) × 100.
[b]Precision = coefficient of variation = (S.D./mean) × 100.

Fig. 2A shows the chromatogram of DTC in urine from a patient whose urinary excretion was studied. A representative standard sample is also shown (Fig. 2B). The results of the incubation study for all seven patients are listed in Table II. An increased concentration of DTC was found in patients B.M. and R.K. after treatment with glucuronidase and sulfatase. The concentrations of DTC found in the urine of these patients were 12–13% higher after

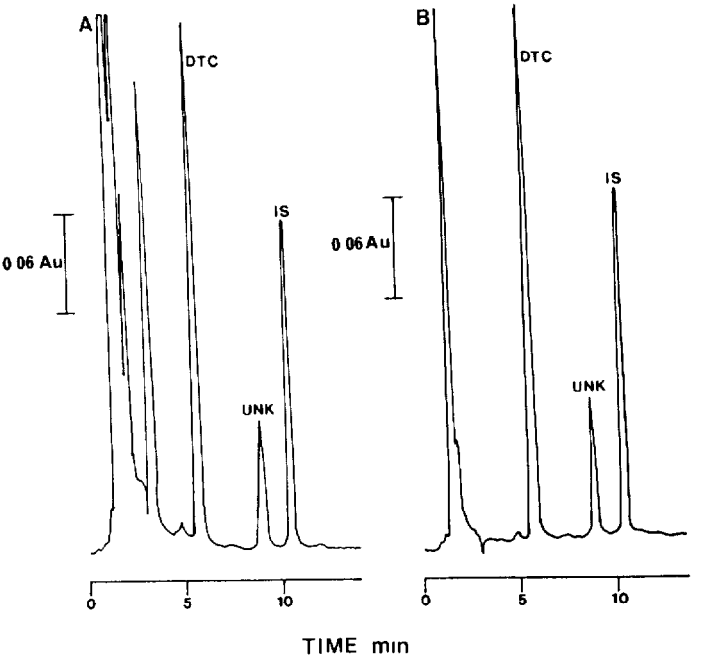

Fig. 2. (A) Chromatogram of urine aliquot obtained over a 24-h period following an intravenous DTC dose of 0.5 mg/kg. The DTC peak is equivalent to 26.1 μg/ml. (B) Chromatogram of urine standard with 25 μg/ml DTC. Both samples contain 14 μg/ml D-chondocurarine (I.S.). Chromatographic conditions as described in the text.

TABLE II

CONCENTRATIONS OF D-TUBOCURARINE IN URINE BEFORE AND AFTER ENZYMATIC HYDROLYSIS

Patient	Concentration (mean ± S.D.) (μg/ml)		
	Control	After glucuronidase and sulfatase	After sulfatase
M.C.	8.51 ± 0.67	8.48 ± 0.76	8 56 ± 0.54
B.M.	12.43 ± 0.75	13.92 ± 1.48[a]	12.29 ± 0.90
P.E.	10.84 ± 0.72	10.84 ± 0.74	10.18 ± 1.08
P.A.	11.34 ± 2.01	12.03 ± 2.98	11.34 ± 2.16
R.K.	15.48 ± 0.86	17.48 ± 2.02[a]	14.40 ± 0.92
L.L.	61.44 ± 2.14	59.27 ± 1.98	60.12 ± 1.59
K.R.	4.49 ± 0.45	4.11 ± 0.51	3.78 ± 0.00

[a]Significantly higher compared to control.

incubation with the combined enzymes. No changes in the DTC concentrations were observed in these or any of the patients' urine after treatment with only sulfatase.

These results provide preliminary evidence that a potential metabolite of DTC is the glucuronic acid conjugate and not the sulfate conjugate. It is important to reiterate that none of the patients had received any preoperative medications that would induce liver enzymes. It is, therefore, possible that in the presence of chronic liver enzyme induction (e.g. by phenytoin) a higher concentration of glucuronidated metabolites may be seen. This hypothesis will be tested in future experiments.

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