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Short Communication

High-performance liquid chromatographic determination of pilocarpine in plasma

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ABSTRACT

A high-performance liquid chromatographic procedure requiring neither derivatization nor complex sample work-up is reported for reproducibly and sensitively determining pilocarpine in plasma. Following stabilization of pilocarpine against *in vitro* hydrolysis using sodium fluoride, plasma samples were extracted and the extracts chromatographed on a 5-μm, low-carbon-load (6%) C_{18} reversed-phase column. The assay was linear between 10 and 300 ng/ml ($r = 0.998$). It had sufficient sensitivity to quantitate pilocarpine at concentrations as low as 10 ng/ml (signal-to-noise ratio ≥ 4) using a 500-μl sample. The assay appears to be the first published specifically for plasma determinations and has proven capable of supporting pharmacokinetics studies of pilocarpine disposition in the anesthetized dog.

INTRODUCTION

Pilocarpine, a cholinergic agonist, has been widely used in clinical ophthalmology as a topical agent for lowering intraocular pressure in patients suffering from glaucoma. It is also known to promote salivation, and oral doses of is nitrate and hydrochloride salts have been administered as an experimental treatment for the xerostomia resulting from drug therapy, salivary gland irradiation or immunological disease [1,2].

While no assays have been published for the determination of pilocarpine in plasma or urine, gas and high-performance liquid chromato-

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graphic (HPLC) assays for its determination in aqueous humor following topical administration are available [3–5]. These methods are selective and sensitive, but each requires derivatization and extensive sample preparation. We report a selective, sensitive assay for pilocarpine in plasma, which requires neither derivatization nor extensive sample work-up, and provides for inactivation of esterases that catalyze pilocarpine degradation in biological samples.

EXPERIMENTAL

Chemicals

Acetonitrile, methanol, methylene chloride and diethyl ether (HPLC grade, J. T. Baker, Phillipsburg, NJ, USA) were used without further purification. Water was purified by distillation followed by treatment with a Milli-Q purification system (Millipore, Bedford, MA, USA) equipped with a 0.45-μm final filter. Mono- and dibasic potassium phosphate salts (HPLC grade, Fisher, Springfield, MA, USA), pilocarpine nitrate, clonidine hydrochloride and sodium fluoride (NaF) (Sigma, St. Louis, MO, USA) and isopilocarpine nitrate (Aldrich, Milwaukee, WI, USA) were all used as received. Pilocarpine nitrate was assayed by HPLC [6] for pilocarpine and its stereoisomer, isopilocarpine, and was determined to be >99% pilocarpine nitrate.

Apparatus

All glassware was borosilicate, silanized with a 1:100 solution of Prosil-28 (PCR, Gainesville, FL, USA) and air-dried. Plasma samples were analyzed using a Varian Model 5020 liquid chromatograph (Varian, Palo Alto, CA, USA) equipped with a Model 441 fixed 214-nm ultraviolet (UV) detector (Waters Assoc., Milford, MA, USA) and a Varian Model 9176 recorder operating at 5 mV full scale. Separations were achieved at 21–23°C on a 150 mm × 4.6 mm I.D. Spherisorb ODS-1 (6% carbon load, particle size 5 μm) reversed-phase column (Alltech, Deerfield, IL, USA) protected by a guard column (1.5 cm × 4.6 mm I.D.) filled with 37-μm C_{18} reversed-phase packing (Corasil, Waters Assoc.). The mobile phase was pumped at 1.2 ml/min (approximately 110 bar).

Mobile phase

The mobile phase was a mixture of 7 m*M* potassium phosphate (pH 4.0)–acetonitrile–methanol (55:30:15). The aqueous component was filtered through at 0.45-μm cellulose filter (Millex HA, Millipore, Bedford, MA, USA) to remove particulate contaminants and degassed under vacuum in an ultrasonic bath to remove dissolved gases prior to mixing with the organic components. It was stored for periods not exceeding one week at 4°C prior to use.

Standard solutions

Aqueous pilocarpine nitrate stock solutions (0.5 mg/ml as pilocarpine base) were used to prepare working solutions each day at concentrations between 1 and 10 μg/ml. Aqueous stock solutions of clonidine · HCl (0.5 mg/ml as clonidine base) were used for daily preparation of an internal standard (I.S.) solution for addition to each analytical sample. Both stock solutions were stored protected from light at 4°C and found to be stable under these conditions for at least one month.

Sample preparation

Blood was collected over an excess of solid NaF (125 mg/ml) to inhibit esterase activity which otherwise quickly destroys pilocarpine [7]. Since the equilibrium solubility of NaF in water at 25°C is about 43 mg/ml, the NaF provided is about a three-fold excess of the amount required to saturate the sample.

A 500-μl volume of plasma sample, an equal volume of I.S. solution and 3.0 ml of methylene chloride were placed in 13 × 100 mm glass tubes, tightly capped with polyethylene extraction tube plugs (Oxford Labs., Foster City, CA, USA), gently shaken for 10 min and centrifuged (2100 *g*, 10 min). The organic phase was transferred to a 15-ml tapered glass centrifuge tube and evaporated to dryness at 40°C under nitrogen. The residue was reconstituted in 150 μl of HCl (0.001 *M*) by vortex-mixing (4 min) and sonication in an ultra-

sonic bath (45 s). The reconstituted sample was washed with diethyl ether (2.0 ml) by vortex-mixing for 2 min, and the phases were separated by centrifugation (2100 *g*, 5 min).

After discarding the ether phase, most residual ether was removed by applying a vacuum to the aqueous phase (10 s). The tubes were again tightly capped and stored overnight at −20°C. When the samples were thawed, each was again briefly exposed to vacuum (10 s) to remove final traces of diethyl ether which were driven from solution by the freeze–thaw process. The final volume of each analytical sample was approximately 110 μl of which 35–100 μl were injected onto the analytical column.

Calibration and reproducibility

Calibration standards were prepared for each set of unknown samples. Standards prepared using drug-free human plasma were indistinguishable from those prepared using dog plasma, and either source should be considered satisfactory. In the present case standards were prepared using between 9 and 45 μl of pilocarpine working solution and enough drug-free human plasma to produce a 600-μl sample volume. After vortex-mixing (15 s), solid NaF (again about 125 mg/ml) was added to inhibit esterase-mediated degradation of pilocarpine, and the sample vortex-mixed (15 s). Samples were then centrifuged (2100 *g*, 10 min) to separate any undissolved NaF, and the supernatant fluid prepared for chromatographic analysis as outlined above.

Five aliquots of each of two plasma standards (30 or 300 ng/ml pilocarpine base) were repeatedly analyzed on the same day to determine intra-day variability of the assay. Similar samples were stored frozen at −20°C and analyzed on five separate days to determine inter-day variability. All such samples contained an excess of solid NaF, like the calibration standards which were similarly preserved.

In vivo study

Analysis of plasma from a singly beagle dog, representative of a group of six dogs administered pilocarpine intravenously, is illustrative. Complete pharmacokinetic details are published elsewhere [8]. This dog (dog No. 2 in ref. 8, age 2 years, weight 8.4 kg, Marshall Farms, North Rose, NY, USA) was initially anesthetized with sodium pentobarbital (Fort Dodge, Fort Dodge, IA, USA, 64 mg/ml, 35–50 mg/kg) and subsequently maintained at a level of anesthesia sufficient to prevent skeletal muscle movements (other than those associated with ventilation) by means of a constant-rate pentobarbital infusion (5.8 mg/ml, 30 mg/h). A patent airway was maintained by a 7-mm cuffed endotrachial tube. Heparinized catheters were placed in both the left cephalic and saphenous veins to enable blood sampling and fluid replacement. The animal was allowed to stabilize for a period of 30–45 min during which time it was monitored for changes in breathing, heart rate and body temperature. A constant-rate intravenous infusion of an aqueous solution of pilocarpine nitrate (240 μg/kg/h base equivalent) into the left cephalic vein was then begun and continued for a period of 2.5 h during which time an infusion of normal saline was provided to maintain hematocrit close to baseline. Heparinized blood samples were drawn every 15–30 min from the right cephalic vein into glass tubes containing NaF and processed as detailed above. The sample was gently agitated, centrifuged at high speed (3 min, 13 000 *g*), and the plasma transferred to polyethylene tubes containing an additional 200 mg of solid NaF. The tubes were immediately sealed with tight-fitting polyethylene caps and stored at −20°C until assay.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms of a drug-free human plasma sample and a plasma sample from the dog taken approximately 1 min following cessation of the 2.5-h intravenous infusion (pilocarpine plasma concentration = 115 ng/ml). Retention times for pilocarpine and isopilocarpine were both 7.2 min while the clonidine internal standard was retained for 12 min. Both were completely resolved from the pentobarbital anesthetic peak at approximately 4.5 min. The 6%

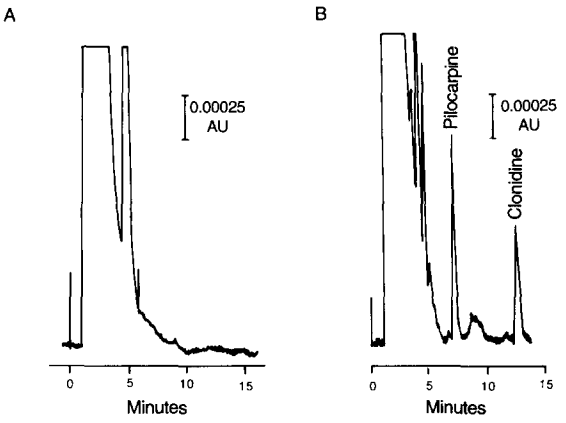

Fig. 1. Chromatogram of extracts of (A) drug-free human plasma and (B) plasma from an anesthetized beagle dog obtained 1 min following cessation of a 2.5-h constant-rate intravenous infusion (240 μg/kg/h); pilocarpine concentration = 115 ng/ml.

carbon load of the analytical column enhances the sensitivity of the assay by reducing the retention time of pilocarpine and sharpening peaks, relative to what was observed with higher-carbon-load columns (*e.g.* Waters μBondapak C_{18}).

The diethyl ether wash used in the sample work-up was effective for removing lipid contaminants. Variability of analyses decreased if the ether-washed extracts were stored frozen overnight, then analyzed after thawing and briefly applying vacuum (to remove residual ether) the following day. Spurious peaks developed if frozen extracts were allowed to remain frozen for periods in excess of 24 h.

Plasma samples containing 300 ng/ml and preserved with NaF lost $< 10\%$ of their pilocarpine when stored frozen –20°C for three months. The minimum detectable concentration of pilocarpine in plasma was found to be 5 ng/ml (signal-to-noise ratio = 2:1) and the lowest quantifiable concentration was 10 ng/ml (signal-to-noise ≥ 4). Calibration curves of peak-height ratio for pilocarpine to internal standard in either dog or human plasma were linear over the concentration range 10–300 ng/ml ($r = 0.998$).

The inter-day coefficient of variation was 2.7% at 300 ng/ml and 4.9% at 30 ng/ml ($n = 5$). Similarly, the intra-day coefficients of variation were 1.7 and 4.3% for the 300 and 30 ng/ml samples, respectively. Overall recovery of pilocarpine was greater than 90% at all concentrations studied. The mean accuracy of the assay was found to be 98% at 300 ng/ml ($n = 5$) and 96% at 30 ng/ml ($n = 6$).

Detailed investigations [8,9] of the pharmacokinetics and pharmacodynamics of pilocarpine in the anesthetized dog using both intravenous infusion and buccal absorption inputs have shown that pharmacokinetically useful levels of pilocarpine can be accurately quantified in plasma using this analytical procedure.

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