Determination of Bethanidine in Plasma by Liquid-Chromatography with a Microbore Reversed-Phase Column

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In this novel method for quantifying bethanidine in plasma, after a multi-step extraction of bethanidine and internal standard from 2.0 mL of plasma, the drugs are separated on a "microbore" C₁₈ reversed-phase column and quantified by their ultraviolet absorbance at 210 nm. The isocratic chromatographic separation takes about 15 min with use of an ion-pairing regent in the mobile phase (acetate buffer/acetonitrile, 9/1 by vol) and a flow rate of 0.25 mL/min. Sensitivity is increased relative to conventional columns, and solvent consumption is reduced by 90%. The standard curve is linear to at least 5 mg/L, and the detection limit is 0.02 mg/L. The within-run precision of the method is excellent (CV 4%) at a midrange concentration of 1.25 mg/L.

Additional Keyphrases: drug assay · tricyclic antidepressants · ventricular tachycardia and fibrillation · cardioactive drugs

Bethanidine (N-benzyl-N",N"-dimethylguanidine) is an old drug for which there may be a new indication. Originally investigated as an antihypertensive drug in the 1960s (1), it never enjoyed widespread use. Other workers noted that bethanidine was effective in suppressing ventricular tachycardia and fibrillation (2); however, because of adverse side effects (persistent orthostatic hypotension), further clinical studies were abandoned. After it was learned that certain tricyclic drugs given in doses well below that for antidepressant efficacy were effective antagonists of bethanidine's hypotensive action (3), Bacaner and Benditt (4) proposed that bethanidine combined with a tricyclic may be an effective oral means of preventing ventricular fibrillation in nonhospitalized patients, a major cause of death. In their recent clinical trial, this approach was used on a series of 23 patients whose condition could not be controlled with any combination of other antiarrhythmic drugs: 18 patients (78%) had complete suppression of spontaneous or electrophysiological inducible tachyarrhythmias. After it was decided to expand these clinical studies, we began the work reported here, to develop a sensitive and specific assay for bethanidine in plasma by reversed-phase, "high-performance" liquid chromatography (HPLC).

Materials and Methods

Samples. Blood drawn into green-stopper (heparin) Vacutainer Tubes (Becton Dickinson, Rutherford, NJ 07070) was centrifuged within 2 h of venipuncture and the plasma stored at -20 °C until assay. Distilled water shaken in these tubes and then carried through the entire procedure gave no extraneous peaks in the chromatogram. Drug-supplemented plasma standards treated similarly and frozen for as long as two weeks were analyzed, with results quantitatively identical to those for freshly prepared plasma standards. We thus concluded that the sampling procedure had no adverse effect on the analysis.

Chemicals. "Distilled in glass" acetonitrile and chloroform from Burdick and Jackson were purchased from BODMAN Chemicals, Media, PA 19063; the distilled water used in all HPLC work was obtained with the "Milli-Q" purification system (Millipore Corp., Bedford, MA 01730). All remaining chemicals used in buffer preparation and the extraction were ACS reagent grade (Fisher Scientific, Fairlawn, NJ 07410).

Standards. Bethanidine hemisulfate was a gift from the manufacturer (A. H. Robins, Richmond, VA 23220). The internal standard, N-methylphenethylamine, was from Aldrich Chemical Co., Milwaukee, WI 53201. Stock solutions of each compound, containing 1 g of the free amine per liter, were prepared in 10 mmol/L HCl and stored at 4 °C.

The 5 mg/L working standard of bethanidine was prepared by adding 50 μL of the stock solution to 10 mL of drug-free pooled plasma. Serial dilutions of this standard, prepared with the same drug-free pooled plasma, were used to construct the standard curve.

The working internal standard solution, 20 mg/L, was prepared daily by diluting 1.0 mL of the stock to 50 mL with distilled water.

Reagents. Sodium acetate trihydrate buffer, 20 mmol/L, containing 0.5 g of disodium EDTA and 2.0 g of tetramethylammonium chloride per liter, was adjusted to pH 5.0 with glacial acetic acid. The working mobile phase (acetate buffer/acetonitrile, 9/1 by vol) was filtered through a 0.22-μm pore-size filter (type GS; Millipore Corp.) and purged with helium to remove dissolved gases.

Apparatus. A Model 324 chromatograph with a Model 155-40 "UV/VIS" variable-wavelength spectrophotometer and Model C-RLA integrator/recorder were all obtained from Beckman Instruments, Inc., Berkeley, CA 94710. The prepacked analytical columns used in the study were a Beckman UltraspHERE ODS-Ion Pair (5-μm particle size), 25 cm × 4.6 mm, and a C₁₈ "Solvent Miser" (5-μm particle size), 25 cm × 2.1 mm microbore column from Alltech Associates, Deerfield, IL 60015.

Sample preparation was facilitated by using a reciprocating shaker and an all-glass rotary mixer.

Procedure. Mix 2-mL aliquots of plasma, standard, or control with 200 μL of the working internal standard solution in 16 × 125 mm screw-capped tubes and extract gently for 12 min in 10 mL of diethyl ether. Following a brief centrifugation to separate the phases, discard the organic layer and adjust the pH of the aqueous layer to pH 13 with 1.0 mL of 5.0 mol/L NaOH. Add 10 mL of chloroform, shake for 15 min, and centrifuge. Transfer about 8.0 mL of the organic (lower) layer into clean tubes containing 2.0 mL of 0.1 mol/L HCl. After mixing and centrifuging as before, transfer the acid layer to a 12-mL glass-stoppered conical centrifuge tube and evaporate under reduced pressure in a 50 °C water bath. Reconstitute the dry extract with 200 μL of the mobile phase, vortex-mix for 5 s, and inject 50 μL into the chromatograph. Use a flow rate of 0.25 mL/min.
with the microbore column and the detector set at a wavelength of 210 nm with a range of 0.1 A full-scale.

Quantification is based on peak-area ratios of bethanidine and the internal standard. From a plot of these peak-area ratios vs concentration for the series of plasma standards, determine the concentration in the patient's sample by simple interpolation.

Results

Figure 1 illustrates representative chromatograms from aqueous bethanidine standards, drug-free plasma extracts, and a plasma extract from a patient receiving bethanidine. In more than 20 plasma specimens known to be bethanidine-free, we observed no extraneous peaks with retention times in the vicinity of bethanidine or the internal standard. Patients in our clinical study received protriptyline, a drug that prevents bethanidine-induced orthostatic hypotension. All other antiarrhythmic drugs were discontinued. Drug-free plasma supplemented with protriptyline (1 mg/L) also gave no interfering peaks when analyzed by the proposed method. Similar results were obtained for added diazepam, which is used to premedicate the patients before cardiac catheterization. The patients' tacharrythmias are drug refractory; i.e., clinical response is poor with all drugs. Thus, the concomitant administration of bethanidine with other antiarrhythmic drugs or tricyclic antidepressants other than protriptyline is not likely in clinical practice and certainly not indicated in clinical research studies. To detect other drug interferences, we obtained blood samples before the loading dose of bethanidine and analyzed them both with and without added internal standard. In none of our patients' samples were peaks observed at the retention times of bethanidine or of the internal standard. A graph of the peak-height ratios of bethanidine to internal standard for bethanidine-supplemented plasma was linear between 0.7 and 5.0 mg/L and passed through the origin with a correlation coefficient of 0.9996. The slope of the line is reproducible from day-to-day with a mean value of 0.948 (SD 0.02, n = 8). Although this range is optimum for our studies, instrument sensitivity is sufficient (25 ng gives a signal-to-noise ratio of 10) to permit lower limits of detection simply by increasing initial sample and injection volumes.

Analytical recovery and precision. The present extraction procedure provides excellent recovery from aqueous solutions, ranging from 98 to 105% (mean 99%, n = 5). The recovery of bethanidine from a plasma sample to which 1.25 mg/L had been added was 100 ± 5% (n = 8). Repeated injections of a single low-concentration extract of plasma (mean 0.19 mg/L) gave a CV of 2% for the precision of the instrument. The precision of the entire chromatographic procedure was evaluated by analyzing eight plasma samples, each with a concentration of 1.25 mg/L. With this sample, representing a typical peak plasma concentration, we obtained a CV of 4%. Day-to-day precision was estimated by analyzing the variance of a bethanidine-supplemented plasma control. Aliquots of the control were stored at −70 °C and analyzed on consecutive days along with freshly prepared plasma standards. A mean value (n = 17) of 0.94 mg/L was obtained with a CV of 6.4%.

Pharmacokinetic data. The doses of bethanidine used in these studies are substantially higher than usually prescribed when indicated for hypertension, and little is known of the pharmacokinetic parameters. From our data on a few patients, the dose–response curve in Figure 2 appears typical, with the peak concentration in plasma occurring 60–90 min after a 20 mg/kg (body wt) oral loading dose, the drug half-life being about 4 h.

Discussion

A search of the literature revealed two existing methods for plasma bethanidine: a fluorometric assay (5) and a gas chromatographic separation with quantitation by flame ionization or multiple ion detection (6). In the fluorometric assay, bethanidine is extracted from plasma into chloroform and the fluorescence is then determined by using a drug-dye complex formed with eosin Y. This assay is not specific for bethanidine and an internal standard cannot be used. The multistep extraction requires uniform timing in a dimly lit room and use of scrupulously clean, foil-covered tubes, and the fluorescence decays rapidly (T1/2 = 2 min) in the excitation light path. Our attempts to duplicate the sensitivity and reproducibility of the method reported in this paper were unsuccessful.

Initial gas–liquid chromatographic studies in our laboratory revealed that undervitized bethanidine does not chromatograph well, and that the trifluoroacyl and pentfluoropropionyl derivatives formed multiple products, which decomposed on removal of the anhydride. The chromatographic method with multiple ion detection reported for the analysis of another guanido-containing drug, guanethidine (6), involves a multistep extraction, removal of the guanido group by alkaline hydrolysis, and trifluoroacetylation of the resulting primary amine. This method permits the use of an internal standard but was not applied to the analysis for
benthainidine in plasma. Our study confirmed the earlier finding that the alkaline hydrolysis of benthainidine to benzyllaline is not complete even after 24 h at 110 °C, and that there is extensive decomposition at higher temperature. For these reasons, we believe both of the existing assays are unsuitable for routine use.

The extraction procedure was based on an earlier assay for guanethidine (6). To optimize the extraction at basic pH, we investigated several solvents, including hexane, ether, ethyl acetate, toluene, and chloroform. Chloroform gave the best overall analytical recovery and phase separation. The initial ether extraction at neutral pH causes negligible loss of the drug, yet results in cleaner chromatograms and decreases emulsion formation in subsequent extraction steps. The final evaporative step is widely used in clinical toxicology laboratories to concentrate an extract easily and quickly. The sample can be both concentrated and placed in the solvent that is most suitable for the chromatographic separation.

The absolute uncorrected recovery of benthainidine, added to plasma to give a final concentration of 1.25 mg/L, averaged 43% (35-48%). Variable amounts of the drug are lost at each step when the entire added volume of an organic extract cannot be recovered owing to emulsion formation, but the use of an internal standard in the plasma standards taken through the extraction procedure fully compensates for this variability. The relative recovery is quantitative and the results are precise.

In the initial development of the present method, we used a conventional 25 cm × 4.6 mm C18 reversed-phase column. The sodium acetate mobile phase was optimized by varying the concentration of acetonitrile to best resolve benthainidine from rapidly eluting plasma contaminant peaks without an undue long analysis time. The addition of an ion-suppression reagent, tetramethylammonium chloride, resulted in improved chromatography by reducing peak tailing. The internal standard, N-methylphenylethylamine, one of many compounds tested for suitability as an internal standard, was chosen because of similar extraction characteristics and chromatographic proximity to benthainidine under the described conditions. Although the wavelength selected provided the best sensitivity, the molar absorptivity was too low to provide the required sensitivity. Attempts to improve the sensitivity by using fluorescence detection of dansyl chloride (DNS) derivatives of benthainidine were unsuccessful because multiple peaks formed. Mono- and di-DNS-benthainidine derivatives were identified by collecting fractions corresponding to chromatographic peaks and obtaining positive ion chemical ionization mass spectra of samples from each fraction, introduced via a solid probe inlet. Additionally, we found that the eluted benthainidine was not easily oxidized or reduced, effectively ruling out the use of an electrochemical detector, which is quite sensitive and selective for some drugs.

Recent reports that demonstrated lower detection limits with the use of microbore (0.5-2.0 mm i.d.) chromatographic columns (7) led us to investigate their use for the present application. With no modifications of pumps, injector, or detector, we observed a fivefold increase in peak area and greatly decreased noise as compared with the same amount of compound injected onto a conventional column. The only changes required were the reduction of the flow rate to 0.25 mL/min and reversing the flow through the spectrophotometric detector to bypass the heat exchanger, to reduce extra-column band broadening. This modification so dramatically improved sensitivity and precision that the present method was adopted for routine use.

We have found that, when in continuous use, the useful life of these analytical columns is about six months and their relatively low cost allows replacement after 500 plasma samples. Lot-to-lot variation in column packing may require adjustment of the proportion of the organic modifier in the mobile phase, to duplicate retention times between different columns.

The value of benthainidine as a clinically useful drug for antifibrillatory prophylaxis remains to be established. Toward that goal, clinical trials in several centers are currently in progress. We believe that a readily available in-house assay for plasma benthainidine will aid in comparing the results from the various institutions regarding therapeutic efficacy. Additionally, the assay may be simply modified to measure both new and existing guanido-containing drugs.

References


