GLC Determination of Phenylephrine Hydrochloride in Human Plasma

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Abstract □ A sensitive GLC method for the determination of phenylephrine in human plasma is described. Its application to *in vivo* phenylephrine levels in humans is currently being investigated. In the procedure, plasma components that interfere with the assay were removed using ion-exchange chromatography. Derivatization of the isolated phenylephrine hydrochloride with trifluoroacetic anhydride produced a sharp symmetrical peak when chromatography was carried out using a QF-1 column and monitored with an electron-capture detector. The method, when applied to plasma containing known amounts of added phenylephrine hydrochloride (12.5–50.0 ng./ml.) gave an average recovery of 80%. The standard deviation based on 15 determinations was 10%.

Keyphrases □ Phenylephrine hydrochloride—GLC using electron-capture detection, human plasma □ GLC, electron-capture detection—analysis, phenylephrine hydrochloride in human plasma

Phenylephrine hydrochloride {(−)-*m*-hydroxy-α-[(methylamino)methyl]benzyl alcohol hydrochloride}, a well-recognized sympathomimetic amine, has been in commercial use for many years. It is a vasoconstrictor and pressor drug chemically related to epinephrine and ephedrine. It is used in surgery, ophthalmoscopic examination, and diagnostic procedures and also has been employed as a nasal decongestant. However, a sufficiently sensitive chemical method has been unavailable for measuring this drug in man.

Various assay methods involving spectrophotofluorometry, UV spectroscopy, and colorimetry have been used to measure phenylephrine in its many available pharmaceutical preparations (1–10). These procedures permit measurement of milligram or microgram quantities of phenylephrine. They do not respond with the sensitivity required to measure phenylephrine in biological systems that may contain <1 p.p.m. of drug.

Two *in vivo* studies in humans were reported using tritium-labeled phenylephrine (11, 12). The tagged drug was administered orally in a normal dosage form as the hydrochloride salt and in a timed-release preparation as a protocolloid tannate complex. These studies provided valuable tritium blood concentration–time profiles using a liquid scintillation counting technique. However, the nonspecificity of the measuring system did not permit any evaluation of the intact phenylephrine concentration. The primary purpose of this work was to develop a sensitive and specific assay procedure for measuring unchanged phenylephrine in human plasma.

GLC previously was used for the determination of other sympathomimetic amines. After derivatization, the amines were chromatographed to produce narrow and symmetrical peaks (13). Acylated halogenated derivatives, particularly the fully trifluoroacylated phenolic amines, were readily measured at the nanogram level with an electron-capture detector. The high detection sensitivity of the trifluoroacetate derivatives formed in the analysis of dopamine (14) and 3-methoxy-4-hydroxyphenylethylene glycol (15) suggested a similar approach for the determination of phenylephrine. In this laboratory the treatment of phenylephrine hydrochloride with an excess of trifluoroacetic anhydride produced a single characteristic peak when chromatographed on a 1.82-m. (6-ft.) column of 3% QF-1. This process served as a basis for developing a sensitive assay procedure. The developed method permitted determination of 25 ng. phenylephrine hydrochloride/2 ml. plasma. Prior to derivative preparation, it was necessary to use a cation-exchange column for removal of plasma components that interfered with the GLC measurement. Details of the method are presented here along with results of a phenylephrine recovery study.

EXPERIMENTAL

Instrumentation—Final measurements were made with a gas chromatograph[1] equipped with an electron-capture detector. Adequate separations were realized on a 1.82-m. (6-ft.) × 2-mm. i.d. glass column packed with 3% QF-1 coated on Gas Chrom Q, 100–120 mesh. The column temperature was maintained at 150°, and the helium flow rate was adjusted to 50 ml./min. The detector and inlet temperatures were kept 40° higher than the column temperature. The meter range of the instrument was set at 1 × 10⁻⁹ amp., and the high voltage was adjusted to 50 v. All derivatized samples were diluted with redistilled ethyl acetate, and a 5-μl. aliquot was applied to the column.

Reagents—Cation-exchange resin[2], 50–100 mesh, was purified and conditioned as follows. Mix 30 g. resin with 100 ml. 6 *M* hydrochloric acid in a 250-ml. beaker. Repeat washes six times, discarding each acid wash. Wash the resin free of acid, using distilled water, and store the resin in distilled water until needed. The glass column containing the resin was described previously (16). The height of the resin in the column was between 4 and 5 cm.

Diatomaceous earth[3] was purified by a standard procedure (17).

Trifluoroacetic anhydride[4] was redistilled. The middle third fraction was collected in a suitable vessel, placed in a desiccator, and refrigerated at 5° until ready for use. Under these pretreatment and storage conditions, the reagent was stable for several weeks.

Ethyl acetate (reagent grade) was redistilled and the middle third fraction was collected.

Standard chromatographic tubes (25 × 200 mm.) were used.

Assay for Plasma—Transfer 1–2 ml. of plasma to a 50-ml. beaker. Adjust the volume to 4 ml. with distilled water and add, with thorough mixing, 1 g. each of sodium carbonate and sodium chloride. Blend the solution with 4 g. diatomaceous earth and transfer the homogeneous mixture to a standard chromatographic tube containing a glass wool plug in its outlet stem. Pack firmly by tapping with a glass rod and then place a glass wool plug on top of the packed column. Position the chromatographic tube directly above a 50-ml. glass-stoppered centrifuge tube containing 4 ml. of 0.1 *M* hydrochloric acid.

Pass 44 ml. of water-washed ethyl acetate through the column and collect the effluent in the centrifuge tube. Shake the stoppered tube for 3 min., centrifuge, and then withdraw and reserve the aqueous acid phase. Reextract the ethyl acetate with 4 ml. of 0.1 *M* hydro-
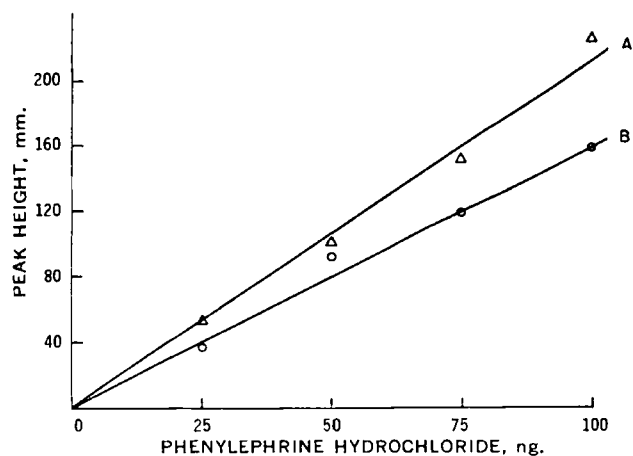
Figure 1—*Response curves for standard phenylephrine hydrochloride (curve A) and for plasma samples containing added amounts of phenylephrine hydrochloride (curve B).*

chloric acid and pool the acid extracts. Transfer the acid pool to a column containing a prepared cation-exchange resin and adjust the flow rate to 1-2 ml./min. Wash the column with three 3-ml. volumes of 0.1 M hydrochloric acid after the sample extracts completely pass into the column. Terminate flow from the column after the last wash just passes into the resin. Discard the effluent volume.

Position the column above a 10-ml. graduated cylinder and then elute phenylephrine with two 3-ml. volumes of 1 M hydrochloric acid. Measure the volume in the cylinder (about 6.3 ml.) and then transfer 2.0 ml. to a 5-ml. beaker. With a slight displacing current of nitrogen and a steam bath, evaporate the contents of the beaker to dryness. Cool the beaker to room temperature and then add 0.2 ml. trifluoroacetic anhydride. Seal the beaker with aluminum foil and place in an oven at 34 ± 1° for exactly 10 min. Remove the beaker to a well-ventilated hood at room temperature. Remove foil, permitting excess trifluoroacetic anhydride to evaporate spontaneously and completely. Dissolve the remaining residue in exactly 0.4 ml. (dry) ethyl acetate. Inject 5 µl. into the gas chromatograph readied for use as described in the *Instrumentation* section. Calculate the peak height measured at the retention time previously established for the acylated phenylephrine derivative.

Assay precision was considerably improved when two 2-ml. aliquots of the 1 M hydrochloric acid effluent were evaporated to dryness, the residues were derivatized, and the measured peak heights were averaged.

Preparation of Standard Calibration Curve—Dissolve phenylephrine hydrochloride reference standard in 0.1 M hydrochloric acid and dilute with 0.1 M hydrochloric acid to provide a solution containing 20 ng./ml. In duplicate, transfer 1.0, 2.0, 2.5, and 3.5 ml. of the standard solution to respective 5-ml. beakers corresponding to 20, 40, 50, and 70 ng. phenylephrine hydrochloride, respectively. Evaporate to dryness the contents of all eight beakers. Add 0.2 ml. trifluoroacetic anhydride to each standard and then continue with the derivatization procedure as directed previously. After removing excess reagent, dissolve the residue with 1.3 ml. (dry) ethyl acetate and introduce a 5-µl. volume into the gas chromatograph. Construct a calibration curve from averaged measured peak height for each standard concentration.

RESULTS AND DISCUSSION

The reaction of trifluoroacetic anhydride with phenylephrine hydrochloride led to the formation of a single derivative, having a retention time of approximately 3.6 min. The derivative is presumed to be that of the fully trifluoroacetylated molecule.

When aliquots of plasma containing increasing amounts of added phenylephrine hydrochloride were carried through the entire assay procedure, the phenylephrine derivative peak height increased in proportion to the amount of drug added (Fig. 1). Processing 2 ml. plasma (blank) through the entire procedure did not give any peaks that interfered with the assay. Figure 2 shows chromatograms resulting from processing 2 ml. plasma and 2 ml. plasma spiked with 25 ng. phenylephrine hydrochloride. The spiked sample peak at this
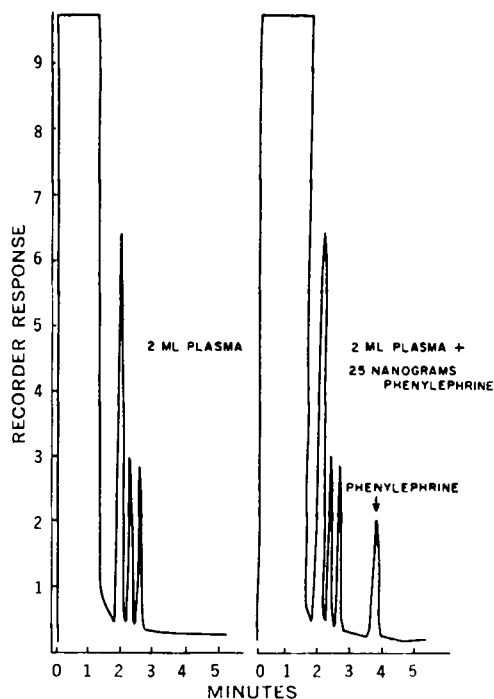

Figure 2—*Chromatogram of 2-ml. plasma sample compared to a parallel sample containing 25 ng. of phenylephrine hydrochloride.*

concentration is readily evident when compared to the blank. When the instrument was set at maximum sensitivity, the phenylephrine peak was always measured on either a positive or a negative sloping baseline. Standards were not carried through the complete assay procedure as described for the plasma samples. Nanogram quantities of standard phenylephrine hydrochloride were subjected only to reaction with trifluoroacetic anhydride and then chromatographed. A standard curve constructed in this manner (Fig. 1) was used to compute the phenylephrine recoveries from plasma spikes. Results conducted on 15 plasma samples containing known amounts of added phenylephrine are shown in Table I. Overall reproducibility was ± 10% and the average recovery was 80%. Repeated injections of 5 µl. of a standard yielded no variable greater than 1.5% and demonstrated that the column did not require priming.

An internal standard, although not employed in this study, may result in improved assay precision for measuring phenylephrine in plasma. A suitable choice might be metanephrine. This particular compound is structurally similar to phenylephrine and produced a similar detector response when derivatized with trifluoroacetic anhydride and chromatographed as directed for phenylephrine. The retention time for the metanephrine derivative was about 7.5 min.

Diatomaceous earth column chromatography, providing 80% recovery, was the most efficient process of those considered for the initial separation of phenylephrine base from the basified plasma solution. For example, two 20-ml. ethyl acetate extractions of the basified plasma solution yielded only a 50-55% recovery of added phenylephrine.

The phenylephrine base contained in the ethyl acetate effluent was extracted quantitatively with two 4-ml. volumes of 0.1 M hydrochloric acid. The complete retention of phenylephrine from these acid extracts on the cation-exchange resin was readily attained. Interfering plasma components were removed by washing the resin column with 0.1 M hydrochloric acid. Phenylephrine then was quantitatively eluted from the resin column using stronger acid. This was readily accomplished using 6 ml. of 1 M hydrochloric acid.

Previous studies involving acylation of primary and secondary amines generally were conducted using the amine in the form of its free base (18-20). In this laboratory, an occasional difficulty was encountered when isolating nanogram quantities of some amine bases, particularly when extracting them from biological systems. Studies indicated that the amines were extracted readily with an organic solvent, but some losses occurred in attempting to concentrate them by solvent evaporation. The problem was thought to be caused by both amine volatility and air oxidation of the base. To

Table I—Precision and Accuracy in Measurement of Phenylephrine Hydrochloride Added to 2 ml. Human Plasma

Number of Samples	Phenylephrine Hydrochloride Added, ng.	Phenylephrine Hydrochloride Found[a], ng.	RSD, %
4	25	19	±11
4	50	42	±7
3	75	57	±10
4	100	78	±11

[a] Mean.

avoid any phenylephrine losses in this study, it was decided to concentrate the drug from plasma and acylate it as its hydrochloride salt. This was readily accomplished after eluting phenylephrine off the resin and evaporating to dryness the 1 M hydrochloric acid effluent. Measurable losses of nanogram quantities of phenylephrine were not realized when the salt residue was subjected to excess heating at 60°. In fact, excess heating is recommended in the procedure to ensure complete removal of any residual amounts of the 1 M hydrochloric acid which, if present, seriously interfere with the subsequent derivatization reaction.

The best conditions found for acylating phenylephrine hydrochloride were 10 min. and 34°. This treatment permitted phenylephrine measurement at maximum sensitivity and reproducibility. Slight changes in the acylating conditions adversely affect the phenylephrine derivatization process and, hence, the reproducibility of the method. For instance, reaction times beyond 10 min. produced erratic results and shorter times yielded responses indicative of incomplete acylation. Furthermore, sensitivity was reduced if the reaction temperature was lowered to 25°. Because of the sensitive nature of the reaction, all samples and standards involved in a single run were derivatized simultaneously and subjected to identical conditions with respect to time and temperature. A fresh set of standards was prepared for each new series of plasma samples assayed.

A problem associated with the acylation of amine salts is the liberation of an equivalent amount of acid. With phenylephrine hydrochloride, this can be represented as shown in Scheme I. The released hydrochloric acid, if not removed, can hydrolyze the ester or amide group of the fully acylated molecule, thereby resulting in a loss of phenylephrine detection sensitivity. If a large excess of trifluoroacetic anhydride were employed, the acylation of phenylephrine was more complete and interferences from the liberated hydrochloric acid were negligible. Both the excess reagent and the liberated hydrochloric acid were effectively removed by spontaneously evaporating the solutions to dryness, preferably in a low humidity environment. The reagent is volatile (b.p. 39°) and the evaporation was complete within a few minutes at ambient temperature. The use of heat or a stream of forced nitrogen to aid the evaporation process should be avoided since both of these procedures result in a loss of the acylated derivative. Nanogram quantities of the derivative in dry ethyl acetate were stable for at least 24 hr.

CONCLUSION

A method was presented for measuring intact phenylephrine in plasma. The lowest amount of the drug measured in this study was 25 ng./2 ml. body fluid (Fig. 2). If desired, this sensitivity can be improved. For example, instead of employing a 2-ml. aliquot resulting from the 6.3 ml. of 1 M hydrochloric acid effluent, the entire 6.3-ml. volume could be used for the assay, producing a threefold increase in sensitivity.

This assay method has not been applied to an actual *in vivo* study of phenylephrine in humans. It is our contention, however, that the proposed analytical procedure, because of its sensitivity, provides
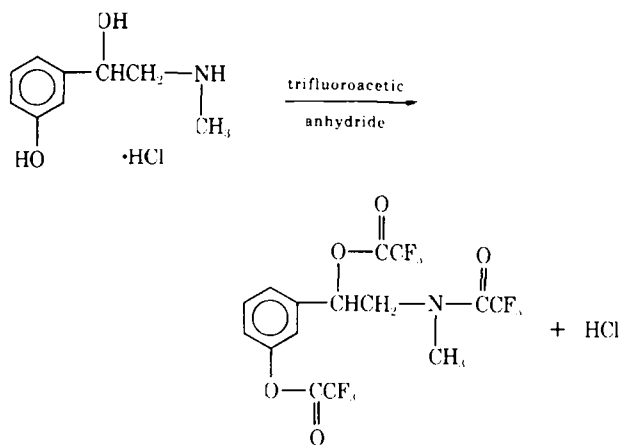
Scheme I

the best approach of those methods currently available for investigating phenylephrine blood levels in man following medication.

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