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High-performance liquid chromatographic determination of phenylephrine and its conjugates in human plasma using solid-phase extraction and electrochemical detection[1]

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Abstract

An HPLC method for the determination of phenylephrine and its conjugates in human plasma was developed and validated. The method for quantitation involved extraction of diluted plasma (subject to hydrolysis with β-glucuronidase for 30 min with 500 units of enzyme per 0.1 ml of plasma at 37°C for the conjugates) on solid-phase weak cation-exchange cartridges followed by elution of the analyte and the internal standard (ethylnorphenylephrine) with 5% triethylamine in methanol. Analysis was carried out on a 15 cm ODS stationary phase using ion-pair reversed-phase chromatography. An electrochemical detector operated at +1.15 V vs. Ag/AgCl was employed for detection. The standard curves were linear in the range 1.0–50.0 ng ml^{-1} for phenylephrine and 25.0–500.0 ng ml^{-1} for phenylephrine obtained from its conjugates. The limit of quantitation was 2.0 ng ml^{-1} (RSD = 17%) and 25.0 ng ml^{-1} (RSD = 18%), respectively. Acceptable accuracy and precision were obtained during intra- and inter-batch analyses for both the assays.

Keywords: Phenylephrine; Conjugates; Ion-pair reversed-phase chromatography; Electrochemical detection; Solid-phase extraction; Enzymatic hydrolysis

1. Introduction

Phenylephrine (1-*m*-hydroxy-α-[(methylamino)methyl]benzyl alcohol) is also known as neosynephrine, *m*-synephrine, adrianol and *m*-sympatol. It is an α-receptor agonist, and therefore used for its vasoconstrictor and mydriatic properties. Phenylephrine is amphoteric in nature with dissociation constants (20°C) of 8.9 (–OH) and 10.1 (–NH). The molecule is ionized at all

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pH values for aqueous solutions, hence extraction by ion-suppression is not possible.

Various methods for the quantitation of phenylephrine in biological fluids have been reported. Fluorescence analysis of phenylephrine in serum was reported by Rubin and Knott [1]. The procedure included a double extraction step and had a sensitivity of 4 μg ml^{-1}. A gas chromatographic method utilizing electron-capture detection was developed for the determination of phenylephrine in human plasma [2]. Derivatization with trifluoroacetic acid was necessary for detection, and the extraction procedure involved laborious preparation of ion-exchange/diatomaceous earth chromatographic columns for individual samples and an organic extraction step. The method gave an average recovery of 80% and sensitivity of 12.5 ng ml^{-1} using 2 ml of plasma. An HPLC method for the determination of phenylephrine in human plasma using fluorescence detection (excitation and emission wavelengths of 270 and 305 nm, respectively) and a solid-phase extraction (phenyl columns) procedure was reported to be sensitive to the 0.5 ng ml^{-1} level [3]. However, the method did not use an internal standard and required variable volumes of plasma (1 or 2 ml) to obtain the desired sensitivity. Phenylephrine was used as an internal standard for the quantitation of ethylnorphenylephrine (etilephrine) in human plasma [4]. The purification involved a two-step procedure of solid-phase extraction on a cation-exchange column followed by extraction with an organic solvent. A review of the available methods indicated that it would be necessary to develop and validate a simple, sensitive and specific method if one were to study the pharmacokinetics of phenylephrine at standard doses. The method for quantitation of phenylephrine in human plasma described here involves single-step solid-phase extraction of plasma followed by HPLC analysis.

2. Experimental

2.1. Chemicals and supplies

Phenylephrine hydrochloride and β-glucuronidase from *Helix pomatia* (enzyme activities 416 800 units g^{-1} of β-D-glucuronide glucuronosohydrolase, 15 400 units g^{-1} of sulfatase) were obtained from Sigma Chemical (St. Louis, MO). Ethylnorphenylephrine hydrochloride was obtained from Boehringer Ingelheim (Ridgefield, CT). Triethylamine (TEA), 1-heptanesulfonic acid (HSA), concentrated hydrochloric acid and sodium acetate were obtained from Fisher Scientific (Springfield, MO). Glacial acetic acid was obtained from J.T. Baker Scientific (Phillipsburg, NJ). Methanol and acetonitrile were of HPLC grade and obtained from Burdick and Jackson Laboratories (Muskegon, MI). Cation-exchange HEMA 1000 CM Resin Extract Clean RC 100 mg solid-phase extraction (SPE) cartridges were obtained from Alltech Associates (Deerfield, IL) and UNIFLO 1.2 μm filters (25 mm diameter cellulose acetate membranes) from Schleicher and Schuell (Keene, NH). Drug-free human plasma was obtained from Biological Specialty (Lansdale, PA).

2.2. Standard solutions

A stock solution of phenylephrine (PE) was prepared by dissolving a precisely weighed 10 mg quantity of phenylephrine base (12.11 mg of phenylephrine hydrochloride) in 100 ml of distilled water (DW). The primary plasma solution prepared by diluting the stock solution with blank plasma was further diluted to prepare calibration standards and validation pools for the assay of phenylephrine. Blank plasma was used for the 0.0 ng ml^{-1} standard. The calibration standards and validation pools for the assay of conjugates of phenylephrine were prepared in the similar fashion as mentioned above. For the sake of distinction, these calibration standards and validation pools will be referred to as being hydrolyzed with β-glucuronidase, or PE-GLU.

The internal standard solution (ethylnorphenylephrine hydrochloride, 500 ng ml^{-1}) was prepared in DW. A 5% TEA–methanol mixture was prepared by diluting 25 ml of TEA to 500 ml with methanol. Methanol–DW (50:50, v/v) was

prepared by mixing equal volumes of DW and methanol. β-Glucuronidase enzyme solution (5000 units ml^{-1}) was prepared by dissolving 12 mg of enzyme in 1 ml of sodium acetate buffer (pH 5.0, 100 mM). The mobile phase for HPLC was prepared by diluting a mixture of 150 ml (130 ml for PE-GLU) of acetonitrile and 7 ml of HSA solution (5.0 g per 25 ml DW) with sodium acetate buffer (pH 5.1; 10 mM) to 1000 ml.

2.3. HPLC system and chromatographic conditions

The HPLC system consisted of a Model 110A pump (Altex, Berkeley, CA), Shimadzu SIL-6B injector operated by a Shimadzu SCL-6B system controller (Shimadzu, Kyoto, Japan), Model LC-4B amperometric detector with LC-22A temperature controller and glassy carbon working electrode (Bioanalytical System, West Lafayette, IN) and Shimadzu C-RIB integrator. An Omniscribe Series B-5000 strip-chart recorder Houston Instruments, Austin, TX) with dual-voltage pens was simultaneously used for the signal measurement. Phenylephrine and the internal standard were separated on an Altex Ultrasphere octadecylsilane (ODS), 5 μm, 150 × 4.6 mm i.d. column (supplier P.J. Cobert, St. Louis, MO) at room temperature using an isocratic mobile phase of acetonitrile–sodium acetate buffer (pH 5.1; 10 mM)–7 mM HSA (15:78:7, v/v/v). The mobile phase used for the separation of PE-GLU contained only 13% acetonitrile. A lower strength mobile phase was required for PE-GLU to facilitate the separation of interfering peaks from the analytes of interest. The flow rate was maintained at 1.0 ml min^{-1} and detection was carried out in the oxidation mode at +1.15 V vs. Ag/AgCl at 30°C. Peak heights were recorded at 50 nA V^{-1}.

The mobile phase employed for the separation of phenylephrine provided about 481 and 257 theoretical plates per centimeter of the column for phenylephrine and the internal standard, respectively. With a capacity factor of 5.0 and 6.8 for phenylephrine and internal standard, respectively, good resolution from each other (phenylephrine–internal standard resolution = 3.1) and the co-extracted compounds was observed. The retention volume for phenylephrine was 7.0 ml and that of the internal standard was 9.1 ml. For PE-GLU, the mobile phase provided about 279 and 272 theoretical plates per centimeter of the column for phenylephrine and the internal standard, respectively. The capacity factor was 8.1 and 11.7 for phenylephrine and the internal standard, respectively (phenylephrine–internal standard resolution = 4.3). Phenylephrine and the internal standard had retention volumes of 10.4 and 14.4 ml, respectively.

2.4. Extraction procedure

2.4.1. Extraction for phenylephrine

The calibration standards and validation pools stored at −20°C were allowed to equilibrate to room temperature, vortex mixed briefly and 1 ml of sample was pipetted into conical tubes. Internal standard (100 μl of 500 ng ml^{-1} ethylnorphenylephrine) and 4 ml of DW were delivered to each sample and the diluted plasma samples were filtered through a 1.2 μm filter. The SPE cartridges were conditioned successively with 2 ml of DW, 2 ml of pure methanol, 2 ml of 5% TEA–methanol, 2 ml of DW, 2 ml of 1.0 N hydrochloric acid and 5 ml of DW. Vac-Elut (Analytichem International, Harbor City, CA) was used to control the flow rate. The filtered plasma was poured on the conditioned cartridges and extracted at a flow rate of 1.0 ml min^{-1}. The SPE cartridges were then washed with 2 ml of DW, 1 ml of methanol–DW (50:50) and 1 ml of methanol. The samples were eluted from the SPE cartridges at a flow rate of 0.5 ml min^{-1} with 2 ml of 5% TEA–methanol into disposable borosilicate culture tubes. The eluate layer was poured into a clean 15 ml conical tube and evaporated to dryness for 60 min at 40°C. The residue was allowed to equilibrate to room temperature, reconstituted in 500 μl of DW and 100 μl of each sample were injected into the HPLC system.

2.4.2. Extraction for PE-GLU

The stored samples (100 μl) were equilibrated to 37°C in a shaker bath. After the addition of 30 μl of 0.1 N HCl and 100 μl of β-glucuronidase solution, plasma samples were incubated at 37°C

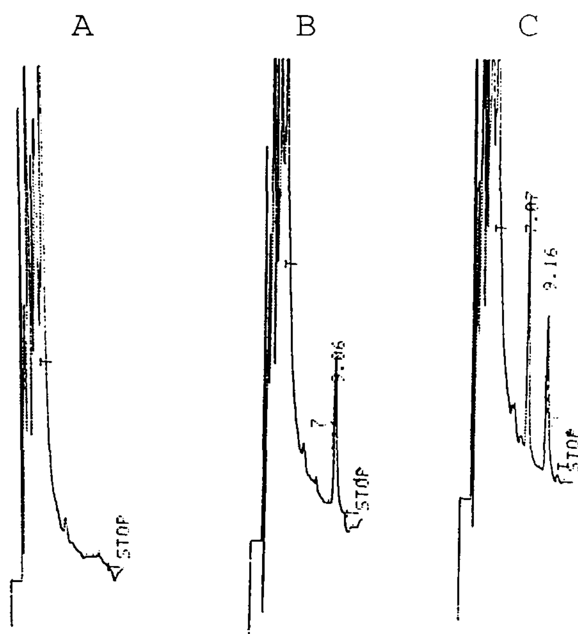

Fig. 1. Typical chromatograms of (A) blank plasma from a pharmacokinetic study and phenylephrine standards, (B) 2.0 ng ml^{-1} and (C) 50.0 ng ml^{-1} of phenylephrine (PE) with 50 ng ml^{-1} of internal standard (IS). PE, 7.0 min; IS, 9.1 min.

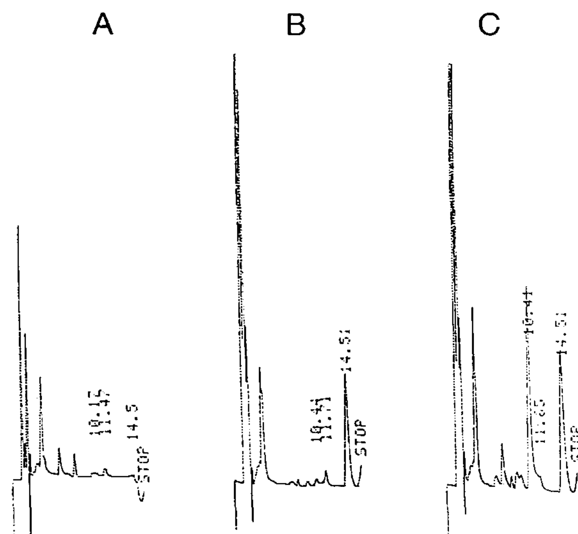

Fig. 2. Typical chromatograms of (A) blank plasma from a pharmacokinetic study and PE-GLU standards, (B) 25.0 ng ml^{-1} and (C) 500.0 ng ml^{-1} of phenylephrine (PE) with 500 ng ml^{-1} of internal standard (IS). PE, 10.4 min; IS, 14.5 min.

for 30 min. Internal standard was added to each sample (100 μl of 500 ng ml^{-1} solution), the samples were vortex mixed briefly and then 1 ml of sodium acetate buffer (pH 5.0; 100 mM) was delivered to each sample. The SPE cartridges were conditioned sucessively with 1 ml of methanol and 1 ml of DW. The remainder of the procedure was the same as above except that the 5% TEA–methanol layer was dried for 40 min at 45°C and the residue was reconstituted in 150 μl of DW and 50 μ were injected into the HPLC system.

2.5. Validation design

2.5.1. Validation design for phenylephrine

Six validation batches were run. Each batch contained duplicate calibration standards at concentrations of 0.0, 1.0, 2.0, 5.0, 10.0, 20.0, 40.0 and 50.0 ng of phenylephrine per ml of plasma. There were three validation pools at concentrations of 5.0, 10.0 and 40.0 ng ml^{-1} plasma. Each validation pool was assayed six times in a batch. The calibration standards and the validation pools were assayed in a random order. Validation pools at concentrations of 1.0 and 2.0 ng ml^{-1} were run eight times in one batch to determine the limit of quantitation of the assay.

2.5.2. Validation design for PE-GLU

Four validation batches were run. Duplicate calibration standards were assayed in each batch at concentrations of 0.0, 25.0, 50.0, 75.0, 100.0, 250.0 and 500.0 ng of phenylephrine per ml of plasma. There were four validation pools, three with known concentrations of 25.0, 100.0 and 400.0 ng ml^{-1} plasma and the fourth being an authentic sample from a dosed subject and of unknown concentration. This fourth validation pool was obtained from an actual pharmacokinetic study and served to evaluate the reproducibility of the hydrolysis procedure. Each batch contained all four validation pools assayed six times each in a random order along with the calibration standards.

2.6. Data evaluation and calculations

Calibration curves were generated by weighted (weight = 1/ratio of phenylephrine to internal

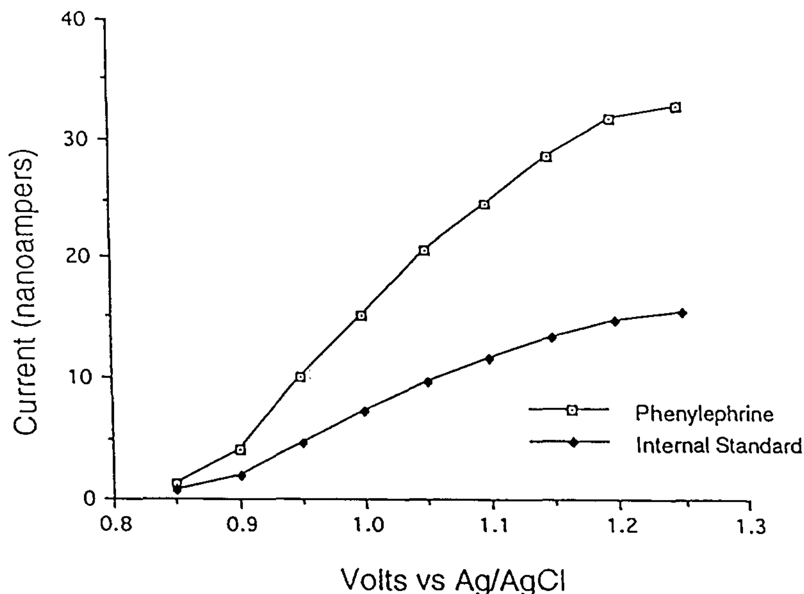

Fig. 3. Hydrodynamic voltammogram of phenylephrine and internal standard.

standard) linear regression of all the calibration standards (excluding the 0.0 ng ml^{-1} standard). Regression statistics, i.e. intercept, slope and correlation coefficient, were calculated for each curve. The relative standard deviation (RSD) was used as a measure of precision; it is the standard deviation (SD) expressed as a percentage of the average computed concentration: RSD (%) = (SD × 100)/average concentration. The analytical recovery (AR) was used to assess the accuracy and is defined as average computed concentration expressed as a percentage of the amount of analyte added: AR (%) = (average concentration × 100)/amount of analyte added. The limit of quantitation (LOQ) was defined as the smallest detectable concentration that can be estimated with an acceptable degree of precision and accuracy. The limit of detection was set at the lowest concentration level that can be determined to be statistically different from an analytical blank (0.0 standard). It was found by taking twice the standard deviation of the 0.0 standard in units of concentration, i.e. ng ml^{-1}. The acceptance criteria for the calibration standards and the validation pool were as described by Shah et al. [5]. The accuracy and precision of the calibration standards and validation pools were to be within 15% of the theoretical concentration. An RSD of 20% was considered acceptable for the lowest validation pool.

3. Results and discussion

3.1. Chromatography

Fig. 1 presents typical chromatograms following a 100 μl injection of extracted standards prepared to contain 50.0 and 2.0 ng ml^{-1} of phenylephrine and 50 ng ml^{-1} of internal standard and a chromatogram of blank plasma. Typical chromatograms following a 50 μl injection of extracted standards containing 500.0 and 25.0 ng ml^{-1} of PE-GLU and 500 ng ml^{-1} of internal standard and a blank plasma are presented in Fig. 2.

3.2. Detection

Detection was done electrochemically in the

Table 1
Regression statistics for phenylephrine and enzymatically hydrolyzed phenylephrine (PE-GLU)

Analyte	Range (ng ml^{-1})	n	Slope	Intercept	Correlation coefficent
Phenylephrine	1.0–50.0	6	31.31 ± 1.50	0.201 ± 0.327	0.998 ± 0.001
PE-GLU	25.0–500.0	4	401.7 ± 21.3	3.39 ± 5.91	0.996 ± 0.002

Table 2
Accuracy and precision for validation pools of phenylephrine and enzymatically hydrolyzed phenylephrine (PE-GLU)

Analyte	Concentration (ng ml^{-1})	n	Mean	SD	RSD (%)	AR (%)
Phenylephrine	5.0	30	4.76	0.41	8.7	95.1
	10.0	29	9.71	0.62	6.4	97.1
	40.0	29	40.3	1.63	4.1	100.7
PE-GLU	25.0	23	24.35	4.47	18.3	97.4
	100.0	22	88.15	10.8	12.3	88.2
	400.0	24	375.8	37.3	9.9	93.9
Phenylephrine from conjugates	Unknown	24	194.3	18.1	9.3	–

oxidation mode on a glassy carbon working electrode at an applied potential of +1.15 V (reference electrode Ag/AgCl). To select the proper detection potential, a hydrodynamic voltammogram was constructed (Fig. 3). A sensitivity of 50 nA V^{-1} was found to be appropriate for all the samples.

3.3 Linearity and reproducibility

The calibration curves were reproducibly linear in the range 1.0–50.0 ng ml^{-1} for phenylephrine and 25.0–500.0 ng ml^{-1} for PE-GLU. Regression statistics from the calibration standard curves during the validation study are presented in Table 1.

3.4. Accuracy and precision

Table 2 presents the parameters used to verify the accuracy and precision of the methods for determination of phenylephrine and PE-GLU. The intra- and inter-batch accuracy and precision were evaluated and were within the acceptable limits. The average precision for phenylephrine was 6.4% and the average accuracy was 97.6% and those for PE-GLU were 13.3% and 93.2%, respectively.

3.5. Limits of quantitation and detection

The LOQ for the assay of phenylephrine was 2.0 ng ml^{-1} with an RSD of 17.0% and an AR of 102.7%. The validation pool at the concentration of 1.0 ng ml^{-1} failed to meet the specified acceptance criteria with RSD and AR of 27.9% and 120.2%, respectively. The LOQ for PE-GLU was 25.0 ng ml^{-1}. The limits of detection for the determination of phenylephrine and PE-GLU in human plasma were 0.40 and 6.8 ng ml^{-1}, respectively.

3.6. Reproducibility and optimization of hydrolysis of the conjugates

The amount of enzyme (β-glucuronidase) required and the length of incubation for the complete hydrolysis were optimized using various amounts of the enzyme and different lengths of time. A sample with an unknown amount of phenylephrine conjugates obtained from a human pharmacokinetic study was used for the optimization procedure. After incubation for an appropriate time with an appropriate concentration of enzyme, the samples were extracted as described above and analyzed by HPLC. The ratio of peak height of phenylephrine to that of the internal

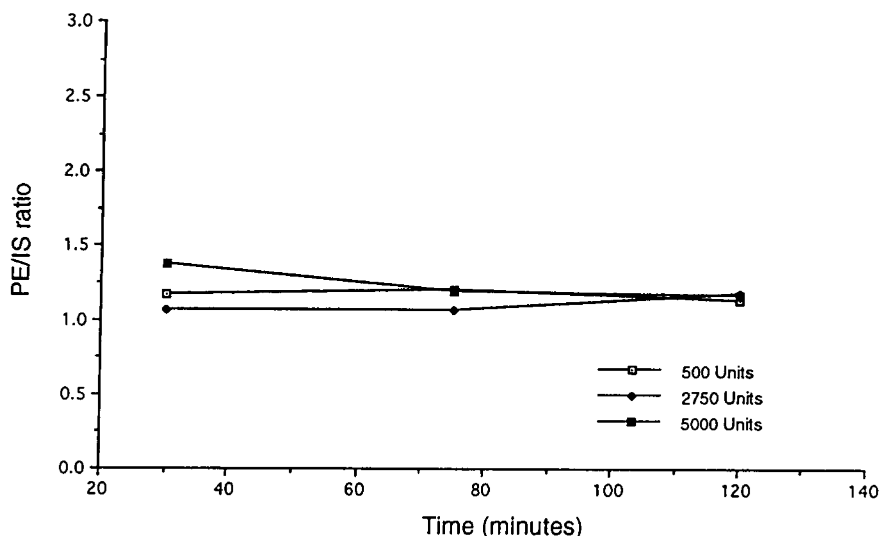

Fig. 4. Effect of varying incubation times (30–120 min) at 37°C and enzyme concentration (500–5000 units per 0.1 ml of plasma) on peak-height ratio of phenylephrine to internal standard (PE/IS).

standard was used as a measure of the extent of hydrolysis, an increase in the ratio indicating an increase in phenylephrine liberated from phenylephrine conjugates.

β-Glucuronidase enzyme (500, 2750 or 5000 units) dissolved in 0.1 ml of 100 mM sodium acetate buffer (pH 5.0) was incubated with 0.1 ml of plasma for 30, 75 or 120 min at 37°C in a water-bath. The results of the optimization procedure are shown in Fig. 4. It was concluded that 500 units of enzyme incubated with 0.1 ml of plasma for 30 min was sufficient for complete hydrolysis of phenylephrine conjugates.

Reproducibility of the hydrolysis was estimated as the RSD (precision) obtained during the validation procedure and the results are presented in Table 2. An RSD of 9.3% indicated that the hydrolysis of phenylephrine conjugates was being carried out reproducibly.

3.7. Recovery

The absolute recoveries of phenylephrine and the internal standard from plasma during the analysis were estimated at 10.0 and 40.0 ng ml^{-1}, respectively. Phenylephrine showed recoveries of 52% and 51% at 10.0 and 40.0 ng ml^{-1} concentrations, respectively, and those for the internal standard were 73% and 81%, respectively.

3.8. Specificity

Specificity was determined by evaluating ten plasma samples devoid of drug to verify the absence of interfering substances present at the retention times of phenylephrine and the internal standard.

3.9. Stability of phenylephrine

The stability of phenylephrine in human plasma stored at −20°C was studied at concentrations of 5.0, 10.0 and 40.0 ng ml^{-1}. Plasma samples spiked with appropriate concentrations of phenylephrine were aliquoted into polystyrene tubes and stored along with blank plasma. At specified intervals of time (1, 2 and 5 weeks), one set of stored spiked plasma was thawed and six replicates were analyzed along with replicates of identical concentrations of freshly prepared samples. The method for the analysis and extraction of phenylephrine was as outlined above. The peak-height ratio (phenylephrine to internal standard) obtained upon analysis of freshly prepared and stored sam-

ples was evaluated to assess relevant and statistically significant degradation [6].

The data obtained from the stability study indicated that phenylephrine stored at −20°C in polystyrene tubes was stable over a peiod of 5 weeks. The results were consistent over the concentration range studied.

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