In the second study carried out on Formulations D, E, F, and G, it was pointed out that the two systems do not arrange the different formulations in the same way, and therefore, the existence of response surfaces proportional for the chosen parameters must be ruled out.

With regards to the general correlations, Figs. 3 and 4 show the regression of E_2 against D_{30} and E_{12} against D_{180}, respectively, for all the formulations studied. In spite of the large differences existing between the various formulations, a significant linear regression is obtained, especially in the case of E_{12} against D_{180}. Even the F value obtained for the regression term, b_0, of the ANOVA of the regression was higher than the quadruple of the critical value tabulated. The high F values indicate the capability of the apparatus employed for the *in vitro* studies.

A study of response surfaces is not necessary for establishing a routine control. Nevertheless, in biopharmaceutics, a knowledge of the technological factors which can interact with drugs, as well as of the physicochemical properties responsible for this interaction, is essential. A mechanistic model which would include all variables, both technologically and physiologically dependent, is not feasible, therefore, the use of response surfaces to quantify the effect produced by the factor studied is necessary. This quantification should be independent of therapeutic implications and should insist on establishing the necessary guidelines for obtaining an exact formulation of the dosage forms.

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Determination of Isoetharine in Plasma by Reversed-Phase Chromatography with Amperometric Detection

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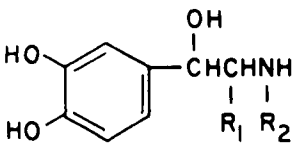
Abstract □ A reversed-phase liquid chromatographic method for the determination of isoetharine in blood plasma, utilizing amperometric detection, is described. Plasma samples were extracted utilizing an ion-pair reagent, di-(2-ethylhexyl)phosphoric acid, to concentrate the catecholamine. Only minor differences were observed in the relative bioavailability of isoetharine hydrochloride and isoetharine mesylate after oral administration to rats. Observed plasma levels, at 1 hr after oral medication, were highly variable in dose-ranging studies at doses of 800–2500 mg/kg/day for 2 weeks.

Keyphrases □ Bioavailability—determination of isoetharine in plasma by reversed-phase chromatography with amperometric detection □ Reversed-phase chromatography—determination of isoetharine in plasma with amperometric detection □ Isoetharine—determination in plasma by reversed-phase chromatography with amperometric detection

Isoetharine (4-[1-hydroxy-2-[(1-methylethyl)-amino]-butyl]-1,2-benzenediol) (I) is a β-agonist which is widely



	R_1	R_2
I	CH_2CH_3	$-CH(CH_3)_2$
II	H	$-C(CH_3)_3$

used as a bronchodilator in inhalation therapy. Reversed-phase liquid chromatography with amperometric detection has been used extensively for the determination of endogenous and exogenous biogenic amines in various biological media (1). The most common methods for sample preparation include various modifications of the method of Anton and Sayre (2) in which the compound of interest is adsorbed to alumina through the catechol moiety. In addition, ion exchange resins (3) and boric acid gels (4) have been used to concentrate the analyte and separate it from interfering substances. In the present study, the catecholamine was extracted into an organic solvent through the formation of an ion-pair with di-(2-ethylhexyl)phosphoric acid (5).

The present report describes an analytical method developed for the determination of isoetharine in blood plasma, and the application of the method to the analysis of rat plasma in studies comparing the oral bioavailability of the hydrochloride and mesylate salts (the two marketed salt forms of isoetharine) and in dose-ranging toxicity studies.

EXPERIMENTAL

Reagents—Isoetharine[1] (hydrochloride and mesylate salts) (I), colterol[2] (mesylate salt, internal standard) (4-[2-[1,1-dimethylethyl)-amino]-1-hydroxyethyl]-1,2-benzenediol) (II), methanol[3], benzene[4], di-(2-ethylhexyl)phosphoric acid[5], were used as received. Water was deionized, distilled, and treated with high-intensity UV radiation[6]. All other chemicals were reagent grade or better and used without further purification.

A 1.5% (or 0.5%) solution of di-(2-ethylhexyl)phosphoric acid in ben-

[1] Breon Laboratories, New York, N.Y.
[2] Sterling-Winthrop Research Institute, Rensselaer, N.Y.
[3] OmniSolve, McB, Cincinnati, Oh.
[4] Nanograde, Mallinckrodt, St. Louis, Mo.
[5] Sigma Chemical Co., St. Louis, Mo.
[6] ORGANICpure, Sybron/Barnstead, Boston, Mass.

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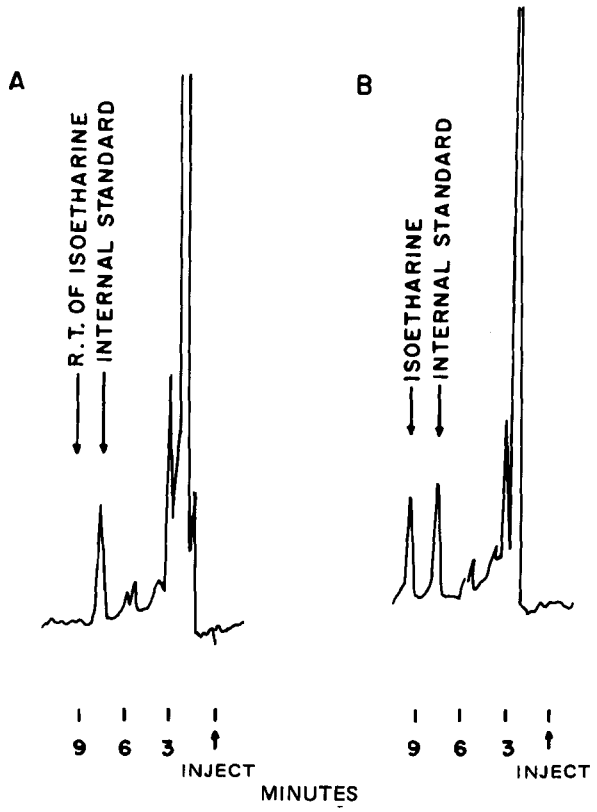

Figure 1—*Representative chromatograms of processed rat plasma: (A) control plasma containing only internal standard; (B) 4 ng/100μl standard. See text for chromatographic conditions. Attenuation 10 nA/V (full scale).*

zene was prepared by diluting 0.6 ml of reagent to 40.0 ml (or 0.5 ml of reagent to 100 ml) with benzene. Phosphate buffer, pH 6.9 (0.2 *M*), was prepared by dissolving 13.6 g of KH_2PO_4 in 300 ml of water, adding 50 ml of 1 *N* NaOH, diluting to 0.5 liter, and adjusting the pH to 6.9 with 1 *N* HCl. Fresh stock standard solutions of isoetharine and colterol were prepared in 0.05 *N* H_2SO_4 at concentrations of 100 μg/ml. All glassware was silanized before use.

Preparation of Standards—The chromatographic system was calibrated by analyzing duplicate plasma standards containing 0, 2.5, 5, 10, 20, 40, 60, 80, and 100 ng/ml of free base. The standards were prepared by adding 100-μl aliquots of isoetharine stock standards (made by serial dilution of the 100 μg/ml stock standard solution with 0.05 *N* H_2SO_4) to 1.0 ml of control human plasma (oxalate anticoagulant). Fresh standards were prepared for each analysis set and analyzed with the prepared samples. Values are reported as isoetharine.

Preparation of Spiked Plasma Samples—Two sets of samples, to be analyzed under single-blind conditions, were prepared in triplicate by adding appropriate volumes of isoetharine stock standard solutions to 1.0 ml of human control plasma. One set was analyzed upon preparation; the other set was frozen for 7 days at −70° before analysis.

Analytical Procedure for Spiked Samples—To each tube containing a sample or standard were added 50 ng of internal standard (100 μl of a 500-ng/ml solution in 0.05 *N* H_2SO_4), 2.0 ml of phosphate buffer (pH 6.9) and 10 ml of 1.5% di-(2-ethylhexyl)phosphoric acid in benzene solution. The tube was shaken and centrifuged. The organic phase was transferred, with methanol-washed disposable pipets, to a tube containing 130 μl of 0.2 *N* H_2SO_4. The tube was shaken and centrifuged, and the organic phase was discarded. The tube was placed in the laboratory freezer (−4°) until chromatographic analysis (usually the next day), at which time the aqueous phase was thawed, and a 100-μl aliquot was injected into the chromatograph.

Chromatography—The HPLC was a modular system constructed of commercially available components: a reciprocating pump[7], syringe-loaded injection valve[8], a 5-μm ODS (25 cm × 4.6-mm i.d.) column[9], and an amperometric (electrochemical) detector with a glassy carbon electrode[10]. The chromatographic conditions were as follows: mobile phase, 0.1 *M* Na_2SO_4 (adjusted to pH 2.8 with phosphoric acid and then adjusted to pH 3.0 with NaOH)–methanol (87.5:12.5, v/v); flow rate, 1.2 ml/min (~150 bar); applied potential, +0.60 V *versus* Ag/AgCl (3 *M* NaCl); at ambient temperature; an injection volume of 100 μl; and approximate retention times of 7.5 min for colterol (internal standard) and 9.3 min for isoetharine.

[7] Milton Roy Minipump, Riviera Beach, Fla.
[8] Model 7120, Rheodyne, Cotati, Calif.
[9] Ultrasphere-ODS, Altex, Woburn, Mass.

Table I—Concentrations of Isoetharine (Free Base) Found in Spiked Plasma Samples

Nominal Concentration, ng/ml	Mean Concentration Found[a], ng/ml	
Minimum Quantifiable Level[b]		
0		
5.5		5.7
	% *SEM*	2.1%
	Mean % Difference	+4.25%
	% *CV*[c]	5.1%
19		19.4
	% *SEM*	0.9%
	Mean % Difference	+1.9%
	% *CV*	2.1%
38		39.4
	% *SEM*	1.3%
	Mean % Difference	+3.6%
	% *CV*	3.2%
88		91.3
	% *SEM*	1.0%
	Mean % Difference	+3.8%
	% *CV*	2.4%

[a] $n = 6$. [b] Mean minimum quantifiable level = 0.92 ng/ml, $n = 2$. [c] Coefficient of variation.

Animal Dosing and Sample Collection—Two groups of six Sprague-Dawley rats[11] each were medicated orally with isoetharine hydrochloride or mesylate. Each animal received the equivalent of 120 mg of isoetharine base *via* a stomach tube as a solution in distilled water. A minimum of 0.2 ml of blood was collected from the orbital sinus in microcentrifuge tubes containing oxalate anticoagulant. Samples were collected prior to medication and at 1, 2, 4, 6, 8, and 10 hr after medication. At 12 hr after medication, blood was collected from the abdominal aorta. The blood was centrifuged and the plasma was separated. The plasma samples were stored in a low-temperature freezer (−70°) until analysis.

In a separate exploratory dose-ranging study, 10 rats/group were medicated orally with isoetharine hydrochloride for 2 weeks at doses of 0, 800, 1400, 2000, and 2500 mg/kg/day. At the end of two weeks, blood samples were obtained from the orbital sinus 1 hr after medication. Plasma was separated and frozen until analysis for isoetharine levels. One rat in the 2500-mg/kg group died during the study.

Sample Preparation—The plasma samples were analyzed by the procedure described for spiked samples with several modifications required because of the small sample volumes. Aliquots of the plasma samples (25–150 μl) were transferred to screw-cap tubes, and 100 μl (5.0 ng) of a solution of colterol (50 ng/ml in 0.05 *N* H_2SO_4) was added as internal standard. Two milliliters of 2% boric acid, 2 g of ammonium sulfate, and 10 ml of 0.5% di-(2-ethylhexyl)phosphoric acid in benzene solution were added. Each tube was shaken and centrifuged, and the organic phase was transferred into a conical tube containing 130 μl of 0.05 *N* H_2SO_4. The tube was shaken and centrifuged; the organic phase was aspirated and discarded. The aqueous phase was frozen until liquid chromatographic analysis on the following day. Duplicate standards containing 0, 0.5, 1, 2, 4, 6, 8, 10, and 15 ng of isoetharine (free base) in 100 μl of control plasma were used for calibration of the chromatograph. Fresh standards were prepared and extracted with each analysis set.

Statistical Analysis—A regression analysis of the peak height ratios (isoetharine/internal standard) obtained for the standards was performed to determine the linearity of the response with respect to concentration. The resulting linear regression was used to estimate the concentrations of isoetharine in the plasma samples. The minimum quantifiable level of the assay was estimated as the concentration whose 80% confidence limit just encompassed zero[12].

[10] Model LC-4 controller and model TL-5 cell, Bioanalytical Systems, West Lafayette, Ind.
[11] Charles River Co.
[12] R. W. Ross and H. Stander, paper presented at the Princeton Conference on Applied Statistics, Dec. 1975.

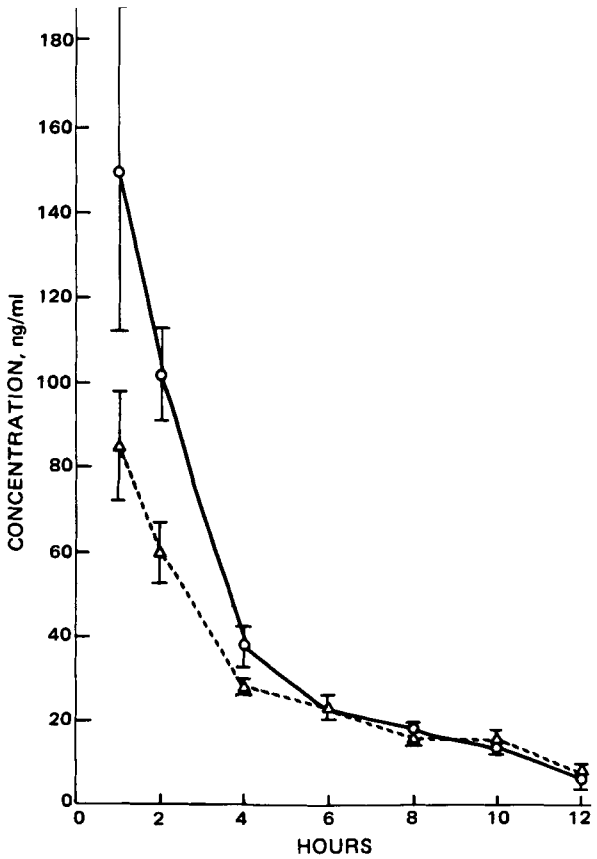

Figure 2—*Plot of mean isoetharine plasma concentration after oral administration of isoetharine hydrochloride or isoetharine mesylate. Key: (○) hydrochloride salt administered; (△) mesylate salt administered. Vertical bars represent the standard error of the mean.*

The assayed levels for the spiked samples were expressed as the percent differences from the nominal values, and a two-way ANOVA with replication was carried out to determine if there was a concentration effect, a time (fresh *versus* frozen) effect, or a concentration times time interaction.

An ANOVA was carried out to determine differences between the two salt forms in terms of maximum observed concentration (C_{max}), the time of the maximum observed concentration (t_{max}), apparent first-order elimination rate constant (K), and the area under the plasma concentration *versus* time curve (AUC). C_{max} and t_{max} were determined by direct observation; the elimination half-life for each rat was determined from a least-squares regression on the logarithm of the 4–12-hr plasma concentration data with time. This assumes first-order elimination kinetics. The AUC was determined from the plasma data by the trapezoidal rule. The data from the dose-ranging study were analyzed by a least-squares regression to determine if observed plasma levels were dose related.

RESULTS AND DISCUSSION

Representative chromatograms of extracted rat plasma standards are shown in Fig. 1. Linear least-squares analysis indicated that peak height ratio (isoetharine/internal standard) *versus* concentration was linear over a range of at least 0 and 0.5–100 ng/ml.

The results of the single-blind analysis of the spiked plasma samples are summarized in Table I. The accuracy of the assay, defined as the mean percent difference from the nominal level was >5%. The estimated assay precision, calculated from the overall mean square error term of the two-way ANOVA, was ±1.9%. The mean (±SE) minimum quantifiable level of the assay was 0.92 (±0.20) ng/ml, n = 2. The mean (±SE) percent recovery of the assay, determined by comparison with unextracted standards, at concentrations of 10, 50, and 100 ng of isoetharine/ml and 50 ng of internal standard/ml, was 78.4 (±2.31%) for isoetharine and 79.1 (±3.6%) for the internal standard.

The two-way ANOVA with replication on the percent differences for the samples indicate neither a concentration effect nor a concentration times time interaction. A highly significant time (fresh *versus* frozen) effect was indicated; however, the trend was to a higher concentration upon freezing. The observed differences were due to a slight difference in the slopes derived from the regression analysis on the standards for the two days, and the difference was statistically significant because of the good precision at each concentration. The observed time effect is considered of little practical significance. The stability of isoetharine and the internal standard in the final acid extract was shown by chromatographing the final acid extract for a set of standards twice, separated by 8 weeks of storage at −4°. The chromatograms obtained on the two days indicated no apparent decomposition of either isoetharine or the internal standard.

The results of the analysis of rat plasma samples, taken after the oral administration of isoetharine hydrochloride and isoetharine mesylate are plotted as means *versus* time of collection in Fig. 2. The mean (±SE) maximum observed plasma concentrations (C_{max}) for the hydrochloride and mesylate salts were 162 (±32) and 92.0 (±9.8) ng/ml, respectively. The mean (±SE) times to C_{max} (t_{max}) were 1.2 (±0.2) and 1.3 (±0.2) hr for the hydrochloride and mesylate salts, respectively.

The area under the plasma concentration *versus* time (AUC) plot was calculated for each rat by the trapezoidal rule using the experimental data. The mean (±SE) AUC was 498 (±56) ng hr/ml for the hydrochloride salt and 344 (±16) ng hr/ml for the mesylate salt.

The apparent first-order elimination rate constant, K, was determined for each animal using the 4- to 12-hr data. The mean (±SE) values of K were 0.21 (±0.04) hr^{-1} for the hydrochloride salt and 0.17 (±0.03) hr^{-1} for the mesylate salt. The corresponding half-lives are 3.3 and 4.1 hr, respectively. It should be emphasized that these are crude estimates of the rate constants since they were determined from data collected over a time period of approximately two half-lives.

ANOVA on the means of C_{max}, t_{max}, and K for the two salts indicated that the differences were not statistically significant ($p \geq 0.05$). The difference between the AUC means for the two salts was significant at the 5% level but not at the 1% level. The hydrochloride salt had the larger AUC (498 ng hr/ml *versus* 344 ng hr/ml).

The observed isoetharine concentrations in rats medicated orally for 2 weeks were highly variable. A least-squares analysis of the data was inconclusive in determining whether the observed plasma levels were proportional to dose in the range of 800–2500 mg/kg.

The formation of an ion-pair with di-(2-ethylhexyl)phosphoric acid provided a simple, rapid means of isolating two exogenous catecholamines from plasma for subsequent quantitation by reversed-phase liquid chromatography. The method is applicable to small (<100 μl) samples and samples up to 2 ml. In this study, the described extraction procedure proved more reliable and gave cleaner chromatograms than adsorption to either alumina or boric acid gels. The usefulness of the assay is demonstrated in that small samples could be analyzed, allowing serial samples to be taken from individual rats, and in the analysis of samples from rats medicated over a 20-fold range of doses.

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