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LIQUID CHROMATOGRAPHIC DETERMINATION OF SOTALOL IN PLASMA AND URINE EMPLOYING SOLID-PHASE EXTRACTION AND FLUORESCENCE DETECTION

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SUMMARY

A liquid chromatographic method using a solid-phase extraction procedure for the quantification of sotalol in plasma and urine is described. Sotalol is eluted from an extraction column with ethyl acetate–acetonitrile (1:2) and, after separation by reversed-phase high-performance liquid chromatography on a μBondapak C_{18} column, is quantified by fluorescence detection at excitation and emission wavelengths of 240 and 310 nm, respectively. The method has been demonstrated to be linear over the concentration ranges 10–6000 ng/ml in plasma and 0.5–100 μg/ml in urine. Mean inter-assay accuracy of the method for plasma ranged from 93 to 100% and for urine from 102 to 114%; precision ranged from 0.5 to 1.6% for plasma over a concentration range of 200–4000 ng/ml and for urine from 0.7 to 2.0% at concentrations of 2–50 μg/ml. Mass spectrometry confirmed the presence of sotalol in isolated chromatographic fractions of plasma and urine extracts from subjects given sotalol orally.

INTRODUCTION

Sotalol, 4′-[1-hydroxy-2-(isopropylamino)ethyl]methanesulfonanilide, is a β-adrenergic blocking agent without intrinsic sympathomimetic activity which exerts both class II and III antiarrhythmic effects [1–3]. Its structure is shown in Fig. 1.

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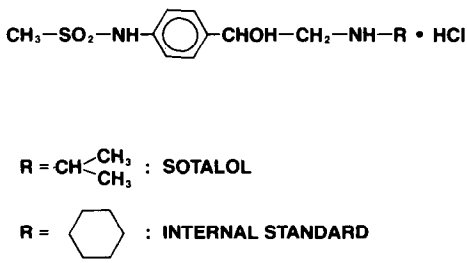

Fig. 1. Structural formulae of sotalol and internal standard.

Early quantification of sotalol utilized measurements of fluorescence following extraction with 1-pentanol–chloroform from both buffered plasma and urine, and subsequent partition into aqueous acid [4]. The method was linear from 0.1 to 10 μg/ml with a lower limit of sensitivity of 0.1 μg/ml of plasma and 2.5 μg/ml of urine. It suffered from the lack of specificity usually encountered with procedures which do not incorporate chromatographic or other purification steps. Sotalol has been determined by gas chromatography (GC) [5] but the method was never employed to support clinical studies.

An assay was described for measuring sotalol in plasma and tissues using high-performance liquid chromatography (HPLC) followed by ultraviolet detection [6]. An internal standard was not used in that procedure. Employing reversed-phase HPLC, Blair et al. [7] and Kärkkäinen [8] reported analytical procedures for determining sotalol in plasma and urine. More recently, liquid–liquid extraction and separation by HPLC followed by fluorometric detection was used to quantify sotalol [9–11]. Fluorescence detection improves the specificity of the method and reduces interference from endogenous components. The method to be described here utilizes solid-phase extraction rather than liquid–liquid extraction; this reduces both sample preparation time and the volume of solvents used, and provides for improved accuracy and precision.

EXPERIMENTAL

Materials

The hydrochloride salts of sotalol and the internal standard, 4′-(2-cyclohexylamino-1-hydroxyethyl)methanesulfonanilide (Fig. 1), were prepared by Bristol-Myers (Evansville, IN, U.S.A.).

Ethyl acetate, acetonitrile and methanol were HPLC grade from Fisher Scientific (Fairlawn, NJ, U.S.A.); sodium hydroxide, electrolytic pellets, were also from Fisher Scientific. Water was double-distilled in glass. Dibasic ammonium phosphate (analytical-reagent grade) was from Mallinckrodt (St. Louis, MO, U.S.A.). Bicine [N,N-bis(2-hydroxyethyl)glycine] was from Calbiochem-Behring (San Diego, CA, U.S.A.).

Human blood was obtained from Ohio Valley Blood Services (Evansville, IN, U.S.A.). The plasma was obtained by centrifugation of Na_2EDTA-treated blood. Plasma units for use as control plasma in preparing standards were combined

and stored frozen. Control urine, obtained from healthy volunteers in the laboratory, was also pooled and stored frozen.

The elution solvent was ethyl acetate–acetonitrile (1:2, v/v). Bicine buffer (2.0 *M*) was prepared by adjusting the pH of a weighed amount of Bicine to 9.3 with 10 *M* sodium hydroxide followed by minimal dilution to the calculated volume. Buffers of lower concentration were prepared by dilution with water.

Primary standard solutions of the hydrochloride salts of sotalol and the internal standard were prepared in methanol at concentrations of 1.0 mg/ml. Secondary standard solutions of sotalol for spiking plasma and urine standards were prepared at several intermediate concentrations by making serial dilutions in methanol. Secondary standard solutions of internal standard were prepared by dilution with methanol at concentrations of 0.1 mg/ml for urine assays and at 0.01 mg/ml for plasma assays. All standards were stored at 4°C when not in use and were prepared monthly. All concentrations of sotalol and internal standard are expressed in terms of the free base.

Solid-phase extraction was accomplished using a Vac Elut vacuum manifold from Analytichem International (Harbor City, CA, U.S.A.) and Baker-10 SPE Octyl (C_8) disposable extraction columns from American Hospital Supply Corporation (McGaw Park, IL, U.S.A.). The eluates were collected in 12×75 mm disposable culture tubes from Fisher Scientific. Solvent was removed with an N-Evap analytical evaporator from Organomation Assoc. (Northborough, MA, U.S.A.) at a water-bath temperature of 30°C and using purified nitrogen.

Instrumentation

The HPLC system consisted of a Model 6000A solvent delivery system from Waters Assoc. (Milford, MA, U.S.A.), a Waters WISP 710B automatic injector, a Model 650-10S fluorescence detector from Perkin-Elmer (Norwalk, CT, U.S.A.), an Omniscribe Model D5217-1A4 dual-pen recorder from Houston Instruments Division (Austin, TX, U.S.A.), and a Model 3357 laboratory automation system from Hewlett-Packard (Sunnyvale, CA, U.S.A.).

A Model 4500 gas chromatographic–mass spectrometric (GC–MS) instrument from Finnigan MAT (San Jose, CA, U.S.A.) equipped with a conversion dynode electron multiplier was used for validation of the method's specificity. An INCOS data system was used to control the gas chromatograph and mass spectrometer, monitor selected ions and store data.

Extraction

The disposable extraction columns were placed in a Vac Elut manifold and washed by rinsing under vacuum (10–14 mmHg) twice with 3 ml of methanol and once with 3 ml of 0.17 *M* Bicine buffer, pH 9.3. A 1-ml volume of plasma, 500 ng (50 μl of a 0.01 mg/ml solution) of internal standard and 0.5 ml of 0.5 *M* Bicine buffer, pH 9.3, were mixed and transferred to a washed column. After sample application, the column was rinsed twice with 2 ml of 0.17 *M* Bicine buffer, with the second rinse drawn through by vacuum. Collection tubes (5 ml) were then positioned in the Vac Elut manifold, and sotalol and internal standard were eluted with successive 2-ml and 1.5-ml volumes of ethyl acetate–acetonitrile

extraction solvent. The aqueous layer in the collection tube was frozen in dry ice–methanol and the solvent layer containing the analytes was decanted into a conical glass-stoppered tube. The solvent was evaporated to dryness under nitrogen at 45 °C, the residue reconstituted in 200 μl of methanol, centrifuged for 5 min at 1000 *g*, and a measured volume injected for liquid chromatographic separation and detection.

For the analysis of urine, 1 ml of sample, 5 μg of internal standard and 0.5 ml of 2.0 *M* Bicine buffer (pH 9.3) were mixed together and transferred to a washed column. Except for the column rinse step, which consisted to two successive 1-ml rinses with 0.5 *M* rather than 0.17 *M* Bicine buffer, the urine procedure was the same as that described for plasma.

Chromatography

The analytes were separated on a 30.0 cm × 3.9 mm μBondapak C_{18} reversed-phase column, 10 μm particle size, from Waters Assoc. using a mobile phase of water–methanol–acetonitrile–0.1 *M* dibasic ammonium phosphate (45:48:6:1, v/v). Chromatography was performed at ambient temperature using a flow-rate of 1.5 ml/min. Under these conditions the k' values for sotalol and internal standard were 8.3 and 14.4, respectively. The eluent was monitored at excitation and emission wavelengths of 240 and 310 nm, respectively, with a chart speed of 0.5 cm/min. Quantification of detector response was based upon measurement of peak-height or integrator response. The analyte/internal standard response ratios from a set of standards were used to calculate a least-squares regression equation which was then utilized to quantify the amount of sotalol present in the samples.

RESULTS AND DISCUSSION

A representative chromatogram of a human control plasma extract is shown in Fig. 2A and one containing 250 ng of sotalol and 500 ng of internal standard per ml of plasma is shown in Fig. 2B. No interfering peaks with the same retention time as sotalol or internal standard were present in control urine.

To further confirm the specificity of this procedure, plasma and urine samples from subjects given sotalol hydrochloride orally in a clinical investigation were extracted as described in the preceding section. The extracts were separated chromatographically, and the material from several separations which eluted with the same retention time as authentic sotalol was collected and pooled separately for each matrix. The sample volume was reduced, 0.5 ml of 2 *M* Bicine pH 9.3 was added, and the aqueous phase shaken with 5.0 ml of diethyl ether. After centrifugation the lower aqueous layer was frozen, the ether phase was decanted, the solvent was evaporated under argon, and the residue submitted for mass spectroscopic analysis. For this evaluation the samples were dissolved in methanol and a portion of each was analyzed using thermospray ionization in a liquid chromatograph–mass spectrometer. The analytes were separated on a μBondapak C_{18} column using a mobile phase of 0.1 *M* ammonium acetate–methanol–acetonitrile (46:48:6) at a flow-rate of 1.3 ml/min. The eluate from the column was routed completely to the interface where it was vaporized and ionized with ammonia

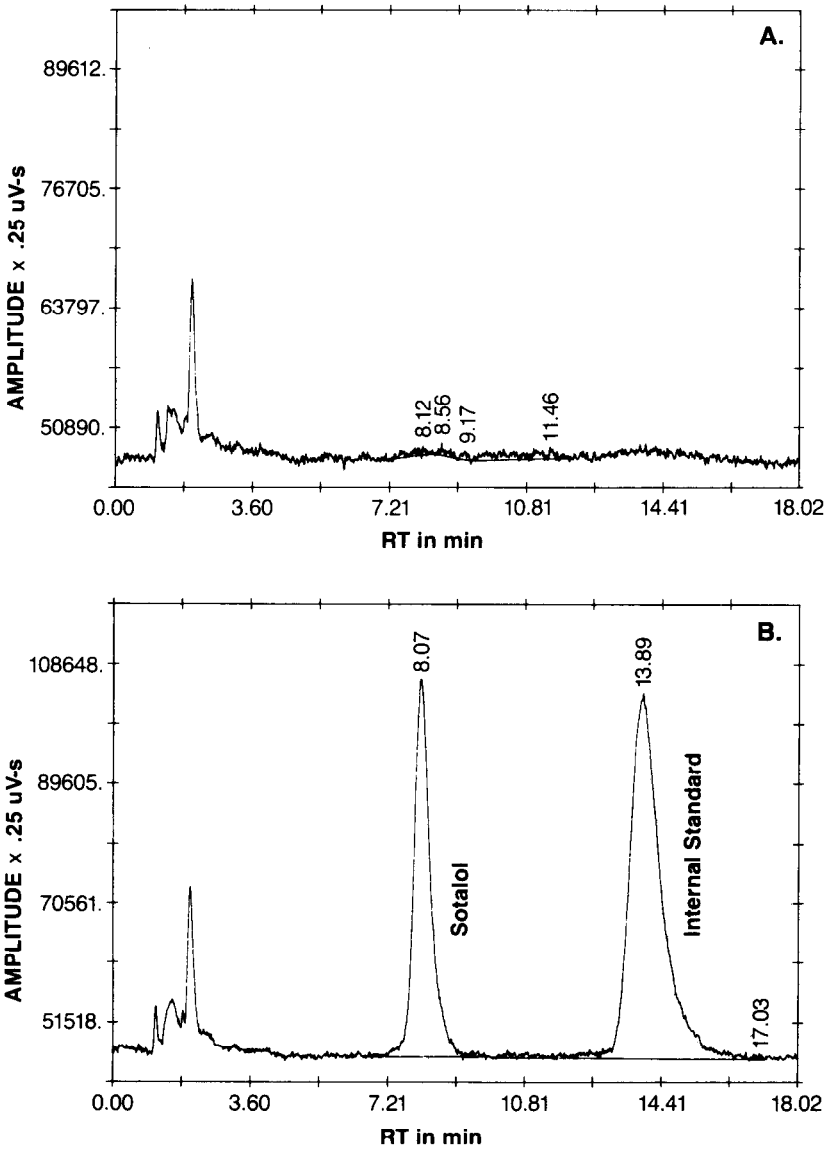

Fig. 2. Chromatograms of human plasma: (A) control; (B) spiked with 250 ng of sotalol and 500 ng of internal standard.

reagent gas. The resulting ions were detected while scanning from 150 to 500 a.m.u. every 2 s. The material quantified as sotalol in the plasma and urine extracts had the same retention time (3 min, 40 s) as that of authentic sotalol. The identity of the material was further evaluated by examination of its fragmentation pattern. Fig. 3 shows the mass spectrum of authentic sotalol and Figs. 4 and 5 exhibit the mass fragmentation patterns of the material in the HPLC fractions from plasma and urine, respectively, from subjects receiving sotalol; the charac-

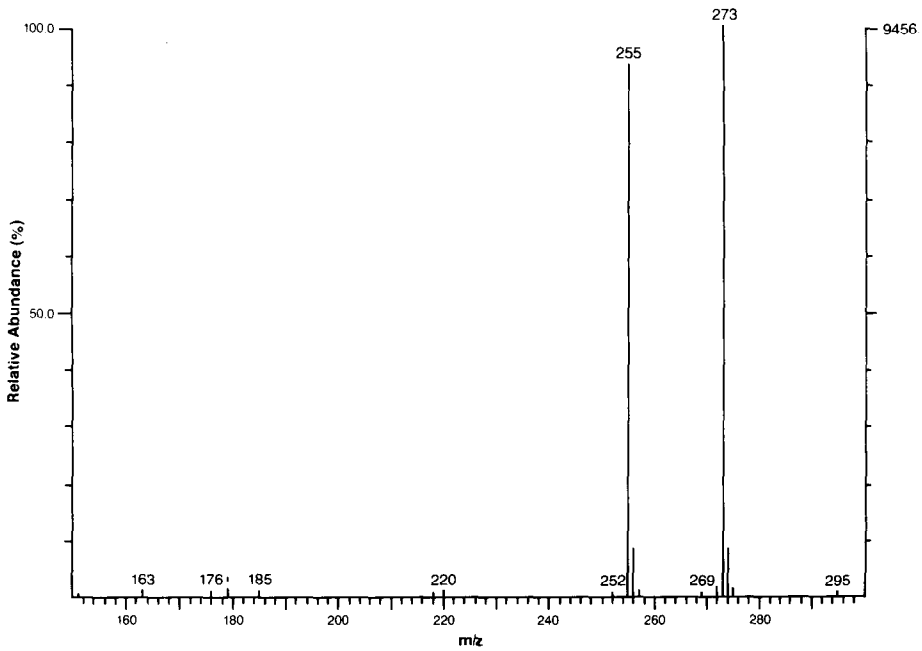

Fig. 3. Mass fragmentation pattern of sotalol standard.

teristic mass spectral peaks at *m/e* 273 and 255 match those of the authentic compound, specifically identifying the material being quantified as sotalol.

A series of sotalol standards ranging in concentration from 10 to 6000 ng/ml in plasma and from 0.5 to 100 μg/ml in urine were analyzed in duplicate. The least-squares regression equations for the calibration curves in plasma and urine were: $y=0.00324x-0.067810$ ($r=0.9999$) and $y=0.36591x+0.050698$ ($r=0.9996$), respectively. The data from each curve were evaluated using a lack-of-fit statistic [12] and were found not to deviate significantly from linearity over the concentration range examined.

The lower limit of detection was established by analyzing, in duplicate, 1-ml plasma or urine samples from eleven different normal volunteers which contained either 0.0 and 10.0 ng of added sotalol per ml of plasma or 0.0 and 500 ng per ml of urine. The actual instrument responses, in μV/s or mm peak height, at the retention time of sotalol were measured and the mean responses are listed in Table I. The mean differences between the blank and the fortified plasma or blank and fortified urine samples were shown to be statistically significant ($p<0.001$) using a paired comparison *t*-test, demonstrating that sotalol may be detected in concentrations as low as 10 ng/ml in plasma and 500 ng/ml in urine.

To establish absolute recovery of sotalol duplicate sets of standards for each sample matrix were prepared, and the solvent was evaporated. A 1-ml volume of plasma or urine was added to one set and these were analyzed for sotalol while the other set was reconstituted with 100 μl of methanol and injected without further treatment. The absolute recovery was calculated as the ratio of the slope of the least-squares regression line for the extracted samples to that of non-

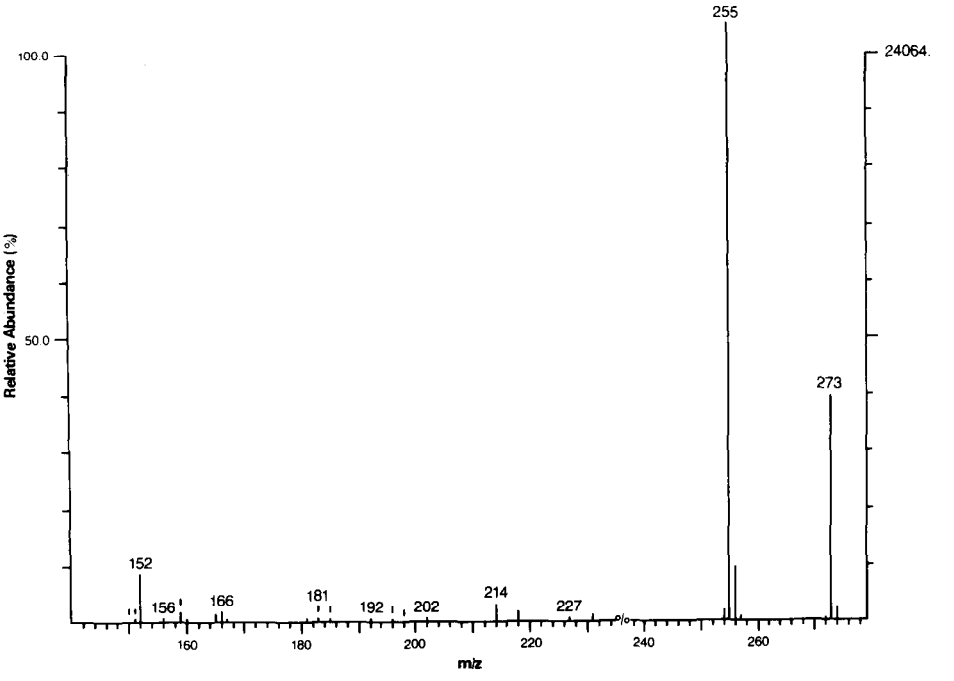

Fig. 4. Mass fragmentation pattern of sotalol HPLC fraction from plasma of a subject receiving sotalol.

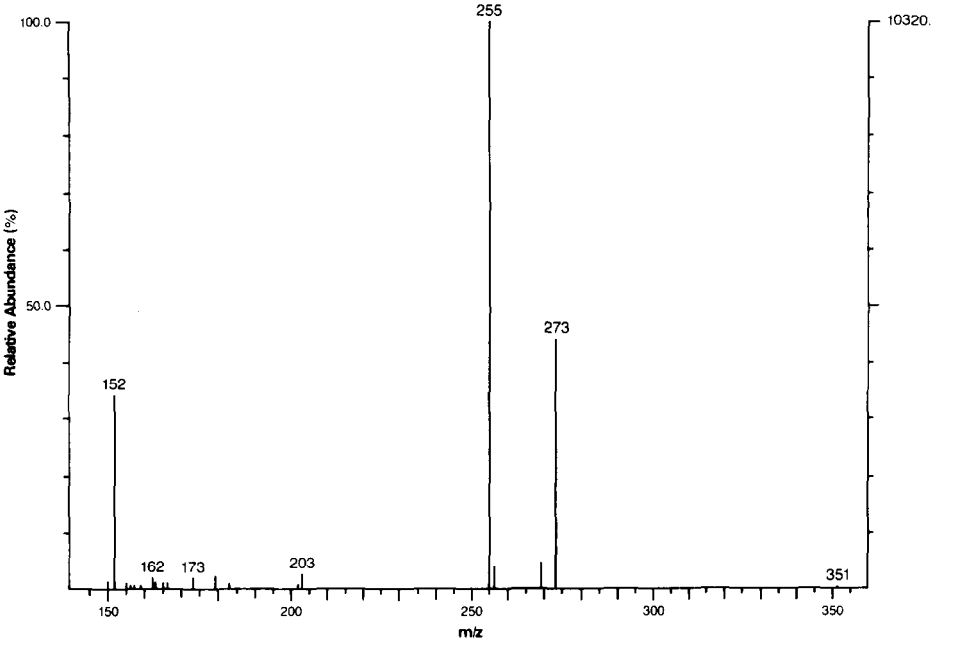

Fig. 5. Mass fragmentation pattern of sotalol HPLC fraction from urine of a subject receiving sotalol.

TABLE I

LOWER LIMIT OF QUANTIFICATION FOR DETERMINING SOTALOL IN PLASMA AND URINE

The fortification level was 10 ng/ml for plasma and 500 ng/ml for urine.

Sample	n	Detector response (mean ± S.D.) (μV/s)		t
		Blank	Fortified	
Plasma	11	1497 ± 481	4794 ± 464	16.78
Urine	10	9657 ± 13216	59311 ± 12799	33.69

TABLE II

INTER-ASSAY REPRODUCIBILITY OF THE METHOD FOR DETERMINING SOTALOL IN PLASMA AND URINE

Sample	Sotalol added (ng/ml)	Amount found (mean ± S.D.) (ng/ml)				R.S.D. (%)	Accuracy (%)
		Assay 1 (n=5)	Assay 2 (n=5)	Assay 3 (n=5)	Total		
Plasma	200	187 ± 6	185 ± 8	185 ± 5	186 ± 1	0.5	93
	800	778 ± 30	799 ± 34	802 ± 14	793 ± 13	1.6	99
	4000	3935 ± 65	4024 ± 119	3943 ± 107	3980 ± 63	1.6	100
Urine	2000	2328 ± 70	2240 ± 70	2300 ± 80	2289 ± 45	2.0	114
	20000	21330 ± 330	20570 ± 380	21100 ± 630	21000 ± 390	1.9	105
	50000	51078 ± 1125	51320 ± 1540	50640 ± 1140	51013 ± 345	0.7	102

TABLE III

STABILITY OF SOTALOL IN FROZEN PLASMA AND URINE

Sample	Weeks of storage	Amount found (mean ± S.D., n=5) (μg/ml)		
		dl-Sotalol	*l*-Sotalol	*d*-Sotalol
Plasma	4	1.01 ± 0.06		
	13	1.00 ± 0.05		
	34	1.09 ± 0.02		
	78	1.21* ± 0.04		
Urine	0		90.3 ± 12.3	86.2 ± 11.4
	5		103.8 ± 8.0	108.7 ± 10.5
	13		103.9 ± 13.8	102.8 ± 14.4
	25		87.5 ± 8.0	85.5 ± 7.3
	52		98.5 ± 9.5	94.4 ± 9.9

*Amount added, 1.2 μg/ml.

extracted samples. For plasma, the regression equations for the extracted and non-extracted standards were $y=270.8x+1683.1$ ($r=0.9999$) and $y=356.1x+446.8$ ($r=0.9998$), respectively, while for urine, the regression equations for extracted and non-extracted standards were $y=42895.7x+3392.8$ ($r=0.9989$) and $y=64344.5x-983.9$ ($r=0.9997$), respectively. The data indicate that the mean recoveries from plasma and urine over the concentrations studied were 76.0 and 66.7%, respectively.

The intra-assay precision and accuracy of the method were estimated by analyzing ten samples at each of two concentrations on the same day. Sotalol concentrations of 0.2 and 4 μg/ml in plasma and 2 and 50 μg/ml in urine were analyzed. The accuracy and precision, as determined by relative standard deviation (R.S.D., %) for determining sotalol in plasma at 0.2 and 4 μg/ml were $93 \pm 3\%$ and $98 \pm 2\%$, respectively; in urine the accuracy and precision at concentrations of 2 and 50 μg/ml were $118 \pm 3\%$ and $104 \pm 4\%$, respectively.

The inter-assay or day-to-day precision and accuracy of the method were determined in plasma and urine by analyzing five replicate samples of each of three concentrations of sotalol on three separate assay days. The three plasma concentrations of sotalol were 200, 800 and 4000 ng/ml and for urine the sotalol concentrations were 2, 20 and 50 μg/ml. The mean results are listed in Table II. The data for the three days were combined to provide an estimate of the inter-assay accuracy and precision of the method. For plasma the accuracy ranged from 93 to 100% and the precision from 0.5 to 1.6%; for urine the values for accuracy ranged from 102 to 114% and those for precision from 0.7 to 2.0%.

The stability in frozen plasma was assessed by preparing a number of samples of control plasma which had been fortified with *dl*-sotalol at a concentration of 1.0 or 1.2 μg/ml. These were stored in a freezer, and five samples were thawed and assayed after 4, 13, 34 and 78 weeks of storage. The results listed in Table III demonstrate that sotalol is stable in frozen plasma for up to 78 weeks. The stability of sotalol in frozen urine was similarly evaluated but using 100 μg of the pure *d*- and *l*-enantiomers rather than the racemic mixture. Assays were conducted after 5, 13, 24 and 52 weeks of frozen storage. The results, also listed in Table III, indicate that sotalol is stable in frozen urine for up to 52 weeks.

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