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DETERMINATION OF BUCINDOLOL AND ITS MAJOR METABOLITES IN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A method for determining bucindolol, a deuterated analogue and two metabolites in plasma samples is described. Analytes are obtained from plasma by liquid extraction of 1 ml of plasma at pH 10.5 with 7 ml of peroxide-free ether—hexane (4:1). Following derivatization with 50 μl of N-methyl-N-trimethylsilyl trifluoroacetamide—pyridine (1:1) for 20 min at 65°C the samples are analyzed by capillary column gas chromatography—mass spectrometry with selected-ion monitoring. Quantification is linear over the range 2–500 ng/ml. Single-day precision was typically 6.2% or better for bucindolol and 13% or better for the metabolites.

INTRODUCTION

Bucindolol, 2-[2-hydroxy-3-{[2-(3-indolyl)-1,1-dimethylethyl]amino}-

propoxy] benzonitrile (I), is a new antihypertensive drug whose chemical and biological properties have been reported [1–5]. Its structure is shown in Fig. 1.

This report provides analytical conditions for extraction and analysis of compound I, a deuterated analogue (II) and two active metabolites, utilizing gas chromatography–mass spectrometry (GC–MS). Plasma concentrations of I range from 0 to 300 ng/ml over the 24-h period following an oral dose of 100 mg, and the method was developed to cover this range. The method has been used for pharmacokinetic studies in which the unlabeled drug, stable-labeled drug and the metabolites are present in a biological sample.

EXPERIMENTAL

Materials

The following compounds were prepared by Bristol-Myers (Evansville, IN, U.S.A.): the hydrochloride salts of I (see Fig. 1); [$^2H_5$] bucindolol (II) (see Fig. 2) and internal standard (III) (see Fig. 3); the hydrate of 6-hydroxyindolyl bucindolol (IV) (see Fig. 4); and the acetate salt of 5-hydroxybenzonitrile bucindolol (V) (see Fig. 5).

Hexane, methanol and methyl-*tert.*-butyl ether were HPLC grade, and isopropanol was ACS-reagent grade, all from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Prior to use the ether was demonstrated to be peroxide-free by an iodine test as follows: 1 ml of ether was shaken with 10 ml of freshly prepared 10% (w/v) aqueous potassium iodide and protected from light for 10–20 min. If no color was produced in either phase when observed against a white background the ether was judged to be peroxide-free. Pyridine and N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) (both silylation grade) were from Pierce (Rockford, IL, U.S.A.). Potassium hydroxide (AR grade) was from Mallinckrodt (St. Louis, MO, U.S.A.). 3-(Cyclohexylamino)propanesulfonic acid (CAPS) 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_2-EDTA-treated blood. Bulk plasma units were combined and frozen for use as control plasma in preparing standards, validation spikes and quality-control samples.

Extraction solvent was a mixture of peroxide-free ether–hexane (80:20, v/v). K-CAPS buffer was prepared by dissolving a weighed amount of CAPS in distilled water, adjusting to pH 10.5 by adding 10 *M* potassium hydroxide and diluting to 1.5 *M* (CAPS) with distilled water.

Primary standard solutions of each analyte were prepared in methanol at 1 mg/ml. Combined secondary standard solutions of I, II, IV and V were prepared at several intermediate concentrations for spiking of plasma standards. A secondary standard solution of III was prepared at 4.0 μg/ml for spiking of samples and standards. The standards were stored at 4°C when not in use and were warmed to room temperature prior to use. All standards were prepared monthly, except compound IV which was prepared weekly. All concentrations are expressed in terms of the free base.

Solvent extraction utilized a Roto-Torque® rotator from Cole Parmer Instrument (Chicago, IL, U.S.A.). Solvent removal utilized an N-Evap analytical

evaporator from Organomation Assoc. (Northborough, MA, U.S.A.) with a water bath temperature of 30°C and an argon gas stream.

Sample extraction utilized clean, washed, 100 × 16 mm Pyrex screw-cap culture tubes from Corning (Corning, NY, U.S.A.). Final samples were derivatized in 100 × 13 mm screw-cap culture tubes from Corning. The tubes were sealed with PTFE-lined screw caps which were cleaned by soaking and rinsing in isopropanol.

Instrumentation

A Model 4500 gas chromatograph–mass spectrometer from Finnigan MAT Instruments (San Jose, CA, U.S.A.) equipped with a conversion dynode electron multiplier was used. An INCOS data system was used to control the gas chromatograph and mass spectrometer, monitor selected ions, store data, integrate areas of peaks on selected-ion chromatograms, generate calibration lines and calculate concentration values.

The gas chromatograph was enhanced by the addition of an on-column injector from J & W Scientific (Rancho Cordova, CA, U.S.A.), which was mounted directly over the standard packed column inlet which was lined with a 6 mm O.D., 2 mm I.D. glass tube and heated to 280°C. Injection of 1 μl of sample over a 2-s period was accomplished using a Model 701RNFS 10-μl on-column syringe from Hamilton (Reno, NV, U.S.A.) with the fused-silica needle protruding about 5 cm into the heated injector space after insertion. Between injections the syringe was thoroughly rinsed with methanol which was pulled through the syringe barrel under vacuum and which bathed the outer surface of the needle.

The chromatographic column was a 3 m × 0.25 mm I.D. fused-silica capillary column coated with DB-5 (bonded 5% diphenyl, 95% dimethylpolysiloxane) at a film thickness of 0.25 μm from J & W Scientific. It was routed directly to the ion source. The velocity of the helium carrier gas was approx. 200 cm/s. The interface oven temperature (between the gas chromatograph and mass spectrometer) was 280°C. The temperature of the gas chromatograph was held at 160°C for 1 min following injection; it was then increased at 20°C/min to 290°C.

The mass spectrometer was operated under electron-impact (EI) ionization conditions at 70 eV. For the full-scan mass spectra of the compounds contained in this report the quality of the mass spectrometer tuning was standardized by analyzing the mass spectral quality-control compound, decafluorotriphenyl phosphine, according to the method of Eichelberger et al. [6] and achieving the published spectral criteria.

For selected-ion monitoring (SIM) experiments the lens and offset voltages and the ion source magnet position were tuned for maximum response at the 314-a.m.u. ion of perfluorotributylamine while maintaining good ion peak shape and approximately unit resolution with the 315-a.m.u. ion. For sample analyte determinations five selected masses were sequentially and repetitively monitored, each for 0.105 s over an 0.25-a.m.u. window at the mass peak apex. The selected masses were 294, 305, 310, 393 and 398 a.m.u. Electron multiplier voltage was set at approx. 1200 V. Quantification was based on the

area under the chromatographic peak on the appropriate background-subtracted selected-ion chromatogram.

Procedures

A standard curve, consisting of duplicates at six concentration levels over the range 2–500 ng/ml, was prepared daily with each set of samples as follows: into extraction tubes were placed 10–200 μl of an appropriate combined methanolic secondary standard solution and the solvent was removed under argon; 1 ml of control plasma was then added to each tube; these standards were then extracted along with the samples.

Quality-control samples were also prepared as above, in multiples, upon receipt of the clinical samples and were stored with the samples under identical conditions to assure the integrity of sample storage and analytical methodology. When a set of clinical samples was withdrawn from storage for analysis a duplicate set of quality-control samples was also withdrawn. The results from these samples were utilized to characterize the accuracy and precision of the assays.

Plasma samples were extracted as follows: samples, quality-control samples and plasma for the standard curve and validation spikes were removed from nominal −17°C storage and thawed at room temperature. After mixing, a 1-ml aliquot of each sample was pipetted into a labeled extraction tube. Internal standard (100 ng) was added to each sample and the tubes were mixed. Then 0.2 ml of K-CAPS buffer was added and the tubes were mixed again. A 7-ml volume of extraction solvent was added and the tubes were sealed with screw caps and rotated on a Roto-Torque for 10 min at the maximum speed which permitted flow from top to bottom of the tube. The layers were separated by centrifugation at 1000 *g* for 2 min followed by freezing of the lower aqueous layer in a bath of dry ice and isopropyl alcohol. The upper organic layer was decanted into a clean 100 × 13 mm screw-cap tube and dried under a stream of argon. Residue on the side of the tube was rinsed to the bottom with 1 ml of extraction solvent and the solvent was again removed under a stream of argon. While still under argon the tubes were sealed with a PTFE-lined screw cap and stored at nominal −17°C for no more than 24 h prior to derivatization.

For derivatization the samples were warmed to room temperature, 25 μl of MSTFA and 25 μl of pyridine were added, then the tubes were capped and heated at 65°C for 20 min. After cooling, 1 μl was injected into the GC–MS system for assay.

The accuracy and precision of the method were characterized by several tests. Intra-assay variability, or within-day error, was studied by analyzing multiple identical spiked plasma samples at two concentration levels on one day. Inter-assay variability, or long-term error, was studied by analyzing multiple identical spiked plasma samples at two concentration levels on each of three days. Quality controls were analyzed in duplicate at two concentration levels on each day of analysis of samples from a clinical bioavailability study. These reflect the very-long-term accuracy and reproducibility of the method.

RESULTS AND DISCUSSION

The 70-eV EI mass spectra of the analytes and their trimethylsilyl derivatives

are presented in Figs. 1—5. An intense fragment ion, resulting from cleavage of the same carbon—carbon bond in each analyte molecule, was chosen for SIM and is responsible for the high sensitivity of the method despite sequential

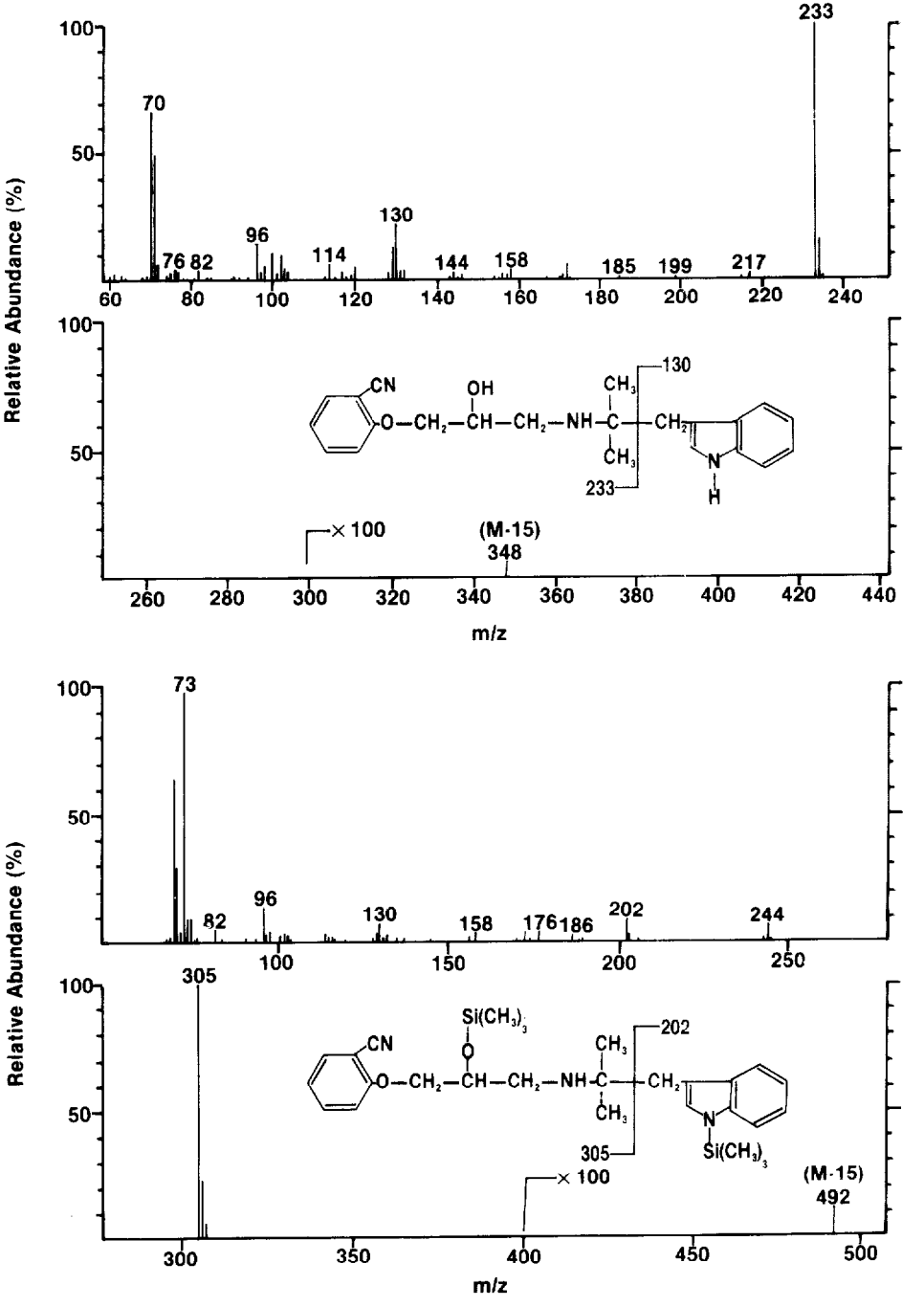

Fig. 1. Electron-impact mass spectra and structures of bucindolol, I (top), and of trimethyl silylated I (bottom).

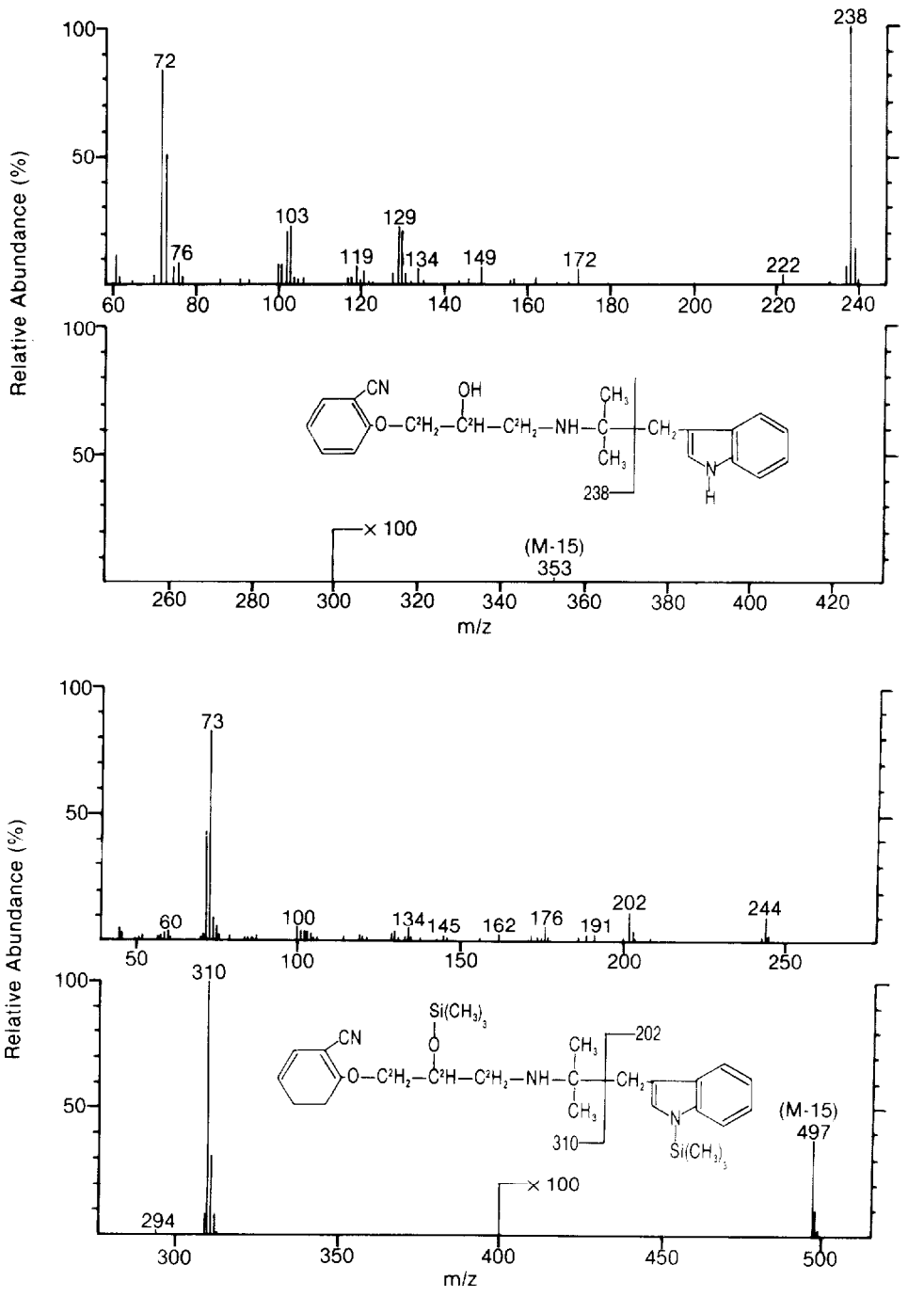

Fig. 2. Electron-impact mass spectra and structures of [$^2H_5$]bucindolol, II, indicating sites of deuteration (top), and of trimethylsilylated II (bottom).

monitoring of five separate ions. Chemical ionization is often regarded as a potentially more specific technique but was found to produce insufficient sensitivity in this instance.

Typical chromatograms from this method are shown in Fig. 6. A 20 ng/ml spiked processed standard is shown in Fig. 6A. The selected ions used for the quantification of each analyte and their respective retention times are also

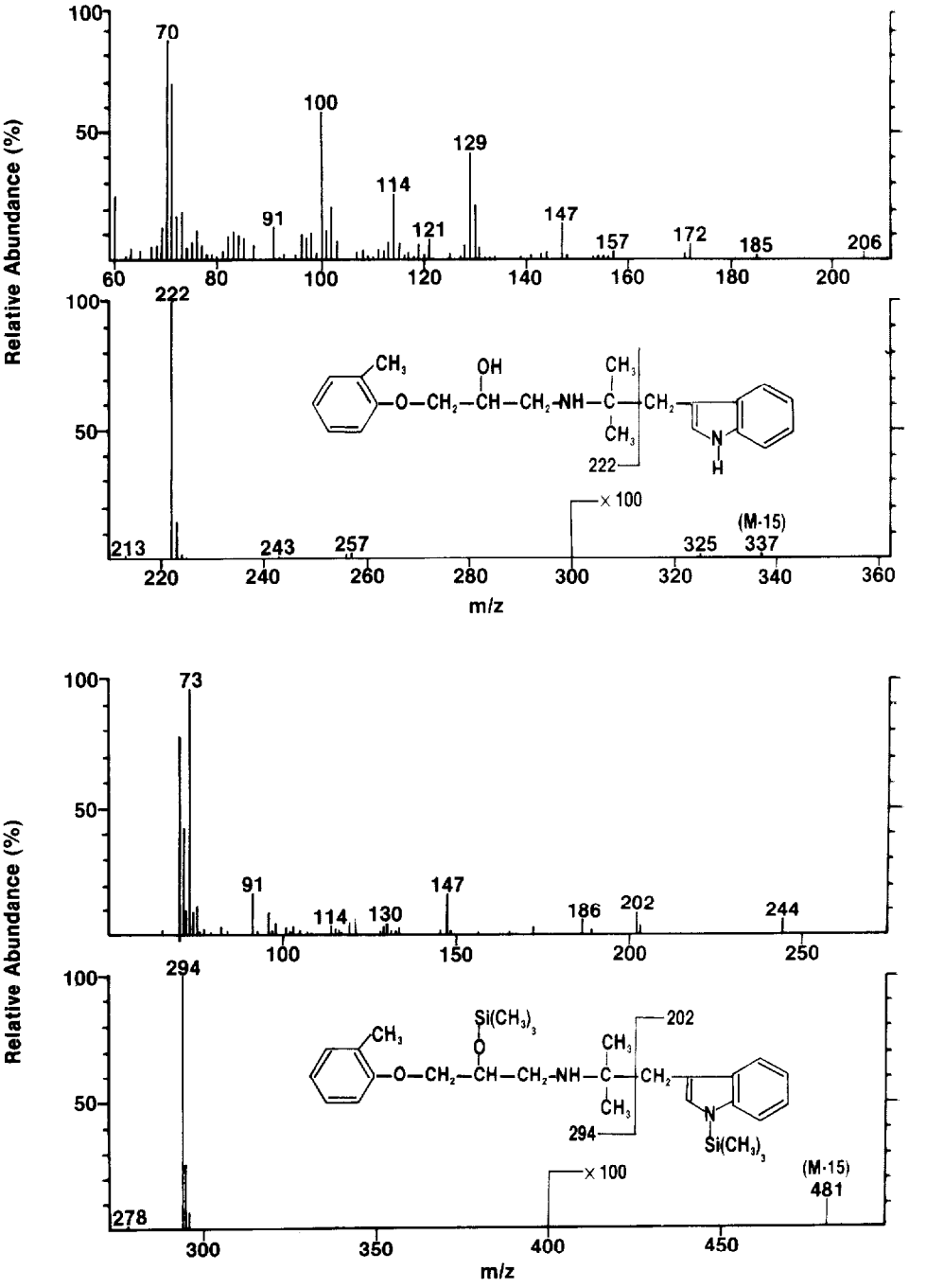

Fig. 3. Electron-impact mass spectra and structures of internal standard, III (top), and of trimethylsilylated III (bottom).

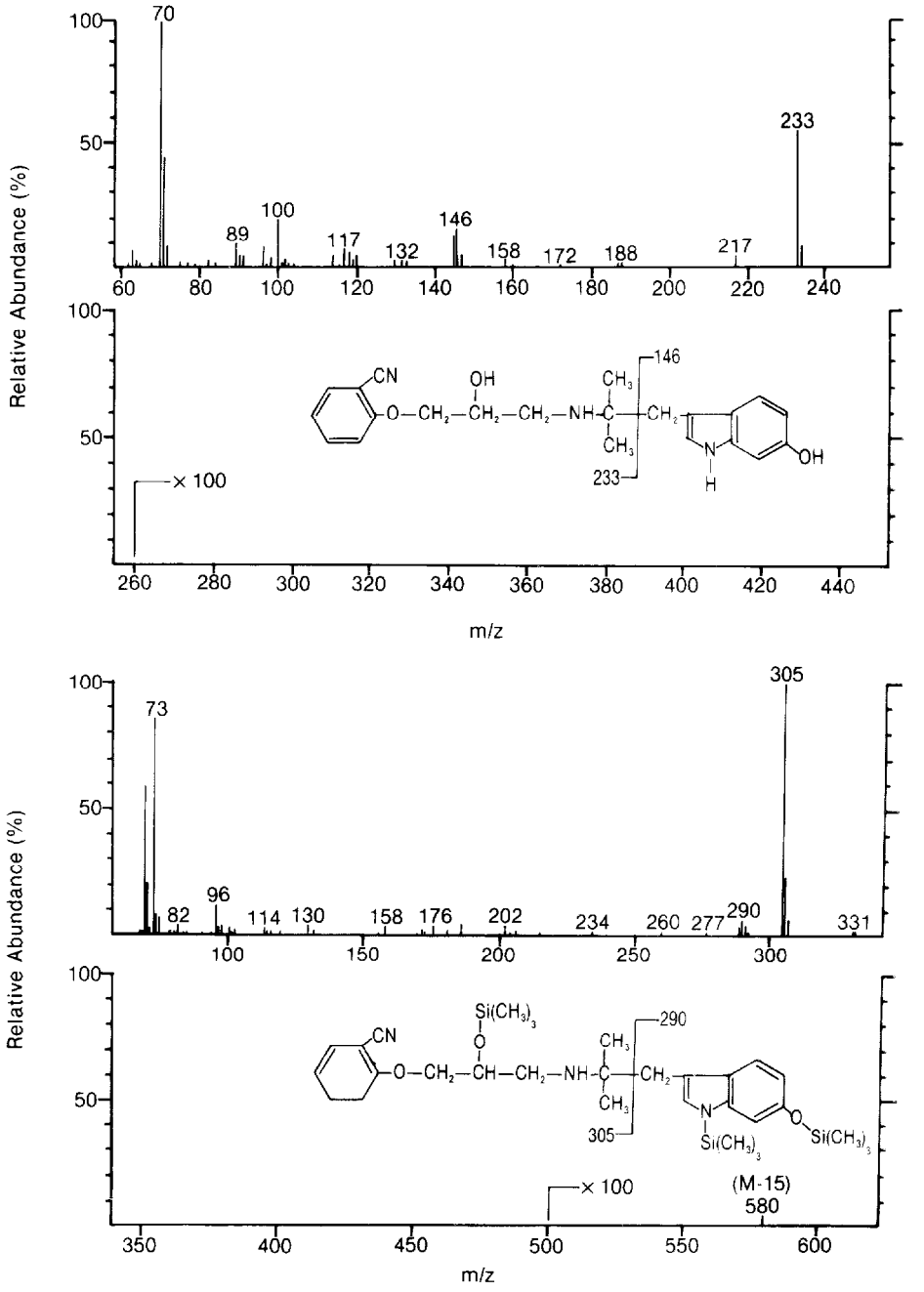

Fig. 4. Electron-impact mass spectra and structures of IV (top) and of trimethylsilylated IV (bottom).

listed. The selected-ion chromatograms at 310 and 398 a.m.u. also served for estimation of the pentadeuterated 6-hydroxyindolyl and 5-hydroxybenzonitrile metabolites of II. A typical blank which was spiked only with internal standard is shown in Fig. 6B.

No significant interfering peaks resulting from endogenous plasma sample constituents or sample preparation artifacts were observed. The typical background-subtracted noise component of the chromatograms can be seen in the figure as it is multiplied many times in contrast to the standard (Fig. 6A).

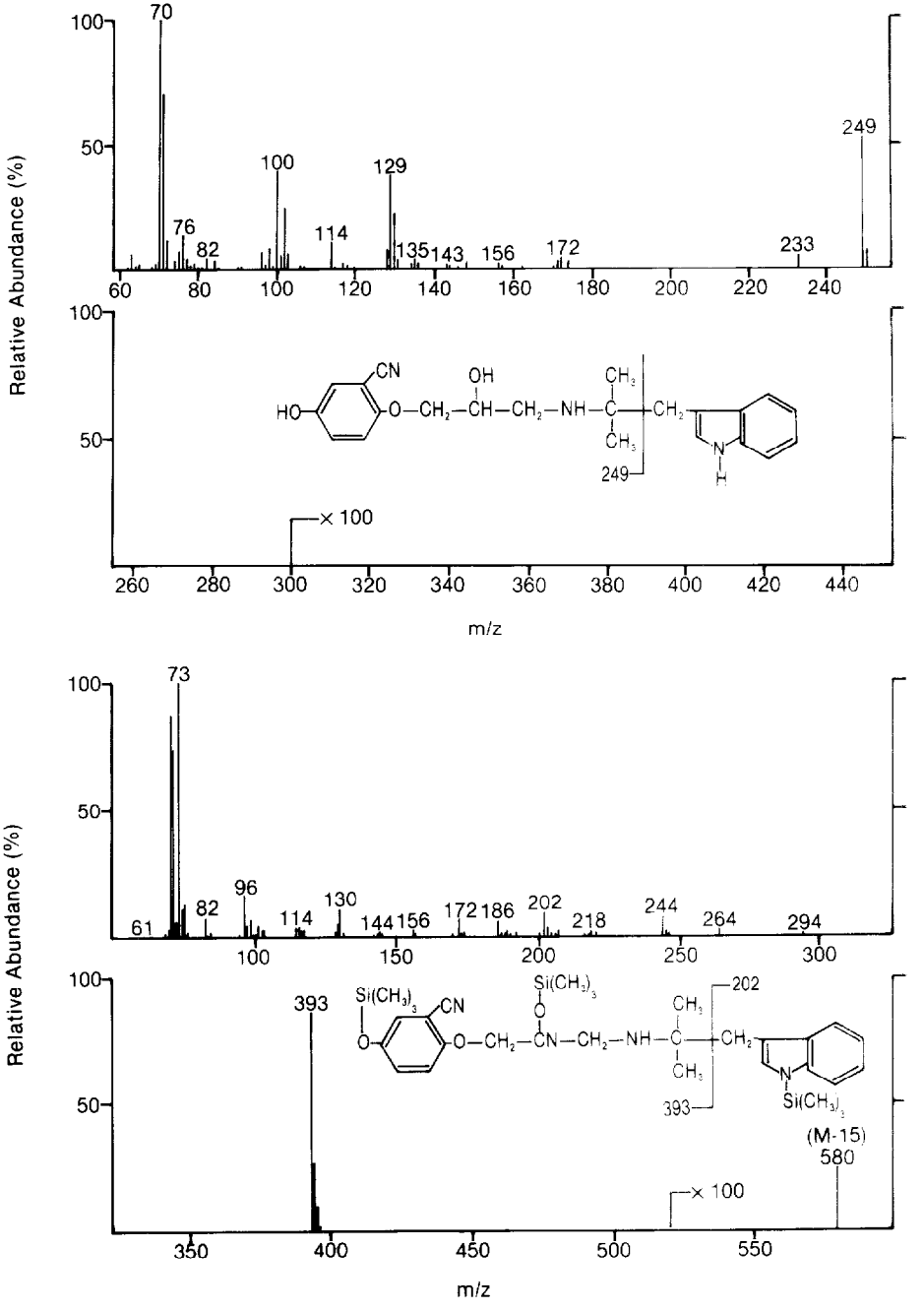

Fig. 5. Electron-impact mass spectra and structures of V (top) and of trimethylsilylated V (bottom).

TABLE I

ACCURACY AND PRECISION OF THE METHOD

Test*	n (X)**	Concentration added (ng/ml)	Concentration observed (mean ± S.D.) (ng/ml)			
			I	II	IV	V
Intra-assay	10 (1)	10	9.9 ± 0.6	9.7 ± 0.6	11 ± 1.4	11 ± 1.1
Intra-assay	10 (1)	100	85 ± 2.5	85 ± 2.8	85 ± 9.2	96 ± 5.1
Inter-assay	5 (1)	10	9.3 ± 0.7	9.8 ± 0.3	9.4 ± 1.2	9.6 ± 0.7
	5 (1)	10	9.9 ± 0.7	11 ± 0.6	11 ± 0.9	11 ± 0.6
	5 (1)	10	10 ± 0.6	10 ± 0.7	10 ± 0.9	9.6 ± 0.7
	15 (3)***	10	9.7 ± 3.8	10 ± 0.4	10 ± 1.0	10 ± 0.7
Inter-assay	5 (1)	100	98 ± 2.5	96 ± 2.3	93 ± 5.3	118 ± 6.3
	5 (1)	100	99 ± 3.8	98 ± 3.7	104 ± 11	115 ± 13
	5 (1)	100	95 ± 4.7	97 ± 5.3	90 ± 7.7	110 ± 9.8
	15 (3)***	100	97 ± 2.1	97 ± 1.0	96 ± 7.4	114 ± 4.0
Quality controls	42 (21)	20	20 ± 1.4	20 ± 1.6	21 ± 3.5	20 ± 2.8
Quality controls	42 (21)	150	148 ± 11	147 ± 10	145 ± 27	149 ± 22

*See text for a description of the tests.

**n is the number of data points over X days.

***Combination of the three days above.

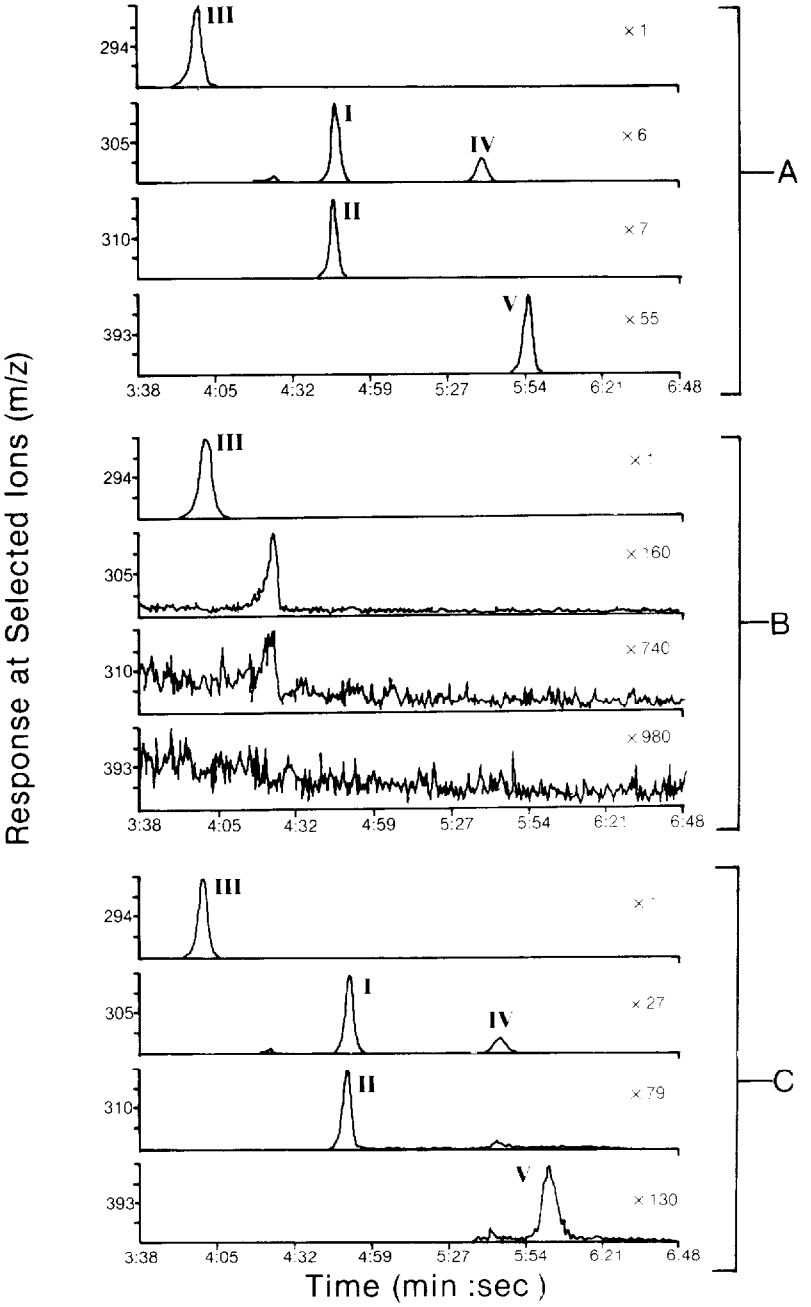

Fig. 6. Selected-ion chromatograms of plasma extracts. (A) Processed standard, spiked with 100 ng/ml internal standard and 20 ng/ml of each analyte. (B) Processed blank, spiked with 100 ng/ml internal standard only. (C) Processed clinical sample, spiked with 100 ng/ml internal standard only; sample concentrations in 5—10 ng/ml range.

Fig. 6C represents an extract from a clinical sample with analyte concentrations in the 5–10 ng/ml range. No additional metabolites were observed which interfere with the analytes despite the monitoring of a fragment ion compared to a potentially more specific molecular ion. Occasional slight shifts in the analyte absolute retention times were observed for the samples compared to the standards but the retention times of analytes relative to internal standard remained constant. This small variation was likely due to differing sample matrix composition and/or concentration.

The absolute recovery of analytes of the extraction procedure was determined over the range 2—500 ng/ml by comparing the responses of unextracted standards and of identically prepared samples. A linear regression of each data set was calculated. The ratio of the slopes of the extracted and unextracted sets was used as an estimate of the mean absolute recovery. The mean recoveries were 75, 72, 52 and 52% for compounds I, II, IV and V, respectively.

Good linearity of response was produced over the range 2–500 ng/ml as evaluated using a lack-of-fit statistic [7]. Typical linear regression equations were as follows: (I) $y = 0.008339x - 0.02150$, $r = 0.99996$; (II) $y = 0.006750x - 0.01688$, $r = 0.99996$; (IV) $y = 0.004512x - 0.00131$, $r = 0.99971$; (V) $y = 0.002303x - 0.01106$, $r = 0.99901$; where y = area analyte/area III and x = concentration analyte (ng/ml).

The data did not deviate significantly from linearity over the concentration range.

Extensive precision and accuracy data were obtained and are presented in Table I. The data indicate integrity of the samples during storage and reproducibility of the method over a number of samples and time periods.

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