The Use of Stable Isotope Labeling and Liquid Chromatography–Tandem Mass Spectrometry Techniques To Simultaneously Determine the Oral and Ophthalmic Bioavailability of Timolol in Dogs

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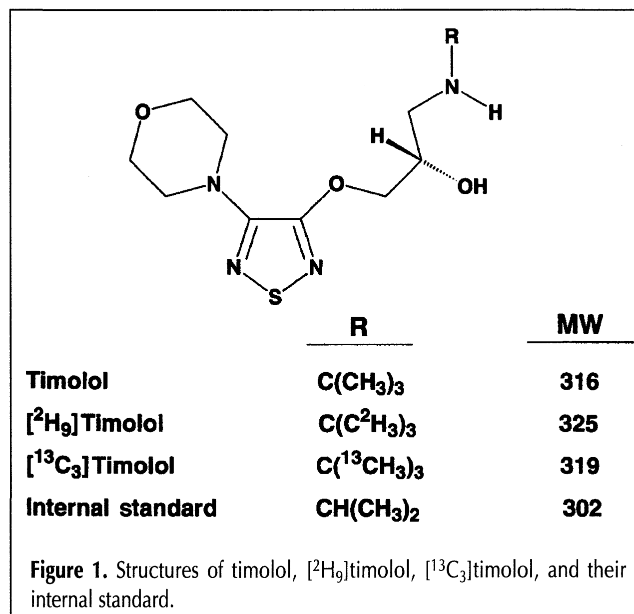
Abstract

Assays have been established for the quantitation of timolol and its $^{13}C_3$- and $^{2}H_9$-stable-isotope-labeled analogs in plasma and urine using liquid chromatography with atmospheric-pressure chemical-ionization tandem mass spectrometry. For the analysis of urine, underivatized timolol and its labeled analogs are monitored while timolol in plasma is assayed down to concentrations of 0.2 ng/mL after derivatization with phosgene. The great power of this technique is illustrated by simultaneously assaying three different species of timolol in plasma and urine obtained from dogs receiving simultaneous ophthalmic, oral, and intravenous doses of unlabeled and [$^{2}H_9$]- and [$^{13}C_3$]-labeled timolol. Thus, the ophthalmic and oral bioavailabilities of timolol are measured in a single experiment rather than as a three-phase crossover experiment. This approach yields accurate and precise analytical data, obviates intrasubject variability, and saves both analytical and animal resources.

Introduction

One of the great advantages of using mass spectrometry as a detection technique in quantitative bioanalytical chemistry is its ability to resolve isotopes. Gas chromatography–mass spectrometry (GC–MS) in conjunction with stable-isotope-labeled drugs has been applied to several types of pharmacokinetic studies (1). For example, drug bioavailability can be determined following simultaneous oral administration of a drug with its stable-isotope-labeled analog given intravenously (2–4). More subtle is the use of stable isotopes as "biological internal standards," in which, in a crossover experiment, two formulations of the same drug are tested, not by direct comparison of their pharmacokinetic parameters, but by comparison of their ratios with respect to a solution of the drug containing a stable isotope given along with each test formulation. By eliminating intra-subject variability, such studies have considerable statistical power that enables significant reduction of the number of subjects studied (5–6).

Such studies were frequently reported when GC–MS was widely used as a bioanalytical tool, but are less common since high-performance liquid chromatography (HPLC) became established as the most universal technique for measuring drugs in biological fluids. In the past few years, HPLC with MS, and particularly tandem MS (MS–MS), detection has become established as a very powerful bioanalytical procedure by providing sensitivity and, most importantly, high specificity to HPLC-based assays. The use of MS detectors in combination with the universal power of HPLC affords new opportunities to use stable isotopes in pharmacokinetic research. As an illustration of the power of tandem MS as an HPLC detection technique for stable-isotope-labeled drugs, we have revisited work conducted 17 years ago by our colleague, Bill VandenHeuvel, who used GC–MS for the quantitation of timolol after simultaneous oral and intravenous administration of timolol and [$^{2}H_9$] timolol, respectively, to human volunteers (4,7). Timolol is a non-selective beta adrenoreceptor antagonist designed for the treatment of cardiovascular disease and also, when formulated



	R	MW
Timolol	$C(CH_3)_3$	316
[$^{2}H_9$]Timolol	$C(C^{2}H_3)_3$	325
[$^{13}C_3$]Timolol	$C(^{13}CH_3)_3$	319
Internal standard	$CH(CH_3)_2$	302

Figure 1. Structures of timolol, [$^{2}H_9$]timolol, [$^{13}C_3$]timolol, and their internal standard.

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as an eye-drop solution, for the treatment of glaucoma. In the present work, we describe methods based on the use of LC–MS–MS which enabled simultaneous determination of the ophthalmic and oral bioavailability of timolol after simultaneous administration of an unlabeled drug ophthalmically, [$^{13}C_3$]timolol orally, and [$^{2}H_9$]timolol intravenously to dogs.

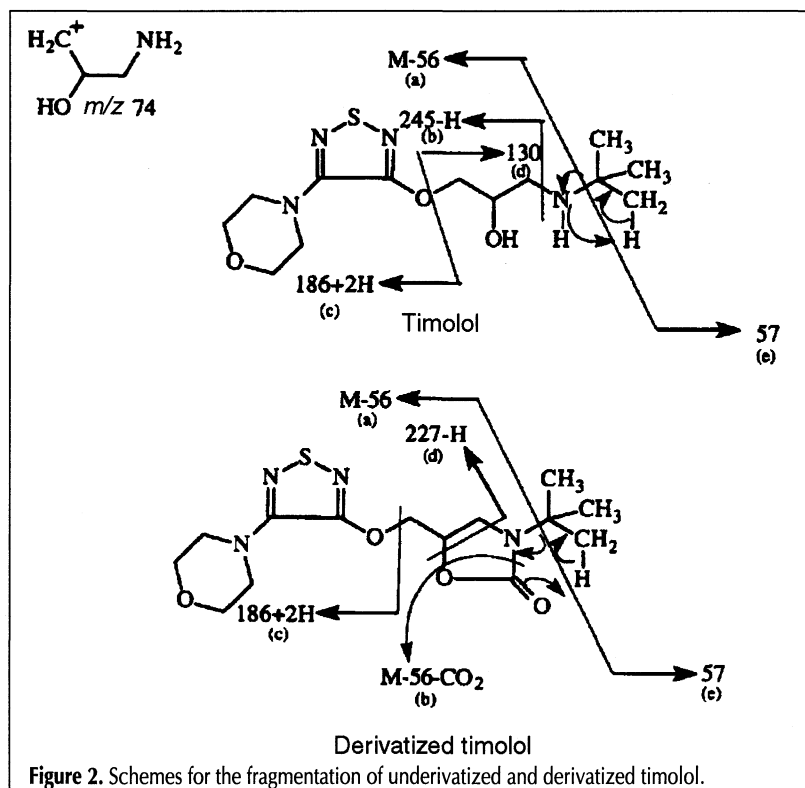

Figure 2. Schemes for the fragmentation of underivatized and derivatized timolol.

Experimental

Timolol, [$^{2}H_9$]timolol, [$^{13}C_3$]timolol, and the *N*-isopropyl homolog of timolol (Figure 1) were synthesized as their maleate salts at Merck Research Laboratories (Rahway, NJ). The isotopic distribution in deuterium-labeled timolol was 99 atom % excess $^{2}H_9$ (M+9) with less than 0.1% (M+0), (i.e., the unlabeled drug). ^{13}C-labeled timolol contained 90 atom % excess $^{13}C_3$ (M+3) with approximately 10% (M+2) and less than 0.1% (M+0).

Acetonitrile and methanol (HPLC grades), potassium hydrogen phosphate, and hydrochloric acid were obtained from Fisher (Fair Lawn, NJ). Tetrahydrofuran (THF) and pyridine (both anhydrous) were purchased from Aldrich (Milwaukee, WI). Phosgene (20% solution in toluene) and trifluoroacetic acid (TFA) were from Fluka (Ronkonkoma, NY) and Sigma (St. Louis, MO), respectively. Air (hydrocarbon-free), nitrogen, and argon (both 99.999% pure)

Table I. Positive Product Ion Mass Spectra of Derivatized and Underivatized Timolol, Its [$^{13}C_3$] and [$^{2}H_9$] Analogs, and the *N*-Isopropyl Homolog Used as the Internal Standard

		Other significant ions (% relative abundance)						Ion combination for SRM
Underivatized	**[M+H]$^+$**	**(a)**	**(b)**	**(c)**	**(d)**	**(e)**	**(a–c)+H**	
Timolol	317	261	244	188	130	57	74	317 → 261
	(62)	(100)	(45)	(32)	(9)	(5)	(60)	
[$^{13}C_3$]Timolol	320	261	244	188	133	60	74	320 → 261
	(48)	(100)	(60)	(30)	(10)	(5)	(50)	
[$^{2}H_9$]Timolol	326	262	244	188	139	66	75	326 → 262
	(62)	(100)	(51)	(17)	(10)	(8)	(35)	
Internal standard	303		244	188	116	58	74	303 → 116
	(100)		(26)	(8)	(79)	(4)	(6)	
Derivatized	**[M+H]$^+$**	**(a)**	**(b)**	**(c)**	**(d)**	**(e)**	**(M-CO$_2$)**	
Timolol	343	287	243	188	226	57		343 → 287
	(24)	(100)	(8)	(5)	(5)	(4)		
[$^{13}C_3$]Timolol	346	287	243	188	226	60		346 → 287
	(27)	(100)	(7)	(4)	(4)	(5)		
[$^{2}H_9$]Timolol	352	288	244	188	226	66		352 → 288
	(32)	(100)	(6)	(3)	(4)	(6)		
Internal standard	329	287	244	189*	226		285	329 → 189
	(100)	(10)	(20)	(75)	(12)		(18)	

* Does not arise via Scheme C.

were obtained from West Point Supplies (West Point, PA). Control dog plasma was purchased from the Biological Specialties Corporation (Lansdale, PA). Bakerbond-C_{18} solid-phase extraction (SPE) cartridges (3 mL × 500 mg) were obtained from J.T. Baker (Phillipsburg, NJ). Water (deionized) was generated using a Barnstead generator (Dubuque, IA).

Dosing solutions

Timolol was administered ophthalmically as Timoptic solution (0.5% timolol). One drop (40 µL, 0.2 mg) was applied to both eyes (0.04 mg/kg). The intravenous dosing solution was [$^{2}H_{9}$]timolol maleate at a concentration of 2.8 mg free base per milliliter of phosphate-buffered saline. The volume injected was 0.1 mL/kg (0.28 mg/kg). [$^{13}C_{3}$]timolol was prepared for oral administration as a solution of its maleate salt in water at a concentration of 0.66 mg free base per milliliter. The dose given was 0.5 mL/kg (0.33 mg/kg).

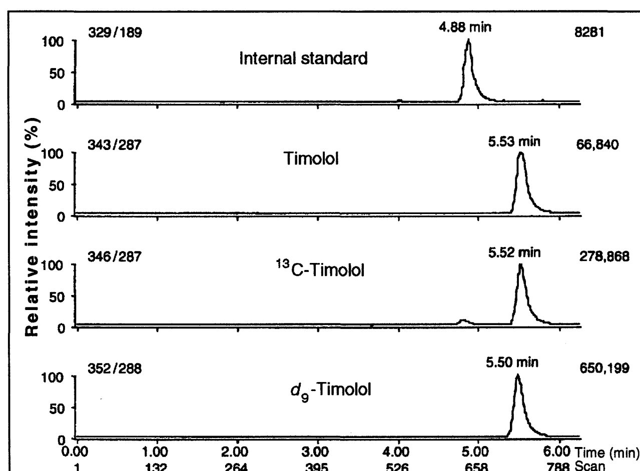

Figure 3. SRM chromatograms of derivatized extracts of plasma from a dog 36 h after dosing ophthalmically with timolol, orally with [$^{13}C_{3}$]timolol, and intravenously with [$^{2}H_{9}$]timolol. The upper channel is that of the internal standard.

Animals

Two male beagle dogs (body weight, approximately 10 kg) received simultaneous doses of timolol (0.4 mg ophthalmically, [$^{13}C_{3}$]timolol [3.3 mg] orally by gavage, and [$^{2}H_{9}$]timolol [2.8 mg] intravenously). Blood was collected in 2-mL amounts before dosage and 5 min, 15 min, 30 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, and 24 h after dosage into a heparinized vacutainer. Plasma harvested after centrifugation was stored at –20°C until taken for analysis. Urine was collected over the 24-h period.

Standard and sample preparations

Standard stock solutions of timolol and its isotopically labeled species were prepared as 1-mg/mL solutions in 0.05M HCl. Aliquots (0.1 mL) of the stock solutions were diluted together in 10.0 mL of 0.05M HCl to yield concentrations of 10 µg/mL of each substance. Following appropriate dilutions, 0.1-mL aliquots were added to 1.0-mL aliquots of control plasma to yield timolol, [$^{2}H_{9}$]-timolol, and [$^{13}C_{3}$]timolol concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 ng/mL. Duplicate plasma quality control samples were prepared at concentrations of 0.5, 2, and 10 ng/mL from independently weighed and diluted samples of each drug.

For the analysis of urine, 0.8 mL of phosphate buffer (0.1M, pH 7.4) was added to 0.2 mL of control urine, and 100 µL of the appropriate standard solutions were added to produce standards containing timolol and its isotopically labeled analogs at concentrations of 50, 100, 250, 500, 1000, and 2500 ng/mL. The concentrations of the urine quality control samples prepared as above were 50, 250, and 1000 ng/mL.

Table II. Intra- and Inter-Assay Accuracy and Precision for the Determination of Derivatized Unlabeled and [$^{2}H_{9}$]- and [$^{13}C_{3}$]-Labeled Timolol in Plasma by LC–MS–MS

Actual concentration (ng/mL)	Timolol accuracy (% found/actual)	Timolol precision (CV [%])	[$^{2}H_{9}$]Timolol accuracy (% found/actual)	[$^{2}H_{9}$]Timolol precision (CV [%])	[$^{13}C_{3}$]Timolol accuracy (% found/actual)	[$^{13}C_{3}$]Timolol precision (CV [%])
Intra-assay (five replicates)						
0.2	98.3	7.5	97.8	3.3	94.5	5.1
0.5	97.1	5.1	98.1	3.8	102.3	5.6
1	99.8	5.6	98.6	4.3	98.8	3.3
2	103.3	4.8	101.8	2.9	102.5	3.5
5	104.2	3.8	104.9	2.9	103.5	4.3
10	98.1	5.9	98.9	5.2	97.8	4.3
20	98.0	3.0	98.4	2.8	98.6	3.5
Inter-assay (five replicates)						
0.5	96.1	4.3	98.4	4.2	96.6	8.1
2	96.2	8.0	98.6	7.4	97.8	6.9
10	97.5	7.8	100.2	6.1	99.2	6.3

Extraction procedures

In a 75 × 12-mm culture tube, 0.1 mL of 0.05M HCl was added to a 1.0-mL aliquot of test plasma. After addition of the internal standard (100 µL of a 0.02 µg/mL solution of the *N*-isopropyl homolog of timolol) and 2.0 mL of potassium hydrogen phosphate in water (0.1M, pH 10.6) to all tubes representing standards, quality control, and test samples, the tubes were briefly vortex-mixed prior to SPE.

To test urine samples (200 µL), 0.8 mL of phosphate buffer (pH 7.4), 0.1 mL of 0.05M HCl, and 100 µL of the internal standard solution were added, followed by brief vortex-mixing. Preparations of plasma and urine (total volume, 1.2 mL) were applied individually to Bakerbond-C_{18} SPE cartridges preconditioned by washing with 2.5 mL methanol and 2.5 mL water. The sample was drawn onto the column with a vacuum applied at approximately 1 mL/min. The column was washed with 2.5 mL of 0.1M potassium hydrogen phosphate (pH 10.6) and then with 2.5 mL of water. After discarding the washings, the drugs were eluted with 2.5 mL of methanol, and the eluate was reduced to dryness in a TurboVap evaporator (Zymark Corporation, Hopkinton, MA) at 50°C. For plasma samples, the residue was transferred to a glass derivatization vial using 0.5 mL of methanol, and solvent was removed under a stream of nitrogen.

Derivatization

The oxazolidinone derivatives of timolol isolated in plasma extracts were prepared according to the procedure previously described (8). A 20-µL amount of a solution of pyridine (1%) in THF was added to the dried plasma extract. After vortex-mixing, 40 µL of phosgene (20%) in toluene was added, and the capped reaction vials were heated at 85°C for 2 h. Excess reagent was removed under a stream of nitrogen. The residue was reconstituted in a solution (100 µL) consisting of acetonitrile–methanol–0.1% TFA in water (36:6:58%, v/v) and was agitated in an ultrasonic bath for 20 min.

Because higher levels of timolol and isotopically labeled timolol were expected in urine, a more sensitive assay was not required. Therefore, the extracts of urine were not derivatized, although the same derivatization procedure could have been followed. Urinary extracts that had been dried as above were reconstituted in 100 µL of a solution consisting of acetonitrile–methanol–0.1% aqueous TFA (27:7:66, v/v) and were agitated for 20 min in the ultrasonic bath prior to analysis.

LC–MS–MS analysis

LC–MS–MS was performed on a Sciex (Thornhill, Ontario) API III*plus* triple quadrupole MS interfaced via a heated nebulizer interface to a Hewlett-Packard 1050 solvent delivery system and autoinjector equipped with a 100-µL loop. For the analysis of derivatized plasma extracts, HPLC was performed using a 50 × 4.6-mm column packed with 3-µm, 100-Å Spherisorb CN (Thomson Liquid Chromatography, Springfield, VA). The mobile phase was acetonitrile–methanol–0.1% aqueous TFA (45:5:50, v/v) at a flow rate of 0.5 mL/min. The volume of sample injected ranged from 10–50 µL. The nebulizer probe and interface temperature settings were 500 and 55°C, respectively. The nebulizing gas (air) pressure and makeup gas (air) were set at 80 psi and 1 L/min, respectively. Chemical ionization was effected by a corona discharge needle set at +4 µA. The curtain gas was nitrogen at 1.7 L/min. The instrument was used in the selected reaction monitoring (SRM) mode in which positive protonated molecular ions were selected by the first quadrupole mass filter with subsequent collision-induced decomposition (argon collision gas [50 eV] at 250 × 10^{12} atoms/cm^2) and monitoring of the appropriate product ions using the third quadrupole filter. The orifice potential and multiplier settings were +55 V and –3.5 kV, respectively. Peak area ratios for the analyte and internal standard were computed using Sciex's MacQuan software. Calibration curves were constructed using a weighted (reciprocal of concentration) linear least squares regression of the standard plasma concentrations and the measured peak area ratios.

Table III. Intra- and Inter-Assay Accuracy and Precision for the Determination of Unlabeled and [$^2H_9$]- and [$^{13}C_3$]-Labeled Timolol in Urine by LC–MS–MS

Actual concentration (ng/mL)	Timolol accuracy (% found/actual)	Timolol precision (CV [%])	[$^2H_9$]Timolol accuracy (% found/actual)	[$^2H_9$]Timolol precision (CV [%])	[$^{13}C_3$]Timolol accuracy (% found/actual)	[$^{13}C_3$]Timolol precision (CV [%])
Intra-assay (five replicates)						
50	85.8	3.7	92.6	1.8	90.0	4.5
100	101.4	7.5	97.3	7.0	99.4	6.0
250	106.0	2.1	102.7	3.5	100.7	1.9
500	106.1	6.0	102.6	4.1	100.9	6.1
1000	100.8	4.1	100.5	2.6	97.7	3.5
2500	90.1	4.5	94.9	3.2	91.0	6.6
Inter-assay (five replicates)						
50	87.1	7.9	89.2	9.1	89.0	7.2
250	111.5	2.0	108.9	5.8	107.8	2.5
1000	102.7	1.4	101.6	4.3	101.0	1.5

Underivatized urine extracts were assayed using a 250 × 4.6-mm Spherisorb cyano column preceded by a 20-mm Supelco Pelliguard LC-CN (Bellefont, PA) guard column. The mobile phase was acetonitrile–0.1% aqueous TFA (30:70, v/v) at 1 mL/min. Other than the precursor/product masses monitored, the MS conditions were as described for derivatized timolol with the exception that the curtain gas flow was 1.0 L/min.

Pharmacokinetic methods

Areas under the plasma concentration–time curves (AUC) were calculated using the trapezoidal rule. AUC [0–∞] was calculated using the following relationship:

$$[AUC]_0^{\infty} = [AUC]_0^t + C_t/K$$

where C_t is the plasma concentration at the last measurable time point and K was calculated using the terminal phase of the ln C-versus-time profiles. The plasma half-lives ($t_{1/2}$) were determined by log-linear least squares regression of the terminal phase of the plasma concentration–time profiles following intravenous administration. Plasma clearance (Cl) values were determined by dividing the intravenous dose by the corresponding AUC [0–∞].

Bioavailability (F) was calculated as

$$\% F = AUC_{(po\ or\ opt)}/AUC_{iv} \times Dose_{iv}/Dose_{(po\ or\ opt)} \times 100$$

where the subscripts po, opt, and iv refer to oral, ophthalmic, and intravenous administration, respectively. This equation assumes that the plasma clearance of the drug is the same in all treatments. This condition was met in the present study design because the oral, ophthalmic, and intravenous treatments were given simultaneously.

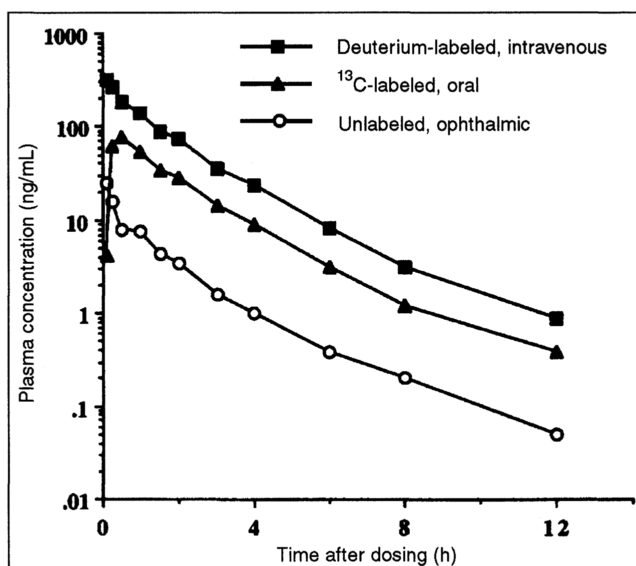

Figure 4. Mean plasma concentration–time curves of labeled and unlabeled timolol following simultaneous ophthalmic, oral, and intravenous administration to dogs.

Results and Discussion

The positive product ion mass spectral fragmentations of both underivatized timolol and the oxazolidinone derivative of the drug are shown in Figure 2. The principal fragment ions of both derivatized and underivatized substances are shown in Table I. The fragmentation pattern of the oxazolidinone derivative of timolol is interesting, showing a base peak in the product spectrum of m/z 287. This occurs by a classical McLafferty rearrangement that can be confirmed by the spectrum of the [$^2H_9$]timolol derivative whose base peak is at m/z 288 because of deuterium transfer. The oxazolidine derivative of the internal standard does not demonstrate this fragmentation pattern. The base peak of its product ion spectrum is m/z 189, which arises by a more energetically favorable mechanism. It is important that the fragment be differentiated from that at m/z 188 (fragmentation C).

The plasma assays were configured using the following precursor/product ion combinations of the oxazolidinone derivatives: timolol m/z 343 → 287; [$^2H_9$]timolol, m/z 352 → 288, [$^{13}C_3$]timolol m/z 346 → 287 and the internal standard m/z 329 → 189.

SRM chromatograms of derivatized plasma extracts taken from a dog dosed with timolol and its stable-isotope-labeled analogs are shown in Figure 3. The cross-reactivities between the SRM channels used were investigated by assaying 10 ng of each derivatized standard (unlabeled, [$^{13}C_3$]- and [$^2H_9$]timolol) and examining the apparent quantities observed on the complementary channels. In all cases, the cross-reactivities were less than 0.1%. That is, the injection of 10 ng of the derivatized unlabeled timolol produced an apparent signal less than 10 pg of the derivatized $^{13}C_3$- and $^2H_9$-labeled timolol species.

The most abundant product ion fragments of underivatized, unlabeled, and labeled timolol are shown in Table I, which also indicates the precursor/product ion combinations used for the analysis of the drug in urine.

Accuracy and precision

The intra- and inter-assay accuracy and precision for the analysis of timolol and its isotopically related species in plasma are shown in Table II. Using a maximum acceptable variation coefficient of 10%, the limits of quantitation (LOQs) for the determination of labeled and unlabeled timolol as their oxazolidinone derivatives in plasma were 0.2 ng/mL. The inter-assay accuracy and precision for the urine assay were also regarded as satisfactory (Table III). The LOQs for underivatized timolol and its analogs in urine were 50 ng/mL.

Absolute recoveries

The absolute recoveries of the internal standard, timolol, [$^2H_9$]- and [$^{13}C_3$]timolol after SPE of urine were 71, 62, 64, and 60%, respectively. For plasma, the recoveries of all species ranged from 99 to 105%.

Plasma and urine analysis

Calibration curves showed good linearity over the ranges examined for both plasma and urine. The mean plasma con-

centration–time curves of labeled and unlabeled timolol after their simultaneous administration to dogs are shown in Figure 4. The elimination half-life and total plasma clearance of [$^2H_9$]timolol after intravenous administration were approximately 1.6 h and 10 mL/min kg, respectively. The average oral and ophthalmic bioavailabilities were 29 and 40%, respectively. The quantity of unchanged drug excreted in 0–24-h urine was calculated. Only 1–2% of the dose was recovered unchanged after oral or ophthalmic administration, whereas 4–10% of the intravenous dose was recovered in urine.

Conclusion

Few pharmacokinetic uses of stable isotopes in conjunction with LC–MS–MS have been published. Barrish and coworkers have described the determination of the oral bioavailability of rizatriptan by simultaneous oral and intravenous administration of the unlabeled and [$^{13}C_2$,$^{15}N_3$]-labeled species to dogs (9). The quantitation of unlabeled and deuterium-labeled indinavir following oral and intravenous administration to man has been reported (10), and the absence of kinetic isotope effects when [$^2H_3$] tenidap was co-administered along with the unlabeled drug to rats has also been described (11).

The present work provides evidence of the usefulness of such experiments in providing measurements of bioavailability by simultaneously administering the labeled and unlabeled species of the drug to dogs. In the present case, the availability of two different stable-isotope-labeled species of timolol enabled simultaneous oral, intravenous, and ophthalmic administration in a single rather than a three-period crossover experiment. To our knowledge, this is the first published account of stable-isotope-labeled drugs being used to evaluate pharmacokinetics after simultaneous administration by three routes.

Whereas relatively high concentrations of timolol could be determined in urine without derivatization, successful measurement of the drug at the lower concentrations found in plasma necessitated the preparation of the oxazolidinone derivatives. Despite the great advances in bioanalytical technology (such as LC–MS–MS), there are still lessons to be learned from earlier days when bioanalysts were chemists as well as chromatographers.

We face great demands on our LC–MS–MS equipment. The use of stable-isotope-labeled drugs can greatly improve estimates of bioavailability by eliminating the intra-subject variability present in conventional crossover experiments. This can greatly reduce the number of human subjects or animals, the number of separate dosing phases, and, of particular importance to the analysts, the number of samples required to be assayed.

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