Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 2, pp. 157–163, 1993
Printed in Great Britain

0731-7085/93 $6.00 + 0.00
© 1993 Pergamon Press Ltd

Determination of the β-adrenergic blocker timolol in plasma by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

T.V. OLAH, J.D. GILBERT* and A. BARRISH

Merck Research Laboratories, West Point, PA 19486, USA

Abstract: A method based on LC–MS–MS has been developed for the determination of timolol in plasma using the $(CD_3)_3$-labelled species as the internal standard. Timolol is isolated from plasma by a simple solid-phase extraction and converted to its oxazolidin-2-one prior to analysis on a 50 × 4.6 mm reversed-phase high-performance liquid chromatography column packed with SynChropak, C_{18}, 5 μm. The column eluate is passed by means of a heated nebulizer interface into a corona discharge atmospheric pressure chemical ionization source where the analyte and its internal standard are detected using multiple reaction monitoring (MRM). The very high specificity of this technique permits chromatographic run times of less than 2 min. The method has a lower quantifiable limit of 0.5 ng ml^{-1}, with intra- and inter-day relative standard deviations less than 10%, and enables the determination of timolol in plasma after ocular administration to volunteers.

Keywords: *Timolol; plasma; analysis; LC–MS–MS; oxazolidinone; phosgene.*

Introduction

Timolol, (*S*)-1-[(1,1-dimethylethyl)amino]-3-[[4-(4-morpholinyl)-1,2,5-thiadiazol-3-yl]oxy]-2-propanol, is a non-selective β-adreno-receptor antagonist [1] designed for the treatment of both cardiovascular disease [2, 3] and glaucoma [4, 5]. Chromatographic methods for the measurement of timolol in the plasma of humans receiving therapeutic oral doses of the drug have been described using various extraction and detection systems. However, none of these can measure timolol in plasma at sub ng ml^{-1} concentrations with acceptable precision (RSD = 10–15%). In several procedures described in the literature, the lower quantifiable limits, which must be substantiated by calculation of precision, can be readily confused with detection limits based only on signal-to-noise measurements. The earliest assay [6], based on gas chromatography (GC) with electron capture detection, has a lower quantifiable limit of 2 ng ml^{-1} (RSD = 40%). Two procedures using GC with electron ionization mass spectrometric (EI-MS) detection have been described [7–9]. The EI-MS of the timolol trimethylsilyl ether derivative used by these investigators is characterized by a molecular ion of very low relative abundance and a base peak at *m/z* 86 due to facile cleavage of the *t*-butyl-methylamine function. Monitoring of such low masses necessitated the use of tedious extraction and back-extraction systems to minimize interference from endogenous plasma components, which is much more probable when ions of lower masses are being monitored [7]. The best sensitivity achieved by GC–MS was 1 ng ml^{-1} with an intra-assay RSD of 16% [8].

Recently the increased use of timolol as an illicit inhibitor of the performance of racehorses has led to the investigation of alternative extraction methods and derivatives such as the acetate [10] and 2-(dimethyl)-silamorpholines [11] for the determination of the drug in urine by GC–MS. These derivatives, which promote more diagnostic fragments of increased molecular weight and hence less potential interference, have not been applied to the determination of timolol in human plasma at low concentrations. Methods based on LC have also been described for the measurement of timolol in plasma. Two of these claim sensitivity of 2 ng ml^{-1} but quote precision only at 10 ng ml^{-1} [12] and 5 ng ml^{-1} [13] using electrochemical and ultraviolet (UV) detection, respectively. A more recent LC procedure [14] with UV detection afforded good

* Author to whom correspondence should be addressed.

inter- and intra-assay precision at 5 ng ml^{-1}, but RSDs at lower concentrations were not reported. A radio-receptor assay for the measurement of timolol in plasma shows excellent precision at concentrations as low as 50 pg ml^{-1}, but requires a complex extraction procedure to ensure specificity with respect to potentially interfering timolol metabolites [15].

The purpose of the present investigation was to establish a method for the reliable and specific determination of timolol in plasma with acceptable accuracy and precision at concentrations approaching 0.2 ng ml^{-1}. The equally important objective of rapid sample throughput prompted the investigation of liquid chromatography with atmospheric pressure chemical ionization (APCI) MS–MS detection [16, 17]. The considerably superior specificity of tandem LC–MS–MS over LC–MS and the consequential gain in assay sensitivity and speed of analysis have previously been noted [18], and such a procedure successfully fulfilled our assay requirements. The $(CD_3)_3$-labelled analogue of timolol was used as the internal standard (Fig. 1).

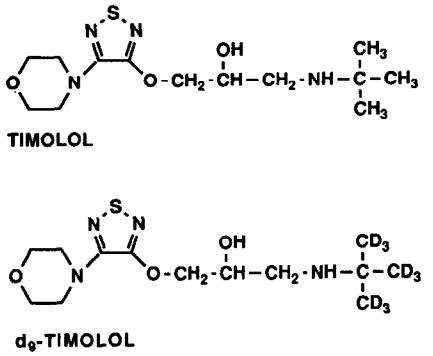

Figure 1
Structures of timolol and its d_9-labelled internal standard.

Experimental

Materials

Timolol maleate and d_9-labelled timolol maleate [G.J. Gatto and H.E. Mertel, unpublished] were synthesized at the Merck Research Laboratories (Rahway, NJ, USA). Both substances were >99% pure as determined by high-performance liquid chromatography (HPLC). The isotopic purity of the internal standard was 99 atoms % [7].

Acetonitrile (HPLC grade) and potassium hydrogen phosphate were obtained from Fisher (Fair Lawn, NJ, USA). Ammonium acetate was supplied by Sigma (St Louis, MO, USA). Pyridine (anhydrous) and hydrochloric acid (analytical grade) were purchased from Mallinckrodt (Paris, KY, USA). Tetrahydrofuran (anhydrous) and phosgene (20% solution in toluene) were from Aldrich (Milwaukee, WI, USA) and Fluka (Ronkonkoma, NY, USA), respectively. Air (hydrocarbon-free), and nitrogen, argon and helium (all at 99.999%) were obtained from Matheson (Morris Plains, NJ, USA). 'Bakerbond'-C_{18} cartridges (6 ml × 500 mg) were purchased from J.T. Baker (Phillipsburg, NJ, USA) and disposable tapered reaction vials (1-ml) and crimp caps were obtained from Rainin Instruments (Woburn, MA, USA).

Standard and sample preparation

Standard stock solutions of timolol maleate and its internal standard were prepared as 1 mg ml^{-1} solutions in M/20 HCl. Standards and quality control samples were prepared by the addition of known amounts of standard solutions (0.1 ml) to 1.0 ml of control human plasma. The concentrations of timolol, as its maleate salt, in standard plasma samples were 0.2, 0.5, 1, 2, 5, 10 and 20 ng ml^{-1}. Duplicate quality control samples were prepared at concentrations of 0.5, 2 and 10 ng ml^{-1} for each batch of *ca* 50 test samples processed. Batches in excess of 50 samples were chromatographed along with two sets of standards and quality controls.

Extraction procedure

A plasma sample (1.0 ml) was placed in a 75 × 12 mm glass test tube. After the addition of 100 μl of a 0.02 μg ml^{-1} solution of internal standard and 2.0 ml of potassium hydrogen phosphate (pH 10.6, 0.1 M) the tube was briefly vortexed. The entire mixture was applied to a Bakerbond cartridge pre-conditioned by washing with methanol (4 ml) and distilled water (5 ml). The sample was drawn into the column (with vacuum applied) at approximately 1 ml min^{-1}. The column was washed with 0.1 M potassium hydrogen phosphate (3 ml) and water (3 ml). After discarding the washings the drug was eluted in methanol (3 ml) into a glass centrifuge tube and the solvent removed at room temperature in a Speedvac Model SVC200H (Savant, Farmingdale, NY, USA). The residue was transferred in acetonitrile (400 μl) into a

microderivatization vial and the solvent removed under a stream of nitrogen.

The oxazolidinone derivatives were prepared using a modification of the method described by Gyllenhaal and Vessman [19, 20]. To the dried residue was added 20 μl of a solution of pyridine (1% v/v) in tetrahydrofuran. After brief vortex mixing, 40 μl of a solution of phosgene (20% in toluene) was added and the capped vials were incubated at 85°C for 2 h. Excess reagent was removed under a stream of nitrogen and the residue reconstituted in 100 μl of mobile phase. The vial was re-capped, agitated in an ultrasonic bath for 20 min, and briefly vortexed to remove air bubbles prior to analysis.

The absolute recovery of timolol from plasma was determined by extracting samples of plasma spiked at concentrations of 2 and 20 ng ml^{-1}, adding 2 ng of internal standard to the extracts and comparing the peak area ratios of the oxazolidinones obtained from the extracted samples with those of unextracted reference solutions containing the same quantities of drug and internal standard. The absolute recoveries of timolol from plasma at concentrations of 2 and 20 ng ml^{-1} were 106 and 99%, respectively.

Chromatographic system suitability

The chromatographic system precision was determined by making 10 successive injections (20 μl) of a solution containing the oxazolidinones of timolol and its internal standard both at concentrations of 0.02 μg ml^{-1}. The RSD of the peak area ratio was 2.4%.

LC–MS–MS analysis

LC–MS–MS was performed on a Sciex (Thornhill, Ontario) Model API III triple quadrupole mass spectrometer interfaced via a Sciex heated nebulizer probe to a liquid chromatograph consisting of a Perkin–Elmer 250 solvent delivery system and a Perkin–Elmer ISS-100 Autoinjector equipped with a 50-μl loop. HPLC was performed using a 50 × 4.6 mm column packed with SynChropak, C_{18}, 5 μm from Synchrom, Inc. (Lafayette, IN, USA). The mobile phase was acetonitrile–ammonium acetate (pH 4.9; 0.05 M) (50:50, v/v) at a flow rate of 1 ml min^{-1}. The volume of extract injected was 20 μl, and samples were chromatographed automatically in batches of 80–100 under the control of a Macintosh II FX Computer running Sciex's RAD (routine acquisition and display) software. The nebulizer probe temperature setting was 500°C. The nebulizing gas (air) pressure and auxiliary flow were set at 80 psi and 1.0 l min^{-1}, respectively. Gas-phase chemical ionization was effected by a corona discharge needle (+5 μA) and positive ions were sampled into the quadrupole mass analyser via a 0.0045″ pinhole aperture. The curtain gas was nitrogen at 1.7 l min^{-1}. Product spectra were recorded with the quadrupole power supplies set for unit resolution. For MRM the resolution was degraded (50% valley) to enhance sensitivity. The mass spectrometer was programmed to admit the protonated molecules $[M + H]^+$ at *m/z* 343 (drug) and *m/z* 352 (internal standard) via the first quadrupole filter (Q1) with collision-induced fragmentation in Q2 (collision gas argon, 50 eV, 250 × 10^{12} atoms cm^{-2}) and monitoring, via Q3, the product ions at *m/z* 287 for timolol oxazolidinone but 288 for its internal standard. The orifice potential and electron multiplier settings were +55 V and −3.3 kV. The dwell time was 400 ms. Peak area ratios, obtained from multiple reaction monitoring of analyte (*m/z* 343 → 287)/internal standard (*m/z* 352 → 288), were computed using Sciex's MacSpec software. The calibration curves were constructed using a non-weighted linear least squares regression of the plasma concentrations and the measured area ratios.

Accurate mass measurements

Accurate mass measurements were performed on a VG MM-ZAB-HF Mass Spectrometer interfaced to a PDP 11/250 data system. Spectra were obtained by Fast Atom Bombardment of the samples in a thioglycerol matrix using 8 keV argon atoms for bombardment. The instrument was optimized at 5000 resolution using narrow range magnetic scanning and peak matching to peaks in the matrix.

Results

The objective of this research was the development of a reliable but expedient assay for the measurement of timolol in plasma at concentrations approaching 0.2 ng ml^{-1}. LC–MS–MS was selected because the ruggedness and high specificity of the technique requires only the most simple of extractions and enables the use of fast liquid chromatography with a consequentially rapid sample throughput [17,

18]. Attempts to chromatographically retain underivatized timolol proved unsuccessful. Although satisfactory chromatography could be effected by ion-pair chromatography using octylsulphonic acid as counter ion, the detector sensitivity was seriously compromised. Accordingly, the drug was chromatographed after conversion with phosgene to its oxazolidinone, which reduced both its basicity and polarity resulting in satisfactory retention and chromatography. Phosgene is a useful reagent for β-blockers since it derivatizes both the amino and hydroxyl functions simultaneously and the product is relatively stable [19]. Timolol, which has a *t*-butyl substituent on the amino group, requires fairly rigorous conditions for derivatization [20]. The efficiency of derivatization was determined by mass chromatography to be essentially quantitative.

Under the ionization conditions used the oxazolidin-2-ones of timolol and its internal standard show protonated molecular ions at *m/z* 343 and 352 (Fig. 2). Accurate mass measurement confirmed these ions had the

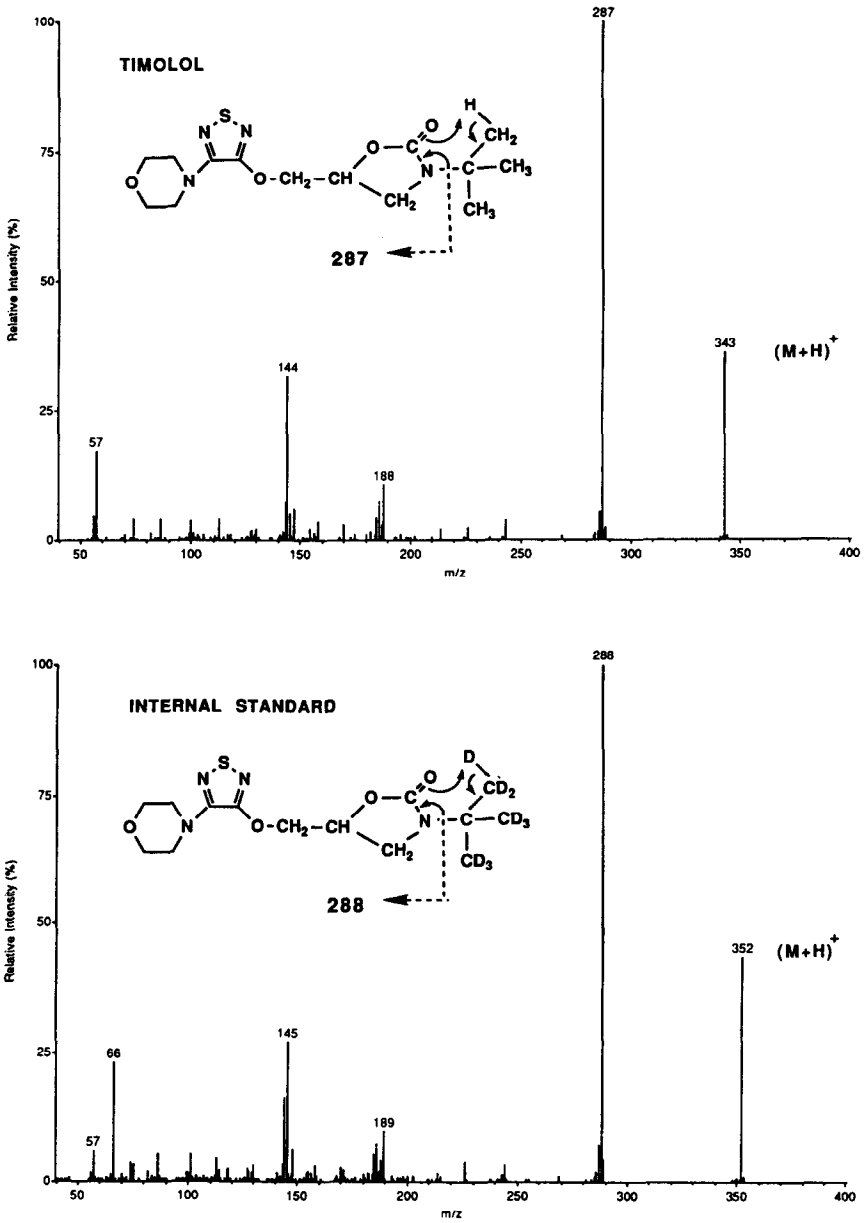

Figure 2
Positive product ion mass spectra (background subtracted) of the protonated molecular ions of the oxazolidinones of timolol (*m/z* 343) and its internal standard (*m/z* 352); 50 ng of each substance analysed by LC–MS–MS.

empirical formulae $C_{14}H_{23}N_4O_4S$ (calculated 343.1440, measured 343.1439) and $C_{14}H_{14}N_4O_4SD_9$ (calculated 352.2004, measured 352.2006), respectively. McLafferty rearrangements yield the corresponding fragment ions at *m/z* 287 and 288. The deuterium transfer is noteworthy.

MRM chromatograms of extracts of plasma from a volunteer following ocular administration of ophthalmic (0.5%) timolol maleate solution are shown in Fig. 3.

Calibration

The data presented in Table 1 demonstrate good linearity and reproducibility of the calibration curves, which show satisfactory fit to the non-weighted linear regression. Calibration standards of plasma containing no drug showed peak area ratios that were essentially zero. Similarly, no interferences from endogenous plasma components were observed when extracts of pre-dose plasma from six human volunteers were processed and chromatographed.

Precision and accuracy

Assay accuracy and intra-day precision were determined by analysis of sets of control plasma containing known quantities of timolol maleate. Inter-day precision was calculated from quality control data obtained during successive analyses. Results are shown in Table 2. Acceptable accuracy, defined as mean % (found/added), intra- and inter-day precision were demonstrated in the assay range 0.5–20 ng ml^{-1}. The RSD at 0.2 ng ml^{-1} was 14.1% with an accuracy of 102.5%.

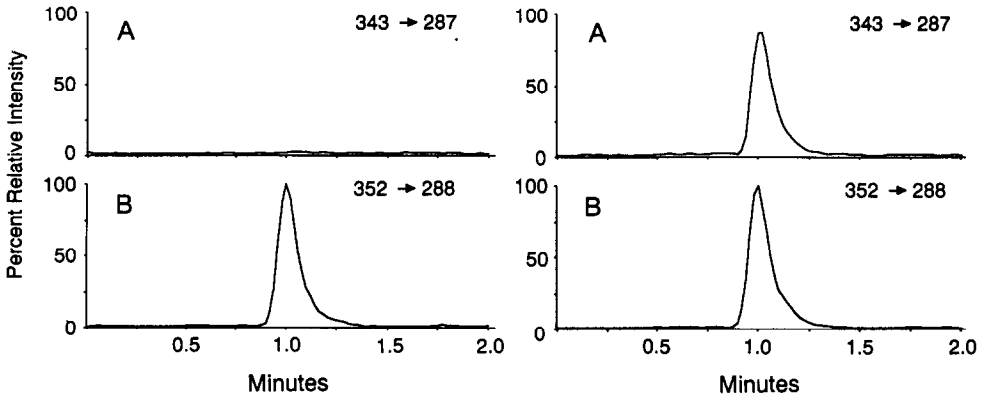

Figure 3
Chromatograms obtained by multiple reaction monitoring of extracts of plasma from a volunteer following ocular administration of ophthalmic (0.5%) timolol maleate solution. Retention times are shown in minutes. Channel A: *m/z* 343 → 287, timolol oxazolidinone. Channel B: *m/z* 352 → 288, internal standard oxazolidinone. Left: extract of a plasma sample collected prior to administration, with internal standard added. Right: extract of a plasma sample, containing 1.3 ng ml^{-1}, collected 2 h after administration.

Table 1
Peak area ratios and curve parameters determined during the construction of five successive calibration lines for the determination of timolol in plasma

Concentration of timolol maleate (ng ml^{-1})	Peak area ratio						RSD (%)
	Curve 1	Curve 2	Curve 3	Curve 4	Curve 5	Mean ratio	
0.2	0.067	0.064	0.055	0.073	0.059	0.064	11.0
0.5	0.128	0.129	0.135	0.121	0.129	0.128	3.9
1	0.232	0.232	0.230	0.219	0.239	0.230	3.1
2	0.436	0.451	0.439	0.429	0.444	0.440	1.9
5	1.083	1.061	1.020	1.025	1.079	1.054	2.8
10	2.097	2.083	2.174	2.113	2.041	2.102	2.3
20	4.202	4.386	4.259	4.159	4.179	4.237	2.2
Slope	0.209	0.217	0.212	0.207	0.207	0.210	2.0
Intercept	0.023	0.006	0.013	0.017	0.023	0.016	43.9
r^2	1.000	0.999	1.000	1.000	1.000	0.9998	0.05

Table 2
Calculation of intra- and inter-day accuracy and precision for the determination of timolol in plasma

	Conc. (ng ml^{-1})	Number of replicates	Accuracy* (%)	Precision (% RSD)
Intra-day	0.2	5	102.5	14.1
	0.5	5	100.8	4.9
	1	5	99.2	3.3
	2	5	99.5	2.0
	5	5	98.8	2.9
	10	5	99.6	2.3
	20	5	101.0	2.2
Inter-day	0.5	4	99.4	7.2
	2	4	91.5	7.1
	10	4	104.0	7.1

*Defined as $\frac{\text{Found}}{\text{Actual}} \times 100$.

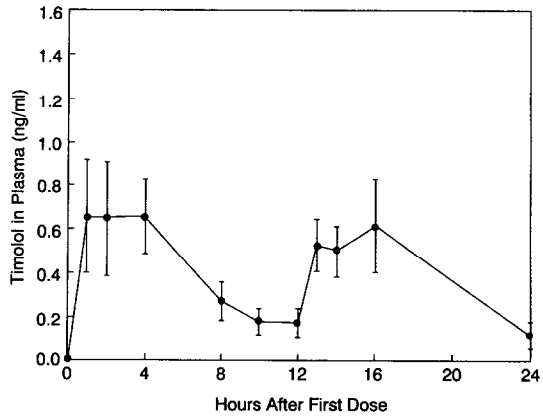

Figure 4
Mean concentration–time profiles of timolol maleate in the plasma of four subjects on the seventh day of administration of ophthalmic (0.5%) timolol maleate (b.i.d. × 7 days).

Discussion

The requirement was for an assay capable of reliably measuring timolol in human plasma at concentrations around 0.2 ng ml^{-1}. The attainment of such sensitivity while maintaining acceptable precision is beyond the capabilities of previous assays based either on HPLC with ultraviolet or electrochemical detection or GC–MS. The latter is capable of measuring timolol at concentrations of 1 ng ml^{-1} with good accuracy and an intra-assay RSD of 16% [8], but necessitates use of a tedious extraction/back extraction procedure prior to derivatization. The chromatographic run time is *ca* 7 min.

In contrast, the present assay uses a simple solid-phase extraction, provides sensitivity to 0.2 ng ml (RSD = 14%) and permits fully automated chromatographic analysis in which 100 extracts can be run in less than 3 h.

An unanticipated complication in the current assay proved the need to derivatize timolol, not to enhance sensitivity but to reduce polarity to ensure chromatographic retention. Although there is no apparent interference by endogenous plasma components in the chromatograms, separation of the analyte from the slug of unretained substances ensures better precision and overall assay performance. Since preparation of the oxazolidin-2-ones proved convenient, quantitative and rapid, no investigation was made of possible alternative derivatives.

The mean plasma concentration–time curve obtained after ocular administration of an ophthalmic solution (0.5%, *ca* 0.2 mg dose^{-1}) of timolol maleate to four volunteers is shown in Fig. 4. Mean plasma concentrations less than 0.2 ng ml^{-1} arise by averaging data points, some of which were zero (i.e. <0.2 ng ml^{-1}). Considerable inter-subject variability is apparent.

Conclusions

This heated-nebulizer interface in conjunction with triple quadrupole LC–MS–MS affords remarkable versatility for the analysis of drugs in biological fluids to sub-nanogram per millilitre concentrations. Of particular importance are the high sensitivity and specificity of the MS–MS detector which, used with conventional HPLC columns, mobile phases and flow rates, enable development of accurate and sensitive assays affording rapid sample throughput [17, 18]. The speed and reliability of the analysis also indirectly improves the quality of data, since the addition of extra quality control samples and standards provides no appreciable burden to the analyst. Such an assay has been developed and successfully applied to measure plasma concentrations of timolol following ophthalmic administration to man.

Acknowledgements — The authors thank Dr W.A. VandenHeuvel for providing the deuterium-labelled timolol maleate, Dr H.G. Ramjit for conducting accurate mass measurements and Dr M.C. Dumasia for helpful advice regarding derivatization procedures.

References

[1] A. Scriabine, M.L. Torchiana, J.M. Stavorski, C.T. Ludden, D.H. Minsker and C.A. Stone, *Arch. Int. Pharmacodyn.* **205**, 76–93 (1973).

[2] R.N. Brogden, T.M. Speight and G.S. Avery, *Drugs* **9**, 164–177 (1975).

[3] The Norwegian Multicenter Study Group, *N. Engl. J. Med.* **304**, 801–807 (1981).

[4] T.J. Zimmerman and W.P. Boger, *Surv. Ophthalmol.* **23**, 347–362 (1979).

[5] G.T. Novack, *Surv. Ophthalmol.* **31**, 307–327 (1987).

[6] D.J. Tocco, F.A. deLuna and A.E.W. Duncan, *J. Pharm. Sci.* **64**, 1879–1881 (1975).

[7] J.R. Carlin, R.W. Walker, R.O. Davies, R.T. Ferguson and W.J.A. VandenHeuvel, *J. Pharm. Sci.* **69**, 1111–1115 (1980).

[8] J.B. Fourtillan, M.A. Lefebvre, J. Girault and Ph. Courtois, *J. Pharm. Sci.* **70**, 573–575 (1981).

[9] W.J.A. VandenHeuvel, J.R. Carlin and R.W. Walker, *J. Chromatogr. Sci.* **21**, 119–124 (1983).

[10] A.M. Duffield, S. Wise, J. Keledjian and C.J. Suann, *J. Chromatogr.* **518**, 215–220 (1990).

[11] M.C. Dumasia and E. Houghton, *J. Chromatogr.* **564**, 503–513 (1991).

[12] M.R. Gregg and D.B. Jack, *J. Chromatogr.* **305**, 244–249 (1984).

[13] M.S. Lennard and S. Parkin, *J. Chromatogr.* **338**, 249–252 (1985).

[14] K. Kubota, H. Nakamura, E. Koyama, T. Yamada, K. Kikuchi and T. Ishizaki, *J. Chromatogr.* **533**, 255–263 (1990).

[15] T. Kaila, *J. Pharm. Sci.* **80**, 296–299 (1991).

[16] H. Fouda, M. Nocerini, R. Schneider and C. Gedutis, *J. Am. Soc. Mass Spectrom.* **2**, 164–167 (1991).

[17] J.D. Gilbert, E.L. Hand, A.S. Yuan, T.V. Olah and T.R. Covey, *Biological Mass Spectrom.* **21**, 63–68 (1992).

[18] J.D. Gilbert, T.V. Olah, A. Barrish and T.F. Greber, *Biological Mass Spectrom.* **21**, 341–346 (1992).

[19] O. Gyllenhaal and J. Vessman, *J. Chromatogr.* **273**, 129–139 (1983).

[20] O. Gyllenhaal and J. Vessman, *J. Chromatogr.* **395**, 445–453 (1987).

[Received for review 22 June 1992; revised manuscript received 26 August 1992]