ELSEVIER

Journal of Chromatography A, 692 (1995) 73–81

JOURNAL OF CHROMATOGRAPHY A

Rapid liquid chromatographic–mass spectrometric assay for oxymetazoline in whole rat blood

Fred J. Hayes*, Timothy R. Baker, Roy L.M. Dobson, Michael S. Tsueda

Miami Valley Laboratories, OTC-Health Care Technology Division, The Procter and Gamble Company, Cincinnati, OH 45239-8707, USA

Abstract

A rapid HPLC–electrospray mass spectrometric assay for the quantitation of oxymetazoline in whole rat blood has been developed. Sample preparation was a single liquid–liquid extraction after addition of a deuterated internal standard (IS) and pH adjustment. An aliquot of reconstituted extract was injected onto a narrow-bore octadecyl reversed-phase column at a flow-rate of 400 μl/min. Using a 20:1 post-column split, 5% of the eluent was introduced into the mass spectrometer interface. Elution of the analyte and IS occurred in less than 2 min. This rapid separation was made possible because of the sample cleanup and the selectivity of the mass spectrometric detection. The $[M + H]^+$ ions for oxymetazoline (*m/z* 261) and $[^2H_6]$oxymetazoline (*m/z* 270) were detected using selected ion monitoring. The linear range of the assay was 0.67–167 ng/g of blood and the limit of quantitation with a 0.30-g sample was 1.0 ng/g. The assay permitted the analysis of nine samples per hour with the requisite sensitivity and selectivity and was used to determine the blood pharmacokinetics of oxymetazoline in rats dosed via intravenous and intranasal routes.

1. Introduction

In recent years, there has been a growing awareness of the potential of intranasal (i.n.) drug administration [1]. Several advantages can be achieved from delivering drugs intranasally [2]. Losses of drug due to degradation in the gastrointestinal tract, gut wall metabolism, and/or hepatic "first-pass" metabolism and elimination can be avoided. Additionally, the existence of a rich vasculature and a highly permeable structure in the nasal membranes can enhance absorption giving plasma concentration vs. time profiles that are comparable to those obtained by intravenous (i.v.) administration.

Various animal models have been evaluated for studying intranasal drug administration; the most common is the rat model [1]. We are using the rat model to study the intranasal absorption of oxymetazoline. This drug has been used safely and effectively as a nasal decongestant for almost thirty years [3]. Practical advantages of this model include ease of handling and cost effectiveness. A disadvantage of using rats is their small blood volumes. Typically, sample volumes are limited to 0.50 ml if serial sampling is performed from the same animal. This can create significant analytical obstacles in an attempt to fully characterize absorption and the pharmacokinetic (PHK) profile of a drug.

We sought to develop a sensitive, non-radioisotopic assay that could be used to accurately

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(94)00630-X

determine the blood pharmacokinetics of i.n.- and i.v.-dosed oxymetazoline. A liquid chromatographic method for the determination of radiolabeled oxymetazoline in rabbit urine, using fraction collection–liquid scintillation counting detection following sample cleanup and preconcentration, has been reported [4]. A method for the determination of unlabeled oxymetazoline in biological fluids has not been reported to date.

Initial efforts were directed toward method development using GC–MS and GC–MS–MS with chemical derivatization and electron ionization. The value of GC–MS-based techniques for the trace-level determination of drugs and their metabolites in biological fluids is well established, particularly when used in conjunction with stable-isotope-labeled internal standards [5]. In addition, recent reports of quantitative GC–MS–MS methodology demonstrate that added selectivity provided by a second stage of mass analysis can result in lower limits of quantitation and shorter analysis times [6,7] than are possible using standard GC–MS. However, the quantitation range required for our application could not be reliably achieved by GC–MS or GC–MS–MS, using electron ionization of trimethylsilated oxymetazoline because of unacceptable interactions between the derivative and active GC sites.

Because of the difficulties encountered with the GC-based approaches, we evaluated the utility of HPLC–MS for low-level quantitation of oxymetazoline. MS has become widely utilized as a selective and sensitive detector for HPLC [8]. Several interfacing technologies have been used to couple the advantages of HPLC separation with the power of MS detection including the moving belt [9], thermospray [10,11], and particle beam [12,13]. The more recent combination of HPLC with MS using electrospray (ES) interfacing [14,15] has led to the use of this technique for new qualitative purposes and routine quantitation. A pneumatically assisted version of ES, called ionspray [16], has demonstrated the instrumental ruggedness required for routine quantitation of pharmaceuticals in biological matrices [17].

Many HPLC–ES-MS features make this technology attractive for use as a bioanalytical technique. For example, although widely varying across compound classes, the sensitivity is excellent in many cases. The ES interface offers mild conditions for the introduction of polar compounds to the gas phase, thereby eliminating the need for derivatization required in many GC-based methods and allowing the analysis of analytes not amenable to GC. The high selectivity of MS detection (especially MS–MS) generally allows for simple sample cleanup and minimizes chromatographic separation requirements. These features can afford significantly shorter sample preparation and analysis times, compared to other bioanalytical methods.

We have developed and describe here a rapid HPLC–ES-MS assay for quantitation of low levels of oxymetazoline in whole rat blood. The assay was applied to studies comparing the blood pharmacokinetics of oxymetazoline in rats dosed via i.v. and i.n. routes. To the best of our knowledge, this is the first reported non-radioisotopic method for the determination of this widely used drug in a biological matrix.

2. Experimental

2.1. Chemicals and reagents

Oxymetazoline [6-*tert.*-butyl-3-(2-imidazolin-2-ylmethyl)-2,4-dimethylphenol] hydrochloride USP Reference Standard was acquired from the United States Pharmacopeial Convention, Rockville, MD, USA. [$^{2}H_{9}$]Oxymetazoline (IS-d_9) internal standard (free base form) was synthesized at Procter and Gamble's Miami Valley Laboratories, Cincinnati, OH, USA. The chemical structures for oxymetazoline and IS-d_9 are show in Fig. 1. [2-^{14}C]Oxymetazoline hydrochloride (specific activity 31 mCi/mmol) was custom synthesized by Amersham, Arlington Heights, IL, USA. Ammonium acetate, acetonitrile and methanol were HPLC grade (Fisher Scientific, Cincinnati, OH, USA). Unless otherwise specified, all other reagents were ACS grade and obtained from standard suppliers. Solutions were made with water obtained from a Barnstead

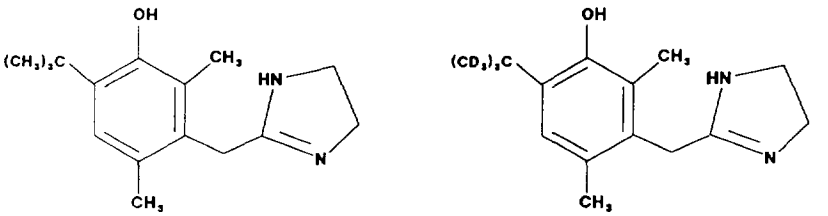

Fig. 1. The chemical structures of oxymetazoline and [$^2H_9$]oxymetazoline.

Nanopure cartridge system (Boston, MA, USA). Heparinized whole rat blood samples were taken from control, i.n.-dosed and i.v.-dosed Sprague-Dawley rats (200–300 g) obtained from Charles River Labs. (Plymouth, MI, USA).

2.2. Extraction recovery experiments

Aliquots of a [^{14}C]oxymetazoline solution in methanol were added with or without IS-d_9 as carrier to 100 × 16 mm borosilicate glass extraction tubes. The solvent was evaporated and 0.30 g of freshly thawed blank whole rat blood was added. The samples were vortexed and allowed to equilibrate for 30 min. The blood samples were then buffered with Na_2CO_3 and extracted once with 4.0 ml of anhydrous diethyl ether. The organic extracts were transferred to clean 100 × 16 mm borosilicate glass culture tubes and evaporated to dryness under nitrogen at 35°C. Dried extracts were reconstituted with 0.20 ml of acetonitrile–5 m*M* ammonium acetate buffer (50:50), transferred to liquid scintillation vials, and analyzed for total counts (Model 2200CA liquid scintillation analyzer; Packard, Meriden, CT, USA). Percent recoveries, based on dpm, were calculated versus aliquots of the [^{14}C]oxymetazoline stock solution.

2.3. Validation and quality control (QC) sample preparation

To evaluate method accuracy and precision, various amounts of the oxymetazoline reference standard (0.56, 2.17, 10.84 or 51.90 ng) were spiked, along with 50 ng IS-d_9, into 0.30 g of freshly thawed blank whole rat blood in 100 × 16 mm borosilicate glass culture tubes. The samples were vortexed, allowed to equilibrate for 30 min, buffered with 0.90 ml of 0.10 *M* Na_2CO_3, and extracted once with 3.0 ml of anhydrous diethyl ether. The volume of ether was reduced to 3.0 ml to minimize solvent usage and losses of the analyte due to absorption to the glass surface. The organic extracts were transferred to clean 100 × 16 mm borosilicate glass culture tubes, evaporated to dryness under nitrogen at 35°C, and stored at −20°C until analysis. On the day of analysis the dried extracts were thawed, reconstituted in 100 μl of acetonitrile–5 m*M* ammonium acetate (50:50), and transferred to autosampler vials. In this way, spiked validation samples were prepared at four levels ($n = 5$) on two separate days except for a 2.17-ng spike that was prepared on three separate days. Similarly, spiked QC samples were prepared at three levels ($n = 3$ each) on days coinciding with PHK sample preparation.

2.4. PHK study sample preparation

Preweighed blood samples (0.10 to 0.50 g) were received frozen in 1.5-ml polypropylene microcentrifuge tubes. These were thawed to room temperature, spiked with 50 ng IS-d_9, vortexed, and allowed to equilibrate for 30 min. The samples were then transferred to 100 × 16 mm borosilicate glass extraction tubes with three separate volumes of 0.10 *M* Na_2CO_3 (each buffer volume was equal to sample volume to

assure the same mequiv. buffer/g blood ratio for each sample) and extracted once with 3.0 ml of anhydrous diethyl ether. The organic extracts were transferred to clean 100 × 16 mm borosilicate glass culture tubes, evaporated to dryness under nitrogen at 35°C, and stored at −20°C until analysis. On the day of analysis the dried extracts were thawed, reconstituted in 100 μl of acetonitrile–5 m*M* ammonium acetate (50:50), and transferred to autosampler vials.

2.5. Preparation of HPLC–ES-MS standards

Non-matrix working standards were prepared in acetonitrile–5 m*M* ammonium acetate (50:50) and analyzed for daily method calibration. Quantitative transfers were made from stock solutions of oxymetazoline hydrochloride (based on mass of the free base form) and IS-d_9 using Wiretrol calibrated micropipettes (Drummond, Broomall, PA, USA) and a P-1000 Gilson Pipetman continuously adjustable pipette (Rainin, Woburn, MA, USA). Working standards ranged from 0.2–50 ng/100 μl with each containing 50 ng/100 μl of IS-d_9 and were stored at 2°C. Based on the reconstitution volume of 100 μl for an extract from 0.30 g blood, this range of standards is equivalent to 0.67–167 ng/g blood. Aliquots were transferred to autosampler vials prior to analysis.

2.6. HPLC–ES-MS conditions

Liquid chromatography was conducted using a Waters 600MS pump in conjunction with a Waters 715 Ultrawisp autosampler (Milford, MA, USA). The column was a 15 cm × 2.1 mm, 5 μm particle diameter, 8 nm pore size, Zorbax Rx-C_{18} (MacMod Analytical, Chadds Ford, PA, USA). The mobile phase consisted of a filtered (0.20 μm) and degassed solution of acetonitrile–5 m*M* ammonium acetate pH 6.5 (60:40), which was pumped at a flow-rate of 400 μl/min at room temperature. Using a 20:1 post-column split, 5% of the flow was directed into the mass spectrometer interface. In all cases, 20 μl of the standards and sample extracts were injected.

All experiments were conducted using a PE-Sciex (Thornhill, Ontario, Canada) API III tandem quadrupole mass spectrometer with the articulated ionspray interface. For quantitation, the instrument was operated with the third quadrupole in the selected ion monitoring (SIM) mode. The collision cell (Q2) used in this work was the original open cell design. Ions at *m/z* 261 and 270 were monitored (0.7 s cycle time) to detect the $[M + H]^+$ of oxymetazoline and IS-d_9, respectively. A nebulizer gas (air) pressure of 42 p.s.i. (1 p.s.i. = 6894.76 Pa) and a curtain gas (nitrogen) flow of 1.2 l/min were used, as well as an indicated orifice voltage of 70 V. The mass spectrometer was calibrated daily using a 10^{-4} *M* solution of polypropyleneglycols (PPGs) that was directly infused with a Harvard Apparatus 22 (South Natick, MA, USA) syringe pump at 10 μl/min.

2.7. Quantitation

Calibration curves were generated from the non-matrix working standards run on each day of analysis. Oxymetazoline levels in QC and unknown samples were determined from weighted ($1/x$) curves derived from the peak area ratios of standard to IS-d_9. All peak areas were selected using the PE-Sciex MacQuan program. The calibrations (eight data points, $n = 1$ each point) were linear over the range of standards used (0.2–50 ng/100 μl). Typical regression data for a daily standard curve generated for analyses of samples (e.g., 40 μg i.n. dose described later) were: slope 0.0465; intercept 0.00429; correlation coefficient 0.99983; standard error of regression for *y* estimate 0.00920. The standard deviations for the slope and intercept from six daily standard curves were 0.00332 and 0.00131, respectively.

3. Results

3.1. Extraction recovery experiments

[^{14}C]Oxymetazoline was used to develop and optimize the extraction procedure for recovery of the drug. Results from preliminary in vitro

Table 1
Effect of Na_2CO_3 amount on recovery of 50 ng [^{14}C]oxymetazoline from rat blood

Na_2CO_3 Concentration (M)	mequiv. buffer/g blood	Percent recovery (± S.D.)
0.00	0.00	12 (± 0.74)
0.01	0.06	32 (± 1.6)
0.02	0.12	67 (± 3.4)
0.04	0.24	77 (± 3.6)
0.06	0.36	78 (± 5.1)
0.08	0.48	82 (± 1.6)
0.10	0.60	83 (± 3.2)
0.15	0.90	83 (± 3.8)
0.20	1.20	87 (± 3.9)

A 0.90-ml volume of buffer was used at each concentration, 0.30 g blood ($n = 3$) at each concentration.

studies indicated that oxymetazoline partitions between plasma and red blood cells in a concentration-dependent manner with higher proportions partitioning into the cells at low ng/g levels. Based on these data (not shown), whole blood was chosen as the target matrix from which to isolate the analyte. Compounds of similar structure [e.g., 2-(2-hydroxyphenyl)imidazoline] have reported pK_a values of approximately 7 and 11 [18]. Therefore, a liquid–liquid extraction with diethyl ether following pH adjustment with aqueous Na_2CO_3 appeared to be the most promising approach to sample preparation.

Results of an experiment to determine the optimum amount of Na_2CO_3 buffer (mequiv./g blood) required for optimal recovery of oxymetazoline from 0.30 g of blood using one extraction with diethyl ether are given in Table 1. Based on these data, 0.90 ml of 0.10 M Na_2CO_3 added to 0.30 g of blood (0.60 mequiv./g) gives good recovery due to adequate pH adjustment without addition of excess reagent that could result in significant salt carryover into the ether extract. Table 2 illustrates the carrier effect of 50 ng IS-d_9 on the recovery of [^{14}C]oxymetazoline from blood using a single extraction over the concentration range of 1.67–333 ng/g. Recoveries with addition of the internal standard were significantly improved for the lower concentrations. This is due to a shift in the total level of oxymetazoline to concentrations where partitioning into red blood cells is essentially constant.

3.2. HPLC–ES-MS results

The electrospray mass spectra of oxymetazoline and IS-d_9 are shown in Fig. 2. The base peak in each spectrum results from the $[M + H]^+$ at m/z 261 and 270, respectively. The presence of a d_8 analogue (m/z 269), a d_7 analogue (m/z 268), and so on, can be seen in the spectrum of the labeled compound, indicating that the *tert.*-butyl group is not fully incorporated with deuterium. This has no effect on the use of this material as an internal standard since no oxymetazoline-d_0 could be detected in this material. For the purposes of this work the d_9 analogue was monitored and the other analogues (d_8–d_5) were ignored. Other peaks seen in the spectra (< 25% relative intensity) are believed

Table 2
Extraction efficiency for [^{14}C]oxymetazoline from blood without and with 50 ng IS-d_9

[^{14}C]Oxymetazoline spike (ng/g)	Recovery (%)	
	Without IS-d_9 (± S.D.)	With IS-d_9 (± S.D.)
1.67	68 (± 6.7)	82 (± 3.4)
10.4	77 (± 2.7)	81 (± 4.4)
41.7	83 (± 5.8)	85 (± 2.4)
167	89 (± 3.5)	82 (± 3.0)
333	88 (± 3.6)	86 (± 3.0)

A 0.90-ml volume of 0.10 M Na_2CO_3 was used for pH adjustment, 0.30 g blood ($n = 3$) at each concentration.

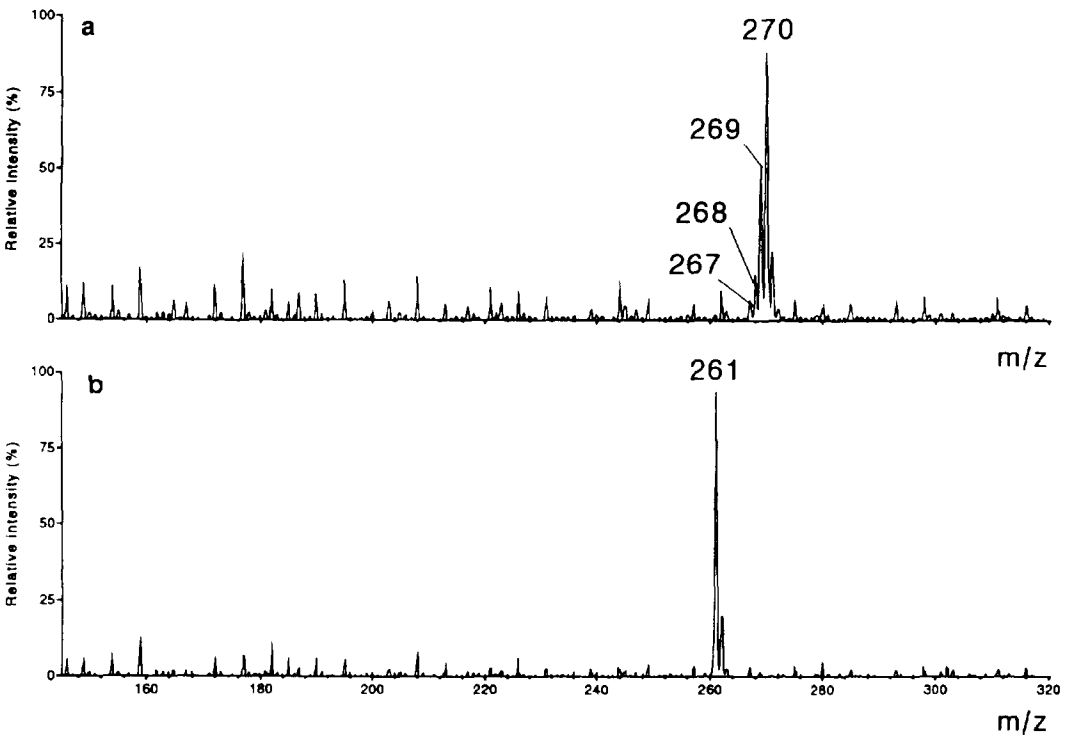

Fig. 2. Electrospray mass spectra of oxymetazoline (b) and its labeled analogue, [$^2H_9$]oxymetazoline (a). Flow injection analysis with acetonitrile–2 m*M* ammonium acetate (50:50) at 40 μl/min.

to be primarily chemical noise from the mobile phase. No evidence of fragmentation of $[M + H]^+$, using these conditions, was noted.

Since the original intent of this work was to detect the analyte using selected reaction monitoring (SRM) MS–MS techniques, daughter ion spectra using these $[M + H]^+$ ions as parents were next obtained (Fig. 3). Unfortunately, daughter ions found in these MS–MS spectra could not be abundantly formed. No experimental conditions, using the open collision cell (Q2), could be found for this highly conjugated compound that would create daughter ions with high enough efficiency to allow sensitive detection using an SRM scheme.

Since MS–MS detection was not feasible, single MS detection (SIM) was utilized. This detection mode, with the described sample preparation, still allowed rapid sample elution and detection. An example of this is illustrated in Fig. 4 where HPLC–ES-MS chromatogram SIM traces for a 2.17 ng/g spike of oxymetazoline with 50 ng IS-d_9 in rat blood are displayed. For comparison, a SIM trace (*m/z* 261) for a blank sample spiked with 50 ng IS-d_9 is superimposed on the 2.17 ng/g spiked sample trace. Although there is noticeable peak tailing (asymmetry factor 2.0, calculated by method of Foley and Dorsey [19]), likely due to interaction of the nitrogen containing ring with residual silanols on the silica packing, use of the Zorbax Rx column permits elution of this basic compound without the use of additional mobile phase modifiers (e.g., triethylamine) that would decrease sensitivity of the $[M + H]^+$ signal.

Table 3 summarizes the results of all validation and QC analyses. The limit of quantitation of the method, with a 0.30-g sample, was 1.0 ng/g.

3.3. Pharmacokinetic results

Typical blood PHK profiles of oxymetazoline in rats generated from i.v. and i.n. dosings of the drug in saline solution are shown in Fig. 5 (40

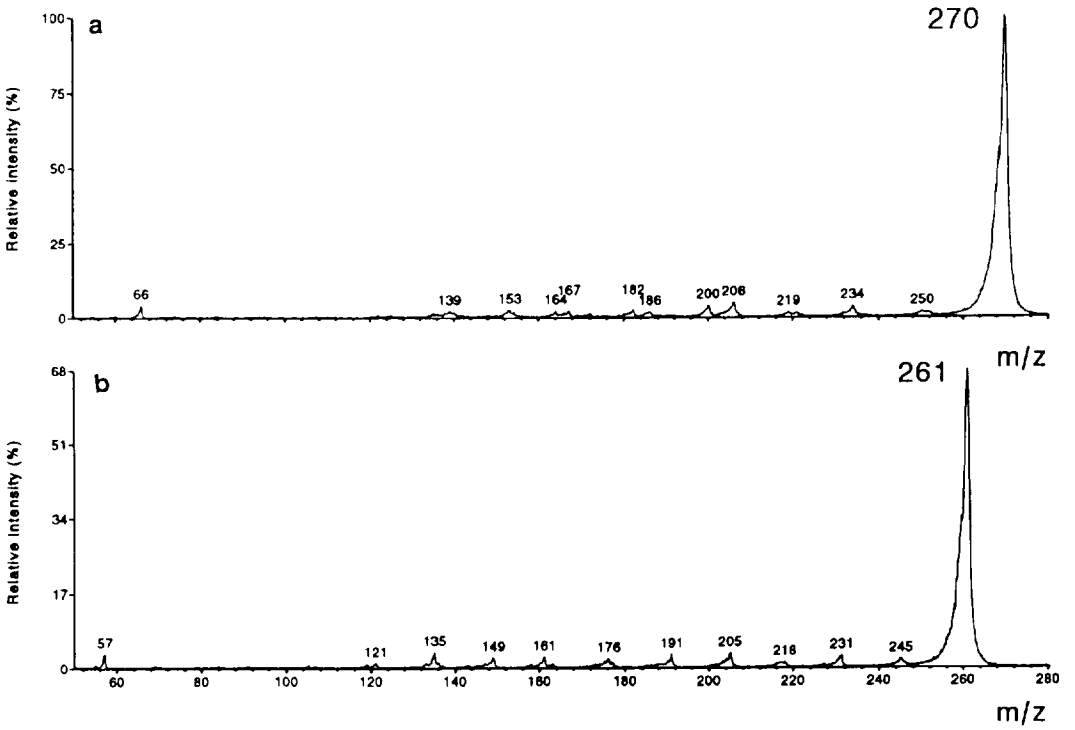

Fig. 3. Electrospray tandem mass spectra (daughters of MH^+) of oxymetazoline (b) and its labeled analogue, [$^2H_9$]oxymetazoline (a). Flow injection analysis with acetonitrile–2 m*M* ammonium acetate (50:50) at 40 μl/min.

μg/dose). Blood samples (typically less than 300 μl) were taken serially following administration of the drug. Fig. 5 illustrates the dramatic differences in blood levels delivered by the two administration routes. The results show that oxymetazoline is not extensively absorbed into the systemic circulation of rats from the intranasally dosed saline solution. The bioavailability of oxymetazoline delivered by the i.n. dosing (n = 4) was 6.8%. This was calculated using areas under the curves (0–90 min time points) for the i.n. and i.v. administrations.

4. Discussion

Although it is not particularly thermally labile, oxymetazoline must be derivatized prior to GC analysis because of its polar characteristics. Even then, the trimethylsilated derivative (TMS_2-oxymetazoline) will interact with the active sites in the heated chromatographic system, thereby becoming a limiting factor for trace-level determinations (data not shown). However, oxymetazoline can be subjected to HPLC separation using column packings that contain less acidic residual silanols (e.g., Zorbax Rx) and introduced to the mass spectrometer via the ES interface in its free-base (underivatized) form.

Although low level detection of oxymetazoline by HPLC–MS–MS using the open collision cell was not feasible, MS detection with SIM permitted development of an assay with the limit of quantitation (1 ng/g in whole blood) necessary for this application. This detection mode, even with the single liquid–liquid extraction, still allowed rapid sample elution and detection without the need for chemical derivatization.

Subsequent to the PHK study described above, additional sample preparation experiments led to a significant improvement in the level of analyte that could be accurately quantitated if larger samples were available. Briefly, a solid-phase extraction with Bakerbond C_{18} solid-

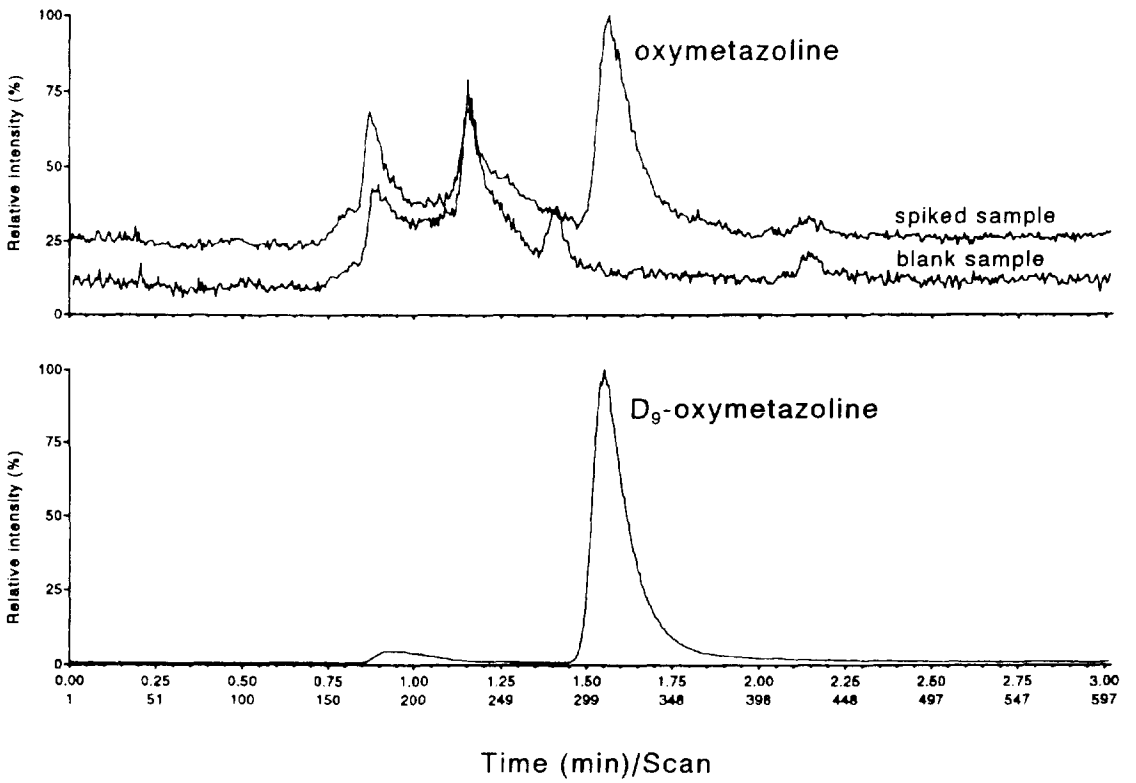

Fig. 4. HPLC–ES-MS chromatogram traces using selected ion monitoring of extracts for 2.17 ng/g oxymetazoline (top, m/z 261) with 50 ng/g [$^2H_9$]oxymetazoline (bottom, m/z 270) spiked into 0.30 g rat blood. The relative intensity signal for the superimposed blank extract trace (top, m/z 261) is offset 12.5% for comparison.

phase extraction cartridges (J.T. Baker, Pittsburgh, PA, USA) was performed following the initial liquid–liquid extraction. Feasibility studies using 2.0-ml samples of human serum (Sigma, St. Louis, MO, USA) showed that 200 pg/ml oxymetazoline could be readily quantitated.

The collision cell (Q2) used in this work was the original open cell design. An improved, closed cell has since become available for the PE-Sciex instrumentation. The possibility of an

Table 3
Accuracy and precision for validation and QC matrix spikes by HPLC–ES-MS

Spiked (ng)	n	Recovered (ng)	Accuracy (%)	R.S.D. (%)
Blank	13	0.053		
0.56	28	0.62	111	19.2
2.17	33	2.30	106	11.9
10.84	28	11.09	102	6.59
51.90	10	51.39	99	3.54

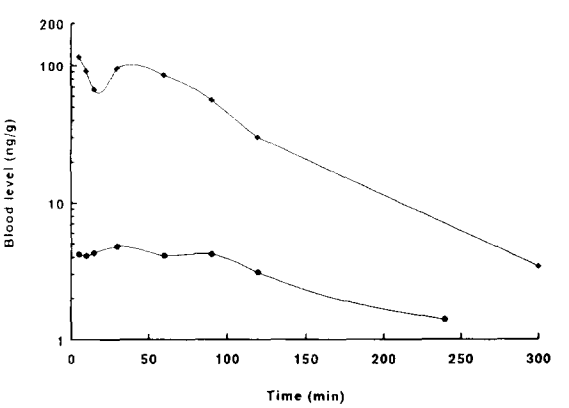

Fig. 5. Pharmacokinetic profiles of oxymetazoline generated from 40 μg i.v. (upper trace) and i.n. (lower trace) solutions administered to rats ($n = 4$).

MS–MS detection scheme for this analyte using the new enclosed collision cell has not been fully explored. However, this improved collision cell does provide enhanced production of product ions. It is reasonable to believe that greater sensitivity could be achieved with an SRM scheme using the new collision cell through better discrimination against matrix interferences.

5. Conclusions

The results of the work described here illustrate the potential of HPLC–ES-MS-based technology for quantitative determination of polar drugs, such as oxymetazoline, in biological matrices. The HPLC–MS approach with ES interfacing permitted the development of a rapid assay without the need for derivatization. The assay provided the requisite sensitivity to accurately define the blood pharmacokinetic profiles of oxymetazoline in rats dosed intravenously and intranasally with the drug. This was especially critical for the i.n. administration route in saline solution because of low sample volumes (typically less than 300 μl) due to serial blood sampling from the same animal and the poor nasal absorption of oxymetazoline in this model.

Acknowledgements

The authors gratefully acknowledge Stanislaw Pikul and Greg A. Switzer for synthesis of the deuterated oxymetazoline internal standard used in this work.

References

[1] S. Gizurarson, *Acta Pharm. Nord.*, 2 (1990) 105.
[2] Y.W. Chien, K.S.E. Su and S. Chang, in J. Swarbrick (Editor), *Nasal Systemic Drug Delivery*, Marcel Dekker, New York, 1989, Ch. 1, p. 1.
[3] D.E. Cowen, *Ear Nose Throat J.*, 45 (1966) 58.
[4] E. Duzman, J. Anderson, J.B. Vita, J.C. Lue, C. Chen and I.H. Leopold, *Arch. Ophthalmol.*, 101 (1983) 1122.
[5] A.P. De Leenheer and L.M. Thienpont, *Mass Spectrom. Rev.*, 11 (1992) 249.
[6] R.L.M. Dobson, D.M. Neal, B.R. DeMark and S.R. Ward, *Anal. Chem.*, 62 (1990) 1819.
[7] R.L.M. Dobson, G.R. Kelm and D.M. Neal, *Biol. Mass Spectrom.*, 23 (1994) 75.
[8] K.B. Tomo and C.E. Parker, *J. Chromatogr.*, 492 (1989) 189.
[9] P. Arpino, *Mass Spectrom. Rev.*, 8 (1989) 35.
[10] C.R. Blakely and M.L. Vestal, *Anal. Chem.*, 55 (1983) 750.
[11] P. Arpino, *Mass Spectrom. Rev.*, 9 (1990) 631.
[12] R.C. Willoughby and R.R. Browner, *Anal. Chem.*, 56 (1984) 2626.
[13] P.C. Winkler, D.D. Perkins, W.K. Williams and R.F. Browner, *Anal. Chem.*, 60 (1988) 489.
[14] C.M. Whitehouse, R.N. Drego, M. Yamashita and J.B. Fenn, *Anal. Chem.*, 57 (1985) 675.
[15] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong and C.M. Whitehouse, *Mass Spectrom. Rev.*, 9 (1990) 37.
[16] A.P. Bruins, T.R. Covey and J.D. Henion, *Anal. Chem.*, 59 (1987) 2642.
[17] T.R. Covey, E.D. Lee and J.D. Henion, *Anal. Chem.*, 58 (1986) 2453.
[18] G.D. Fasman (Editor), *CRC Handbook of Biochemistry and Molecular Biology*, Vol. 1, CRC Press, Boca Raton, 3rd ed., 1976, p. 334.
[19] J.P. Foley and J.G. Dorsey, *Anal. Chem.*, 55 (1983) 730.