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Journal of Chromatography B, 693 (1997) 431–436

JOURNAL OF CHROMATOGRAPHY B

Sensitive reversed-phase high-performance liquid chromatographic method for the determination of atevirdine and its N-desethyl metabolite in human saliva or cerebrospinal fluid using solid-phase extraction

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Received 16 April 1996; revised 30 December 1996; accepted 24 January 1997

Abstract

A sensitive reversed-phase high-performance liquid chromatographic method for the determination of atevirdine and its primary metabolite in human saliva or cerebrospinal fluid using solid-phase extraction is described. Samples mixed with internal standard and sodium phosphate buffer were applied to an activated C_{18} solid-phase extraction column. The reconstituted eluate was injected onto a Zorbax RX C_8 column utilizing a mobile phase of 100 m*M* ammonium acetate (pH 4.0)–isopropyl alcohol–acetonitrile (55:20:25, v/v/v). Fluorescence detection was employed with excitation at 295 nm and emission at 456 nm. Quantitation was achieved using peak-height ratios. The detection response curve was linear from 2 to 850 n*M* for atevirdine in both human saliva and cerebrospinal fluid and from 2 to 250 n*M* for the metabolite in human saliva. The method was utilized to analyze cerebrospinal fluid and saliva samples from clinical studies.

Keywords: Atevirdine; N-Desethylatevirdine

1. Introduction

The non-nucleoside bis(heteroaryl)piperazine compounds (BHAPs) developed at Pharmacia and Upjohn have demonstrated potent inhibitory activity in vitro against the reverse transcriptase (RT) enzyme expressed by the human immunodeficiency virus type 1 (HIV-1) [1–4]. One of these BHAPs is atevirdine mesylate (Fig. 1a), which has been under investigation for the clinical treatment of acquired immunodeficiency syndrome (AIDS) [5–8]. Since HIV-1 crosses the blood–brain barrier, an effective drug for the treatment of HIV-1 and AIDS should distribute to the cerebrospinal fluid (CSF). A high-performance liquid chromatographic (HPLC) method employing solid-phase extraction (SPE) for the sensitive and quantitative determination of atevirdine in human CSF was validated and applied to analyze study samples [9]. The procedure was also employed to quantitatively determine atevirdine and its primary metabolite, an N-desethyl metabolite (Fig. 1b), in human saliva. The method utilized an atevirdine analog, U-85961 (Fig. 1c), as an internal standard.

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PII S0378-4347(97)00090-X

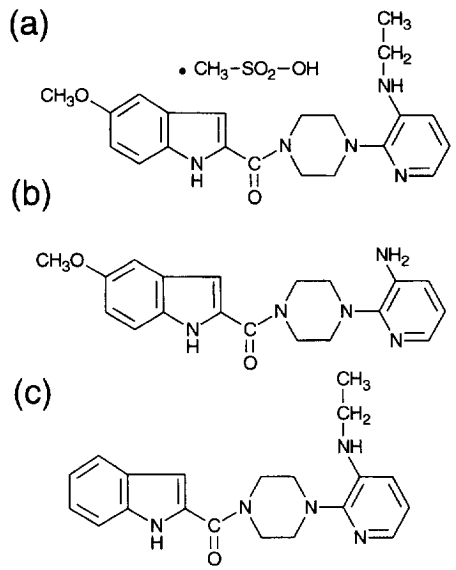

Fig. 1. (a) Atevirdine mesylate, U-87201E (M_r 475.57). (b) Metabolite, U-89255 (M_r 351.41). (c) Internal standard, U-85961 (M_r 349.44).

2. Experimental

2.1. Materials

Atevirdine mesylate, U-87201E, 1-[3-(ethylamino)-2-pyridinyl]-4- [(5-methoxy-1*H*-indol-2-yl)-carbonyl]piperazine, monomethanesulfonate, the N-desethyl metabolite of atevirdine, U-89255, 1-(3-amino-2-pyridinyl)-4 - [(5-methoxy-1*H*-indol-2-yl)-carbonyl]piperazine and the internal standard, U-85961, 1-[3-(ethylamino)-2-pyridinyl]-4-[(1*H*-indol-2yl)carbonyl]piperazine, were prepared at Pharmacia and Upjohn (Kalamazoo, MI, USA) [4]. Analytical grade glacial acetic acid, ammonium hydroxide, 50% sodium hydroxide solution (w/w), and sodium chloride crystals were purchased from Mallinckrodt (Paris, KY, USA). Sodium phosphate (monobasic, monohydrate) and ammonium phosphate (dibasic) were purchased from J.T. Baker (Phillipsburg, NJ, USA). All water was filtered by a Milli-Q water system from Millipore (Bedford, MA, USA). HPLC UV-grade acetonitrile, isopropyl alcohol, and methanol were obtained from Burdick and Jackson (Muskegon, MI, USA). Drug free human saliva and serum were obtained from normal volunteers and drug free human CSF was obtained from recently deceased cadavers. However, due to the limited supply of human CSF, simulated CSF was prepared for the standard curve and quality control samples.

2.2. Reagents

The 100 m*M* ammonium acetate buffer was prepared with glacial acetic acid and ammonium hydroxide was employed to adjust the solution to pH 4.0. The 100 m*M* sodium phosphate buffer was adjusted to pH 7.4 with the sodium hydroxide solution. An isotonic 20 m*M* ammonium phosphate buffer was prepared with dibasic ammonium phosphate and 7.6 g/l sodium chloride, and adjusted with either ammonium hydroxide or phosphoric acid until pH 7.4 was attained. Simulated CSF was prepared by mixing human serum with the isotonic 20 m*M* ammonium phosphate buffer at a ratio of 1 part serum to 99 parts buffer, so the protein and salt content would be comparable to human CSF [10]. Mobile phase for the HPLC was prepared by mixing 100 m*M* ammonium acetate (pH 4.0), isopropyl alcohol and acetonitrile (55:20:25, v/v/v), while filtering through a 0.2 μm nylon-66 Rainin filter (Woburn, MA, USA). The mobile phase was degassed by slowly sparging with high purity helium for approximately 5 min.

2.3. Instrumentation

The HPLC instrumentation consisted of an LDC Constametric III isocratic pump (Thermo Separation Products, Fremont, CA, USA), a Perkin-Elmer ISS-100 autosampler (Norwalk, CT, USA) with the autosampler tray connected to a circulating temperature bath (Endocal-Neslab Model RTE-5B, Newington, NH, USA), a Perkin-Elmer LC240 Fluorescence Detector, a Perkin-Elmer Nelson 941 interface (data collection system) and a Harris NightHawk superminicomputer (Fort Lauderdale, FL, USA). The autosampler tray was operated at about 12°C. The analytical column was a Zorbax RX C_8 column, 150×4.6 mm I.D., 5 μm particle diameter (MAC-MOD Analytical, Chadds Ford, PA, USA) protected by a Brownlee MPLC RP-8 NewGuard column, 15 mm×3.2 mm I.D., 7 μm (Applied Biosystems, Foster City, CA, USA) and was employed at room temperature. The mobile phase flow-rate was 1.0

ml/min which produced a column backpressure of about 13.8 MPa. The fluorescence detector monochronomater excitation was set at 295 nm and the emission monochromater was set at 456 nm with a response of 4 and a fixed factor of 10. Typical retention times of about 9 min for atevirdine, 4 min for the metabolite, and 11 min for the internal standard were observed (Fig. 2).

2.4. Preparation of stocks, standard solutions and quality controls

Stock solutions containing atevirdine and its primary metabolite for the standard curve and the quality control (QC) samples were prepared by dissolving appropriate amounts of atevirdine mesylate and metabolite in methanol. Six standard curve working solutions were prepared by dilution of the stock solutions with methanol, resulting in concentrations ranging approximately from 0.05 to 20 μg/ml for atevirdine mesylate and from 0.035 to 4.5 μg/ml for the metabolite. The signal-to-noise ratio at the lower limits of quantitation for atevirdine (2 n*M*) and its metabolite (2 n*M*) were approximately 9 and 12, respectively. Three QC working solutions were prepared by serial dilution of the QC stock solution with methanol, resulting in concentrations of 1, 10 and 100 μg/ml for atevirdine and 0.39, 3.9 and 39 μg/ml for the metabolite. The QC samples in saliva or CSF were prepared by transferring 0.1 ml of the

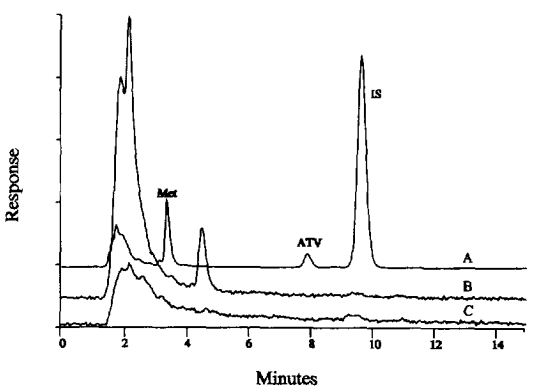

Fig. 2. Comparison of drug free human and simulated CSF. Chromatogram A is a human CSF sample, including the Met, ATV and I.S.. Chromatogram B is drug free human CSF. Chromatogram C is drug free simulated CSF. Chromatograms B and C are scaled at 10× of chromatogram A.

QC working solutions to a 50 ml volumetric flask and diluting to volume with either human saliva or simulated CSF. The stability of atevirdine and its metabolite in matrix when stored at approximately −20°C was at least 3 months. Heating samples at 56°C for 30 min has been shown to deactivate HIV [11]. Atevirdine spiked human CSF samples were also stable when heated at approximately 58°C for 30 min. The 0.1 mg/ml internal standard (I.S.) solution, U-85961, was prepared in a methanol–water (50:50, v/v) solution.

2.5. Sample preparation

Calibration standards were prepared in duplicate by accurately dispensing 0.010 ml of MeOH working solutions to the bottom of 12×75 mm polypropylene tubes and adding 0.5 ml of drug free human saliva or simulated CSF. The calibration standards were then treated in the same manner as the study or QC samples. Study and QC samples (0.5 ml) were transferred to the bottom of 12×75 mm polypropylene tubes, but samples with analyte concentrations exceeding the upper limit of quantitation were diluted by adding 0.4 ml of drug free saliva or simulated CSF to a 0.1 ml sample volume (1/5 dilution). To each sample, 0.025 ml of the U-85961 I.S. solution and 0.5 ml of 0.1 *M* sodium phosphate were added, tubes were capped, and samples were vortex mixed for at least 5 s. A C_{18} SPE column (100 mg/ml, Bond Elut, Analytichem International, Varian, Harbor City, CA, USA) was used for sample extraction. The SPE columns placed on the vacuum manifold system (Supelco Visiprep, Bellefonte, PA, USA) were activated by conditioning the cartridge sequentially with 1 ml of ACN, 1 ml of water and 1 ml of 0.1 *M* sodium phosphate using low vacuum (approximately 15 kPa) and always leaving about 1–2 mm of liquid above the column frit to prevent the sorbent from drying out. Each sample mixture was loaded onto an individual SPE column using low vacuum (approximately 15 kPa). The sample tube was rinsed with 0.5 ml of 0.1 *M* sodium phosphate and loaded onto the respective SPE column using low vacuum (approximately 15 kPa). The column was then washed with 2 ml of water using higher vacuum (approximately 50 kPa), but not allowed to dry. The analytes were eluted from the column with

1 ml of ACN–water (80:20, v/v) using low vacuum (approximately 15 kPa) into a clean 12×75 mm polypropylene tube. The eluate was then evaporated to dryness under nitrogen (approximately 80 kPa, Zymark TurboVap LV nitrogen evaporator, Hopkinton, MA, USA) at 40°C for approximately 50 min. The sample was reconstituted with 0.2 ml of mobile phase, vortex mixed for at least 10 s, and 0.17 ml was injected onto the HPLC system for analysis.

3. Results and discussion

The large volume of drug free CSF matrix necessary for assay development and the applied assays exceeded its availability. The alternative matrix, referred to as simulated CSF, was prepared by diluting human serum with a phosphate buffered saline solution. Serum was used instead of plasma, since fibrinogen is found in plasma, but not in human CSF. Evidence from the analysis of fortified human CSF and simulated CSF samples demonstrated quantitative recovery of atevirdine. No chromatographic interferences with atevirdine or the internal standard were observed for simulated CSF or blank human CSF as shown in Fig. 2. Also, fluorescence detection is a more specific method of detection compared with ultraviolet detection, minimizing the possibility of chromatographic interferences from drugs commonly co-administered to patients receiving atevirdine.

Results from three validation assays, each with three concentrations of QC samples in triplicate and at least six calibration standards in duplicate, indicated the atevirdine standard curve to be linear in CSF from 2 to 850 n*M* with correlation coefficients greater than 0.9998. The mean slope was 12.86 with a standard deviation (S.D.) of 0.14 and the intercept was insignificant. Intra-assay and inter-assay accuracy and precision for the atevirdine QC samples were acceptable, with less than a 7% bias or relative standard deviation (R.S.D.), as shown in Table 1. The method was applied to the analysis of atevirdine in human CSF samples, in which AIDS dementia patients received 600 mg of atevirdine mesylate, administered orally three times a day for twelve weeks [12]. Observed CSF levels ranged from 2.87 to 186 n*M*, demonstrating that atevirdine was able to cross the blood–brain barrier in AIDS patients. This method did not include the quantitation of the N-desethyl metabolite in CSF for the samples analyzed. However, chromatograms from the study samples do provide evidence of this metabolite in human CSF in significant amounts, identified by retention time. Chromatograms of a predose human CSF sample and a postdose study sample (after 12 weeks of dosing) are presented in Fig. 3.

Table 1
Intra-assay and inter-assay accuracy and precision data for atevirdine in quality control samples prepared in simulated cerebrospinal fluid

Concentration (n*M*)	*n*	Assay mean (n*M*)	R.S.D. (%)	Bias (%)
7.66	3	7.88	1.4	+2.9
	3	7.29	2.2	−4.8
	3	7.18	3.0	−6.3
	Overall	7.45	4.8	−2.7
30.7	3	29.4	1.2	−4.2
	3	29.3	0.8	−4.6
	3	28.6	1.1	−6.8
	Overall	29.1	1.6	−5.2
153	3	151	1.5	−1.3
	3	149	0.5	−2.6
	3	147	0.9	−3.9
	Overall	149	1.5	−2.6
307	3	297	1.4	−3.3
	3	292	1.6	−4.9
	3	301	0.5	−2.0
	Overall	296	1.7	−3.6

Likewise, three validation assays, each with three concentrations of QC samples in at least triplicate and six calibration standards in duplicate, were performed in human saliva for atevirdine and the metabolite. The results indicated the atevirdine standard curve to be linear in human saliva from 2 to 850 n*M*, with correlation coefficients greater than 0.9995. The mean slope was 13.24 with a S.D. of 0.26 and the intercept was insignificant. Intra-assay and inter-assay accuracy and precision for the atevirdine QC samples were acceptable, with less than a 13% bias or R.S.D., as shown in Table 2. The standard curves for the N-desethyl metabolite were linear from 2 to 250 n*M*, with correlation coefficients greater than 0.9994. The mean slope was 29.32 with a S.D. of

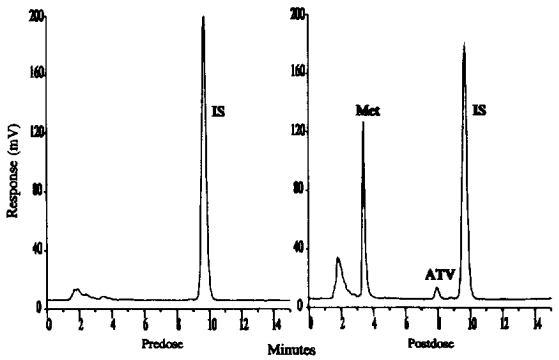

Fig. 3. Representative chromatograms from a human subject who received multiple oral doses of 600 mg atevirdine mesylate every 8 h (CSF concentration of ATV was 15.8 n*M* after 12 weeks of dosing). Met=metabolite; ATV=atevirdine; I.S.=internal standard.

0.67 and the intercept was insignificant. Intra-assay and inter-assay accuracy and precision for the metabolite QC samples were acceptable, with less than a 13% bias or R.S.D., as shown in Table 3. This procedure was applied to human saliva study samples, in which healthy male volunteers received a single oral dose of 400 mg atevirdine mesylate. The observed saliva levels of atevirdine ranged from 7.11 to 4830 n*M*, while the observed N-desethyl metabolite levels ranged from 19.0 to 168 n*M*. Chromatograms of a predose human saliva sample and a 3 h postdose study sample are presented in Fig. 4. No

Table 2
Intra-assay and inter-assay accuracy and precision data for atevirdine in quality control samples prepared in human saliva

Concentration (n*M*)	*n*	Assay mean (n*M*)	R.S.D. (%)	Bias (%)
4.25	4	3.95	8.9	−7.1
	3	4.54	2.3	+6.8
	3	4.54	3.1	+6.8
	Overall	4.30	8.7	+1.2
42.5	4	37.3	3.4	−12.2
	3	41.6	2.8	−2.1
	3	38.8	1.3	−8.7
	Overall	39.1	5.4	−8.0
425	4	376	1.7	−11.5
	3	392	0.6	−7.8
	3	378	1.1	−11.1
	Overall	382	2.2	−10.1

Table 3
Intra-assay and inter-assay accuracy and precision data for the N-desethyl metabolite in quality control samples prepared in human saliva

Concentration (n*M*)	*n*	Assay mean (n*M*)	R.S.D. (%)	Bias (%)
2.23	4	2.12	6.7	−4.9
	3	2.45	0.8	9.9
	3	2.09	7.0	−6.3
	Overall	2.21	8.9	−0.9
22.3	4	22.3	3.9	0.0
	3	24.9	1.3	+11.7
	3	23.0	5.7	+3.1
	Overall	23.3	6.0	+4.5
223	4	226	1.8	+1.3
	3	250	2.9	+12.1
	3	231	2.8	+3.6
	Overall	234	5.1	+4.9

chromatographic interferences with atevirdine, the N-desethyl metabolite, or the internal standard were observed for human saliva.

4. Conclusion

The HPLC procedure described in this article was used to determine atevirdine in human CSF and atevirdine and the N-desethyl metabolite in saliva with acceptable accuracy and precision. The methods

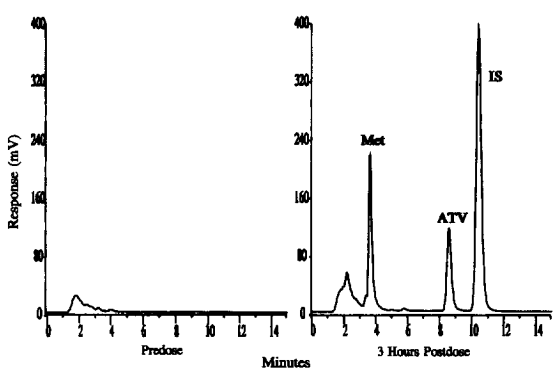

Fig. 4. Representative chromatograms from a human subject who received a single oral dose of 400 mg atevirdine mesylate (saliva concentrations of ATV and Met at 3 h postdose were 123 and 104 n*M*, respectively). Met=metabolite; ATV=atevirdine; I.S.=internal standard.

were free of interference from endogenous matrix components and appear to be useful for the assessment of atevirdine exposure in clinical trials.

Acknowledgments

The authors would like to thank Dean W. Knuth for acquiring the drug free human cerebrospinal fluid and Paul A. Bombardt for his guidance in preparing the simulated CSF.

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