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Determination of travoprost and travoprost free acid in human plasma by electrospray HPLC/MS/MS

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Abstract

A quantitative method for the analysis of AL-5848, the (+)-enantiomer of fluprostenol (FP), in human plasma is described. Plasma was spiked with a tetradeuterated analog of travoprost free acid (AL-5848X) as internal standard (IS) and acidified with 0.1 M formic acid. Sample clean up was performed using reversed phase solid-phase extraction. Following elution of the compounds of interest and evaporation to dryness, the residue was reconstituted in methanol:water (1:1) and chromatographed on an octadecylsilica (C18) column with negative ion electrospray ionization tandem mass spectrometry. The $[M–H]^-$ ions at m/z 457 and 461 for the analyte and IS, respectively, were subjected to collisional fragmentation with argon to yield the same intense 3-trifluoromethylphenolate (m/z 161) product ion. The validated concentration range was 0.010–3.00 ng/ml based on a 1.0 ml plasma aliquot. Fully adequate accuracy, precision, specificity, recovery and stability for routine use in clinical pharmacokinetic studies were demonstrated. Analysis of a second plasma aliquot following incubation with rabbit esterase allows the isopropyl ester pro-drug, travoprost (AL-6221), to be determined by difference.

Keywords: Fluprostenol; Prostaglandin; Electrospray MS–MS

1. Introduction

Travoprost free acid, a potent and selective agonist of the prostaglandin $F_{2\alpha}$ receptor, is the (+)-enantiomer of fluprostenol (+FP), a marketed veterinary product for induction of estrus in mares. The isopropyl ester of +FP, travoprost (AL-6221, Fig. 1), has been developed for reducing intraocular pressure in the clinical treatment of glaucoma. Following topical ocular administration, the ester pro-drug crosses the cornea and is rapidly hydrolyzed to +FP, which distributes to ocular tissues and the systemic circulation. The pharmacokinetics of the radioactive biotransformation products of racemic FP in horses following administration of ^{3}H-FP have been reported [1].

Due to the low doses used for topical administration, a highly sensitive method was required to characterize the clinical pharmacokinetics of +

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FP. Techniques for prostaglandin bioanalysis include radioimmunoassay (RIA) [2,3], enzyme immunoassay (EIA) [4–6], gas chromatography/mass spectrometry (GC/MS) with selected ion monitoring (SIM) [7–9] as well as gas chromatography with tandem mass spectrometry (GC/MS/MS) [10]. More recently, HPLC tandem mass spectrometry (HPLC/MS/MS) [11,12] has been used for trace level bioanalysis of prostaglandins. This technique allows highly sensitive determination of prostaglandins without the need for derivatization as required for GC/MS. It is also specific against metabolites and structurally similar eicosanoids. This is frequently not the case with immunoassays, where cross-reactivity with closely related analogs can positively bias results.

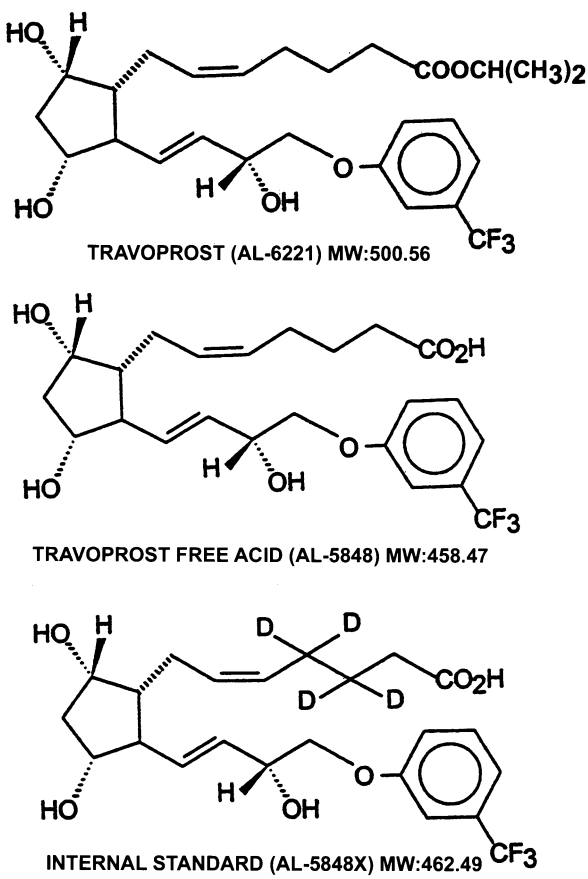

Fig. 1. Structures of travoprost, travoprost free acid (+ FP, AL-5848) and internal standard (IS) (AL-5848X).

In this study, an HPLC/MS/MS method for determination of + FP in human plasma was developed and validated. A tetra-deuterated analog of + FP was used as internal standard (IS) and sample cleanup was performed using reversed-phase solid phase extraction (SPE), with methyl formate used in the elution step. This solvent was previously shown [13] to give good recovery of prostaglandins from biological matrices in reversed-phase SPE.

2. Materials and methods

2.1. Reagents and chemicals

Reference standards of travoprost, travoprost free acid and AL-5848X IS were prepared by Alcon Research, Ltd. The structures are shown in Fig. 1. Methanol, water, toluene, acetonitrile and dichloromethane were of HPLC grade and obtained from EM Science (Gibbstown, NJ, USA). Reagent grade formic acid, 99%, ammonium formate and methyl formate were obtained from Sigma (St. Louis, MO, USA) as was esterase from rabbit liver (100 U per mg). Dulbecco's phosphate buffered saline (1 ×), without calcium or magnesium, was obtained from Hyclone Laboratories (Logan, UT, USA). Control human tripotassium EDTA plasma was obtained from Carter Blood Bank (Fort Worth, TX, USA). Ultra pure carrier grade nitrogen was used for sample extract evaporation and as drying gas in the electrospray source. (Air Products, Allentown, PA, USA). High purity argon (also from Air Products) was used as the target gas for collisional fragmentation.

2.2. Equipment

HPLC/MS/MS analysis was performed with a Micromass Quattro LC (Micromass Ltd, Altrincham, UK) with an electrospray source and Windows based software. Mobile phase was delivered using a Waters Alliance Separations Module, model 2690 (Waters Corporation, Milford, MA, USA) with electronic valve actuator, model EVA 7010. Chromatographic separations were per-

formed using a Phenomenex Columbus C18 column, 5 μm particle size, 100 Å pore size, 150 × 2.0 mm i.d. (Phenomenex, Torrance, CA, USA). SPEC 3 ml MP1 15 mg nonpolar reversed-phase/strong cation exchange SPE cartridges (Ansys, Inc Irvine, CA, USA) were used to isolate the compounds of interest from plasma. An Analytichem International Vac Elut 10 port vacuum manifold with vacuum controller and gauge (Analytichem International, Harbor City, CA, USA) was used for SPE extraction steps. Sample extracts were dried under nitrogen using a Zymark Turbo-Vap evaporation manifold (Zymark Corporation, Hopkinton, MA, USA).

2.3. Preparation of stock solutions, calibration standards and Quality Control (QC) Samples

A certified stock solution of 625 μg/ml + FP in 1:1 acetonitrile:water was obtained from the Alcon Research Analytical Chemistry Department. Serial dilutions of this stock were used to prepare four intermediate spiking solutions over the concentration range 0.150–250 μg/ml. Serial dilutions of the intermediates were performed to give six working solutions over the range 0.500–100 ng/ml. For each validation run, plasma standard curves are prepared in duplicate over the range of 0.010–3.00 ng/ml with a typical spiking volume ranging from 15 to 30 μl into 1.0 ml of plasma. Each curve included two unspiked plasma aliquots as matrix blanks. For QC preparation, a separate set of spiking solutions was prepared from the same certified stock solution. QC samples were prepared in 40 ml lots at 0.040 (low), 1.25 (medium) and 2.25 (high) ng/ml concentrations. Separate 1.0 ml aliquots were frozen for routine use. Duplicate QC samples at each level were thawed and processed with each run. IS was diluted to a working concentration of 20 ng/ml. Standard and IS solutions were stored at approximately 4 °C for up to 6 months.

2.4. Sample preparation procedure

Plasma samples were thawed and centrifuged to remove particulates. One ml aliquots were then transferred into polypropylene tubes and spiked with 15 μl of AL-5848X Working Solution (20 ng/ml). The sample was then acidified (to approximately pH 4) by addition of 1 ml each of 0.1 N formic acid and water and mixed thoroughly.

SPEC 3 ML-MP1 15.0 mg cartridges were conditioned by aspirating 500 μl of methanol under vacuum, followed by similar treatment with 500 μl of 0.04 N formic acid. Care was taken not to allow the adsorbent bed to dry between conditioning steps. The acidified plasma was then added, followed by an aqueous rinse of the polypropylene tube (0.5–1.0 ml) to ensure quantitative transfer. The sample was then aspirated through the cartridges under vacuum.

Two separate rinse steps were employed to remove endogenous components. The first used water (single 500 μl portion), while the second used toluene/dichloromethane (60:40 v/v, two separate 500 μl portions). After aspiration of each of the two rinse solvents, air was aspirated under increased vacuum to dry the adsorbent bed. Analyte and IS were then eluted with 600 μl of 1:4 toluene:methyl formate and evaporated to dryness at 30 °C under nitrogen. The residue was reconstituted in 125 μl of 1:1 methanol:water.

For determination of AL-6221 by difference, a separate plasma aliquot was spiked with 20 U of rabbit esterase (40 IU in 0.1 ml buffered saline) and incubated at 37 °C for 45 min to quantitatively hydrolyze any AL-6221 present to + FP. Sample preparation was then performed as described above.

2.5. Chromatographic and mass spectrometry conditions

Thirty five microliters of the reconstituted plasma extracts were injected onto the HPLC column under isocratic conditions using a mobile phase of ammonium formate 5 mM, pH 6.3 per methanol 30/70 at 0.2 ml/min. The separation was performed at ambient temperature (approximately 22 °C). Under these conditions, the analyte and deuterated IS coeluted at approximately 5–5.5 min.

The mass spectrometer was equipped with a Z-spray electrospray ionization source. Typical source conditions were as follows, capillary 3.0

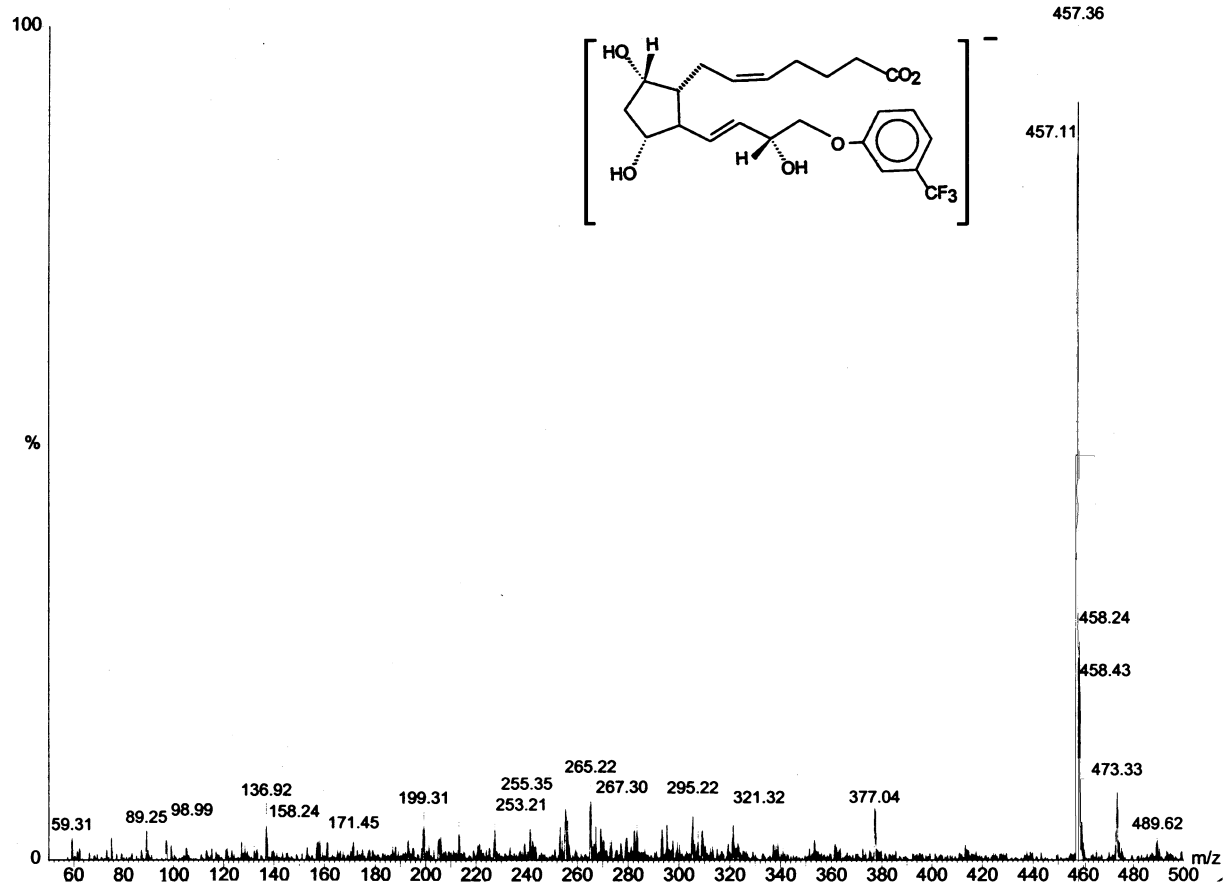

Fig. 2. Mass spectrum of + FP parent $[M–H]^-$ ion.

kV, sample cone 40 V, extraction cone 2 V, RF lens 0.3 V, source temperature 125 °C, and drying gas temperature 250 °C. MS1 parameters were LM resolution 14; HM resolution 14; and ion energy, 1.2 V. Entrance and exit were set to 0 and 1, respectively, with collision energy of 30 eV. MS2 parameters were LM resolution 15.0; HM resolution 15.0; and ion energy 1.2 V. The multiplier was set at 650 V. Nebulizing and drying gas flow rates were 75 and 570 l/h, respectively. The negative ion multiple reaction monitoring (MRM) experiments were conducted with an interchannel delay of 0.03 s, a mass span of 0 Da and an acquisition time of 9.0 min. Ions were collisionally fragmented with argon at 1.5×10^{-3} mbar. The MRM transitions of m/z 457 → 161 and 461 → 161 were monitored for analyte and IS, respectively. Data were collected using MassLynx, Version 3.4 (Micromass). Chromatograms were integrated and smoothed 2–4 times using a Moving Mean algorithm with a two scan window. The calibration curve (peak area ratio, y-axis versus spiking concentration, x-axis) was fitted to a first-order linear model with intercept and $1/x$ weighting.

2.6. Assay validation

Validation was performed using four separate validation curves, each consisting of duplicate spiked plasma standards at nine concentrations over the range 0.010–3.00 ng/ml. Each curve was run on different day and included duplicate QC samples at low, medium and high concentrations. Control plasma from individual donors was

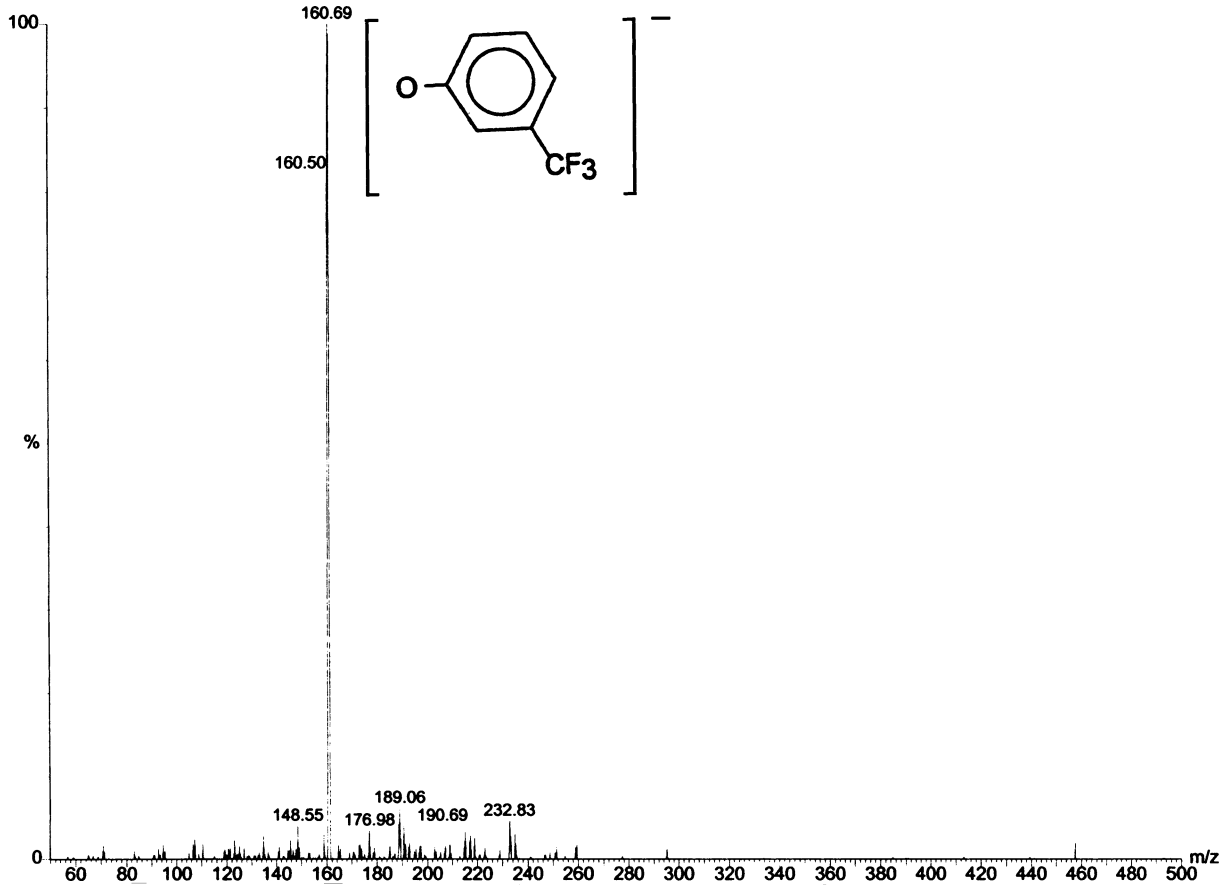

Fig. 3. Product ion mass spectrum showing 3-trifluorophenolate (m/z 161) product ion signal.

screened for potential endogenous interferences. Experiments to assess sample stability under various conditions, as well as absolute recovery of analyte and IS, were also included in the validation runs.

3. Results and discussion

3.1. Mass spectrometry

The standard and IS gave deprotonated parent ions $[M–H]^-$ at m/z 457 and 461, respectively. Fig. 2 shows the mass spectrum for the FP+ parent ion. Fragmentation of these ions using collision activated dissociation (CAD) gave a single intense product ion at m/z 161 corresponding to the 4-trifluoromethylphenolate ion (Fig. 3). No other product ions of significant intensity were seen for either compound, most likely due to the extreme charge stabilization of this ion from delocalization within the phenol ring coupled with the electron withdrawing effect of the trifluoromethyl group. MRM of the m/z $457 \rightarrow 161$ and $461 \rightarrow 161$ transitions allowed selective detection of the two co-eluting compounds with chromatograms of control plasma spiked with IS showing no detectable interference with the analyte MRM signal. Also, chromatograms of control human plasma from ten individual donors showed no interfering signals for either +FP or IS. Fig. 4 shows representative chromatograms of blank and spiked plasma as well as from plasma collected from a volunteer 30 min following topical ocular administration of travoprost.

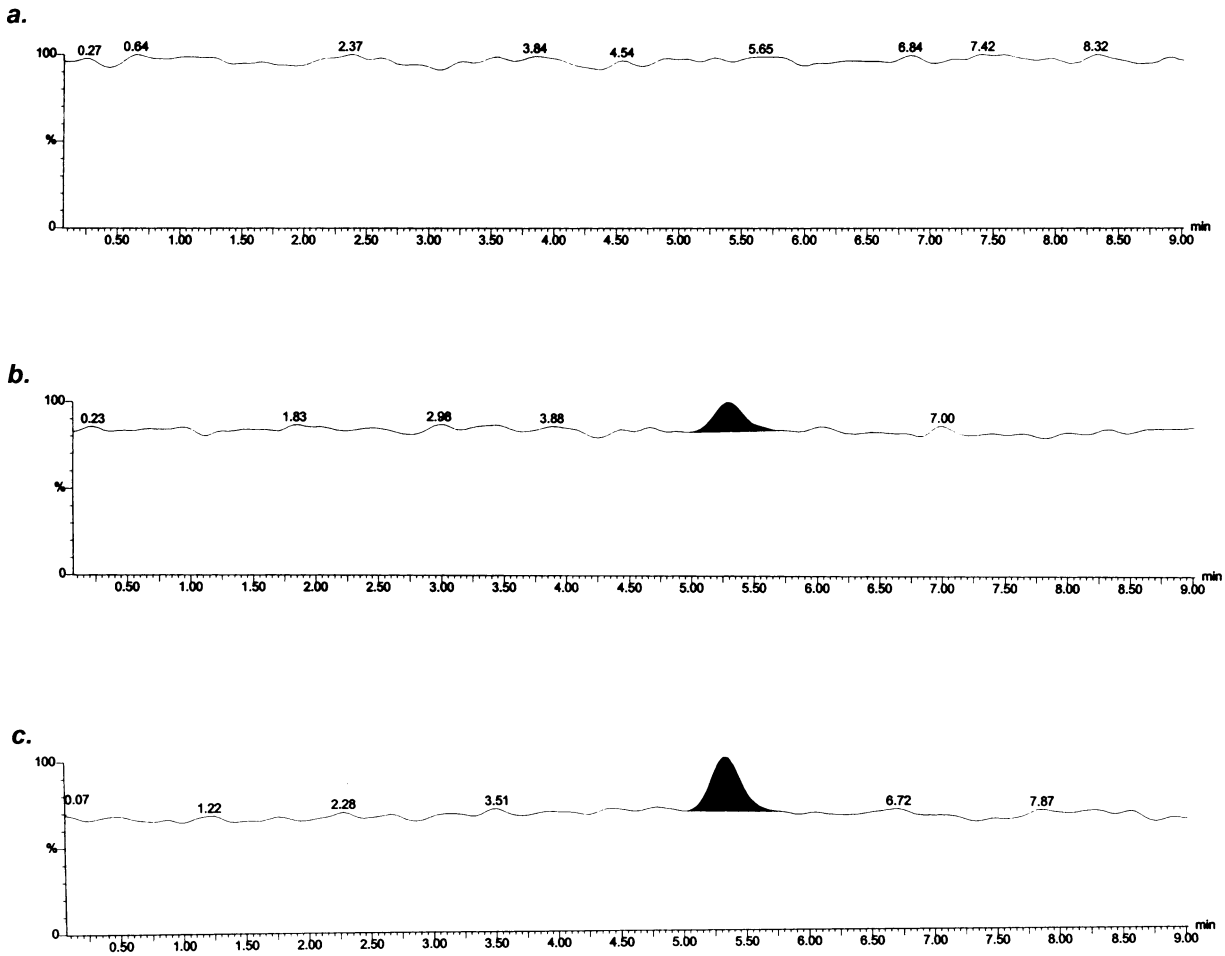

Fig. 4. Ion chromatograms of (a) control human plasma; (b) 0.010 ng/ml quantitation limit spiked standard and (c) plasma sample from a healthy volunteer collected 30 min after topical ocular administration of travoprost showing an AL-5848 concentration of 0.019 ng/ml. Shaded areas show peak integration for AL-5848.

Table 1
Back-calculated AL-5848 concentrations (ng/ml) for spiked human plasma calibration standards

	AL-5848 Spiking concentrations (ng/ml)								
	0.010	0.015	0.025	0.250	0.750	1.50	2.00	2.50	3.00
Mean	0.010	0.014	0.025	0.258	0.752	1.51	2.04	2.49	2.95
N	8	7[a]	8	8	8	8	8	8	8
S.D.	0.0013	0.0015	0.0014	0.009	0.015	0.05	0.07	0.06	0.06
R.S.D (%)	13.1	10.5	5.60	3.39	1.99	3.43	3.31	2.41	2.03
Accuracy (%)	100	95.2	100	103	100	100	102	99.6	98.3

[a] One standard failed acceptance criteria (back-calculated value greater than $\pm 20\%$ of nominal).

Travoprost did not form a negative ion. Nor could adequate analytical sensitivity be obtained in positive mode to quantify the expected extremely low parent drug levels in plasma. Therefore, it was decided to analyze any travoprost present by difference after esterase hydrolysis.

3.2. Analytical performance

Back-calculated concentration data for the validation runs (Table 1) showed overall good agreement with theory. Only 1 out of 72 calibration standards had to be rejected due to a back-calculated response greater than ± 20% from nominal.

Based on previous work in rabbit plasma, a quantitation limit of 0.020 ng/ml for a 0.5 ml sample volume appeared feasible, equivalent to 0.010 ng/ml for a 1 ml aliquot. To determine if this was the actual lower limit of quantitation (LLQ) in human plasma, spiked standards ($n = 5$) were prepared at 0.005, 0.010 and 0.015 ng/ml. The response at 0.005 ng/ml concentration was highly variable, with only two of the replicates showing measurable peaks for + FP. At 0.010 and 0.015 ng/ml the percent R.S.D. was 8.37 and 11.9%, respectively. The mean back-calculated + FP concentration at the 0.010 ng/ml nominal level was 0.0098 ng/ml with 8.37% R.S.D. Based on the acceptable precision and accuracy demonstrated at 0.010 ng/ml and the inability to obtain reproducible response at half this concentration, 0.010 ng/ml was determined to be the effective quantitation limit.

Mean intra-day QC results are presented in Table 2. The corresponding inter-day results are shown in Table 3. Intra-day precision ranged from 1.18 to 5.21% R.S.D., while inter-day precision ranged from 3.07 to 7.94% R.S.D. All individual QC assays were within ± 15% of their nominal concentrations.

Mean absolute recovery of + FP from human plasma over the calibration range was 94.9 ± 4.7% (range, 86.9–99.5%). Mean recovery of the IS at its nominal 0.30 ng/ml spiking concentration was 95.7 ± 6.8% (range, 84.2–103%).

Table 4 summarizes stability of + FP in human plasma. The results demonstrate that the compound is stable in human plasma through at least three freeze/thaw cycles, at room temperature in unextracted plasma for at least 18 h and at − 80 °C for at least 18 months. In addition, stability in reconstituted extracts at room temperature for at least 24 h was shown, as was stability in dried extract residues at room temperature, also for 24 h. Note that the long-term − 80 °C QCs were prepared at higher nominal concentrations than those used in other stability experiments. This is because only a less sensitive single quadrupole

Table 2
Intra-day quality control assay results for AL-5848 in human plasma

QC	Low (0.040 ng/ml)	Medium (1.25 ng/ml)	High (2.25 ng/ml)
Mean	0.040	1.27	2.28
N	6	6	6
CV (%)	5.25	1.18	2.32
Accuracy (%)	100	102	101
N	6	6	6

Table 3
Inter-day quality control assay results for AL-5848 in human plasma

Run	Low (0.040 ng/ml)	Medium (1.25 ng/ml)	High(2.25 ng/ml)
Mean	0.039	1.30	2.28
N	8	8	8
CV (%)	7.94	3.85	3.11
Accuracy (%)	97.5	104	101

Table 4
Stability QC results for +FP in human plasma

Nominal concentration (ng/ml)	Freeze/thaw (3×)	Plasma at R.T. 18 h	Reconstituted sample at R.T. 24 h	Dried residue at R.T. 24 h	Nominal concentration (ng/ml)	18 months at −80 °C
0.040	0.041	0.036	0.044	0.045	0.200	0.219
0.040	0.039	0.040	0.043	0.043	0.200	0.227
1.25	1.29	1.27	1.30	1.29	1.20	1.27
1.25	1.29	1.31	1.32	1.28	1.20	1.27
2.25	2.25	2.31	2.38	2.31	2.25	2.23
2.25	2.24	2.23	2.32	2.35	2.25	2.23

instrument was available at the time these QCs were prepared and tested.

An in vitro experiment was performed to determine the extent of AL-6221 hydrolysis which would be expected during sample collection and handling. Control human plasma at room temperature was spiked with AL-6221 at 1.25 and 2.25 ng/ml and immediately spiked with IS and extracted as described above. The resulting + FP assay results showed approximately 8% of the parent ester hydrolyzing in this short time period. These results demonstrated that determination of the AL-6221 plasma concentration at the moment of sample collection was unfeasible. The purpose of the reanalysis with esterase incubation to determine AL-6221 by difference was to ensure that exposure to all potentially active drug-related material was measured. In light of these findings, no long-term frozen stability data for AL-6221 in human plasma was generated.

3.3. Clinical sample analysis

The validated method was used to determine + FP plasma concentrations following topical ocular administration of either 0.0015 or 0.004% AL-6221 ophthalmic solution to normal volunteers once each morning for 1 week. On days 1 and 7, blood samples were collected into vacuum blood collection tubes containing tripotassium EDTA as anticoagulant. Blood collections were performed immediately prior to dosing and at 5, 10, 15, 30 min and 1, 2, 4 and 8 h post-dose. Each sample was immediately mixed by manual inversion to disperse the anticoagulant and placed on ice. Within 30 min after collection, samples were centrifuged and the plasma fraction transferred to a polypropylene cryogenic storage tube and frozen at −70 °C. Samples were maintained at or below −70 °C until analyzed.

The results showed quantifiable AL-5848 concentrations in 38 out of 355 samples with values ranging from 0.010 to 0.025 ng/ml. All quantifiable results came from samples collected within 30 min after dosing. Fig. 2 shows a typical chromatogram for a subject exhibiting quantifiable + FP plasma concentrations. Reanalysis of high dose (0.004% AL-6221) samples showed a greater incidence of + FP concentrations in incubated samples being slightly higher than the corresponding non-incubated samples. This suggests that trace amounts of parent drug may have been present in the samples. The concentrations, however, would have been extremely low.

The validation results have shown the LC/MS/MS procedure for + FP in human plasma to have accuracy within 97.5–104% of nominal and precision within 6.92% R.S.D. or better, based on the inter-day QC results. Sensitivity is sufficient to demonstrate quantifiable systemic exposure following extremely low topical ocular doses of the parent ester pro-drug. The technique is relatively selective and robust in comparison to immunoassay procedures and does not require derivatization steps, as do GC-based methods for prostaglandins.

References

[1] D.I. Chapman, M.S. Moss, P.W. Tomlinson, M.P. Harrison, P.J. Simmons, The disposition and metabolism of the synthetic prostaglandin fluprostenol (ICI 81 008) in the horse, Xenobiotica 10 (1980) 715–723.

[2] G. Kominami, M. Nakamura, M. Mizobuchi, K. Ueki, T. Kuroda, A. Yamauchi, S. Takahashi, Radioimmunoassay and gas chromatography/mass spectrometry for a novel antiglaucoma medication of a prostaglandin derivative, S-1033, in plasma, J. Pharm. Biomed. Anal. 15 (1996) 175–182.

[3] S. Basu, B. Sjoquist, Development of a radioimmunoassay for latanoprost and its application in a long term study in monkeys, Prostaglandins, Leukotrienes Essential Fatty Acids 55 (1996) 427–432.

[4] P. Pradelles, J. Grassi, J. Maclouf, Enzyme immunoassays of eicosanoids using acetylcholinesterase, Methods Enzymol. 187 (1990) 24–34.

[5] D.K. Miller, S. Sadowski, D. DeSousa, Development of enzyme linked immunosorbent assays for measurement of leukotrienes and prostaglandins, J. Immunol. Methods 81 (1985) 169–185.

[6] S. Yamamoto, Y. Hayashi, T. Yano, N. Ueda, Enzyme immunoassay of TXB_2 and 6-keto-$PGF1_\alpha$ as a substitute for radioimmunoassay, Adv. Prostaglandin Thromboxane Leukotriene Res. 11 (1983) 181–184.

[7] M. Mizugaki, T. Hishinuma, H. Yamaguchi, K. Kanno, Y. Hirose, H. Nakamura, Microdetermination of the 6,15-diketo-13,14-dihydro-prostaglandin $F1_\alpha$ in human plasma using gas chromatography/selected ion monitoring with [^{18}O] 6,15-diketo-13,14-dihydro-prostaglandin

$F1_{\alpha}$ as an internal standard, Prostaglandins Other Lipid Mediators 58 (1999) 187–193.

[8] T. Obata, T. Nagakura, H. Maeda, K. Yamashita, K. Maekawa, Simultaneous assay of prostaglandins and thromboxane in the cerebrospinal fluid by gas chromatography–mass spectrometry–selected ion monitoring, J. Chromatogr. B 731 (1999) 73–81.

[9] A. Burke, J.A. Lawson, E.A. Meagher, J. Rokach, G.A. FitzGerald, Specific analysis in plasma and urine of 2,3-dinor-5,6-dihydro-isoprostane $F_{2\alpha}$-III, a metabolite of isoprostane $F_{2\alpha}$-III and an oxidation product of γ-linolenic acid, J. Biol. Chem. 275 (2000) 2499–2504.

[10] H. Schweer, B. Watzer, H.W. Seyberth, R.M. Nusing, Improved quantification of 8-epi-prostaglandin $F_{2\alpha}$ and F_2—isoprostanes by gas chromatography/triple-stage quadrupole mass spectrometry, partial cyclooxygenase-dependent formation of 8-epi-prostaglandin $F_{2\alpha}$ in humans, J. Mass Spectrom. 32 (1997) 1362–1370.

[11] A. Margalit, K.L. Duffin, P.C. Isakson, Rapid quantitation of a large scope of eicosanoids in two models of inflammation: development of an electrospray and tandem mass spectrometry method and application to biological studies, Anal. Biochem. 235 (1996) 73–81.

[12] H. Li, J.A. Lawson, M. Reilly, M. Adiyaman, S.W. Hwang, J. Rokach, G.A. FitzGerald, Quantitative high performance liquid chromatography/tandem mass spectrometric analysis of the four classes of F_2—isoprostanes in human urine, Proc. Natl. Acad. Sci. 96 (1999) 13381–13386.

[13] W.S. Powell, Rapid extraction of arachidonic acid metabolites from biological matrices using octadecyl silica, Methods Enzymol. 86 (1982) 467–477.