Determination of 6-keto prostaglandin F1α and its metabolites in human plasma by LC–MS/MS

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ABSTRACT

An HPLC–MS/MS method was developed and validated for the quantification of 6-keto prostaglandin F1α, the stable hydrolysis product of prostacyclin, and its metabolites 2,3-dinor-6-keto prostaglandin F1α and 6,15-diketo-13,14-dihydro prostaglandin F1α in human plasma. For sample preparation, a solid phase extraction step was combined with a column switching approach for analytes enrichment and further sample clean-up of the processed sample. The assay was validated in the concentration range 50.0–5000 pg/mL for 6-keto prostaglandin F1α and 6,15-diketo-13,14-dihydro prostaglandin F1α, and 100–10,000 pg/mL for 2,3-dinor-6-keto prostaglandin F1α. The inter-batch precision was better than 12.7%, 9.2%, and 9.4% for 6-keto prostaglandin F1α, 2,3-dinor-6-keto prostaglandin F1α, and 6,15-diketo-13,14-dihydro prostaglandin F1α, respectively. The inter-batch accuracy was between 97.3% and 100.8% for 6-keto prostaglandin F1α, between 97.5% and 103.0% for 2,3-dinor-6-keto prostaglandin F1α, and between 92.0% and 100.0% for 6,15-diketo-13,14-dihydro prostaglandin F1α. Further it has been demonstrated that the analytes were stable in plasma for 20 h at room temperature, during three freeze-and-thaw cycles, for 96 days at −25 °C storage temperature, and 50 h in the autosampler tray at room temperature.

1. Introduction

Prostaglandin I2 (PGI2), or prostacyclin, is a major metabolite of arachidonic acid (AA) produced by vascular endothelial cells [1], and a key natural short-acting vasoactive substance with vasodilatory, platelet inhibitory, and anti-proliferative properties [2,3]. The finding that patients with pulmonary arterial hypertension (PAH) show decreased levels of prostacyclin suggests that it may play an important role in PAH pathophysiology including vasoconstriction, vascular proliferation, remodeling of the vascular wall, and *in situ* thrombosis [4,5]. These findings have resulted in the development of therapeutic corrective approaches and continuous infusion with epoprostenol, a synthetic form of prostacyclin, approved by the FDA in 1996 for the treatment of severe cases of PAH, and is nowadays generally regarded as the most effective treatment option [6].

Epoprostenol is unstable and undergoes spontaneous hydrolysis into 6-keto prostaglandin F1α in solution under physiological conditions. In order to maintain its in-use stability for several hours, epoprostenol is administered by continuous intravenous infusion in an alkaline solution. However, once it enters into the systemic circulation (pH 7.4) epoprostenol is spontaneously hydrated into 6-keto prostaglandin F1α with a half-life of several minutes [7]. For this reason, 6-keto prostaglandin F1α has been used as a surrogate of prostacyclin blood concentrations in clinical studies. So far, several enzyme-linked immunoassays [8–10] or radio immunoassays [11,12] have been developed and are commercially available for the determination of 6-keto prostaglandin F1α in human plasma and urine. The major problem encountered is that non-esterified fatty acids can interfere with the immunoassay resulting in lack of selectivity [13,14]. A combined HPLC-RIA method has been described for the determination of both major metabolites of prostacyclin namely 6-keto prostaglandin F1α and 2,3-dinor-6-keto prostaglandin F1α in human urine [15]. To date, only a few liquid chromatographic (HPLC) methods have been published for the determination of 6-keto prostaglandin F1α in human plasma [16,17]. Chappella et al. have developed a very sensitive HPLC–MS assay for the determination of endogenous levels in the range of 2–3 pg/mL of 6-keto prostaglandin F1α in human plasma using an anti-6-keto prostaglandin F1α antibody and 0.5 mL of plasma for analyte enrichment [17], but no HPLC–MS assay has been reported for the simultaneous determination of 6-keto prostaglandin F1α, 2,3-dinor-6-keto prostaglandin F1α, and 6,15-diketo-13,14-dihydro prostaglandin F1α. The lack of a sensitive and

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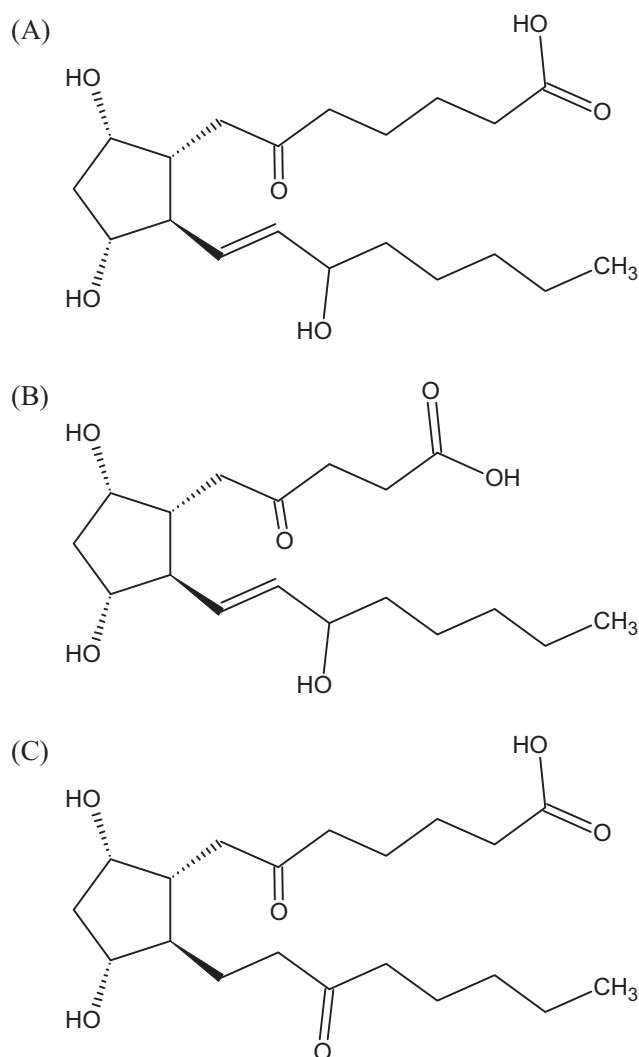*E-mail address:* christoph.siethoff@swissbioquant.com (C. Siethoff).

(A)

(B)

(C)

Fig. 1. Chemical structure of 6-keto prostaglandin F1α (A), 2,3-dinor-6-keto prostaglandin F1α (B), and 6,15-diketo-13,14-dihydro prostaglandin F1α (C).

accurate assay resulted in poor characterization of the pharmacokinetic profile of epoprostenol and its metabolites.

For proper monitoring the pharmacokinetics of 6-keto prostaglandin F1α, 2,3-dinor-6-keto prostaglandin F1α, and 6,15-diketo-13,14-dihydro prostaglandin F1α in a clinical trial, an analytical method was developed and validated for the simultaneous determination of the three compounds in human plasma using solid phase extraction and an HPLC column switching technique followed by mass spectrometric detection. The chemical structures of the three analytes are depicted in Fig. 1.

2. Experimental

2.1. Chemicals and materials

Acetonitrile, methanol, ammonia solution (24.5%), and formic acid were purchased from J.T. Baker (Phillipsburgh, NJ, USA). Purified water was prepared by a Purelab Ultra Ionic purification system (ELGA, Celle, Germany). 6-Keto prostaglandin F1α, 2,3-dinor-6-keto prostaglandin F1α, and 6,15-diketo-13,14-dihydro prostaglandin F1α and their internal standards 6-keto prostaglandin F1α-D4, 2,3-dinor-6-keto prostaglandin F1α-D9, and 6,15-diketo-13,14-dihydro prostaglandin F1α-D9 were purchased from Cayman Chemical (Ann Arbor, MI, USA). All solvents

were of gradient grade quality. Human plasma was obtained from the Blutspendezentrum SRK beider Basel (Basel, Switzerland) from healthy volunteers and K3-EDTA was used as an anticoagulant. Indomethacin and arachidonic acid (AA) were purchased from Sigma–Aldrich, Buchs, Switzerland. For solid phase extraction step, an Oasis HLB μElution Plate (Waters Corporation, Milford, MA, USA) was used.

2.2. Preparation of standard solutions

For the preparation of calibration samples, stock solutions with a concentration of 1.00 mg/mL of 6-keto prostaglandin F1α, 2,3-dinor-6-keto prostaglandin F1α, and 6,15-diketo-13,14-dihydro prostaglandin F1α were combined and further diluted in methanol. For the preparation of QC samples, a second stock solution with the same concentrations was used. Working solutions were prepared in methanol to concentrations 100 times higher than the corresponding concentrations in plasma. The concentrations were calculated taking into consideration purity and salt factors, where applicable.

For 6-keto prostaglandin F1α and 6,15-diketo-13,14-dihydro prostaglandin F1α, the concentrations were in the range of 50.0–5000 pg/mL (50.0, 100, 250, 500, 1000, 2500, 3750, and 5000 pg/mL for the calibration samples, and 50.0, 150, 500, and 3750 pg/mL for the QC samples). For 2,3-dinor-6-keto prostaglandin F1α the concentrations were in the range of 100–10,000 pg/mL (100, 200, 500, 1000, 2000, 5000, 7500, and 10,000 pg/mL) for the calibration samples, and 100, 300, 1000, and 7500 pg/mL for the QC samples.

2.3. Preparation of internal standard solutions

A solution of 6-keto prostaglandin F1α-D4, 2,3-dinor-6-keto prostaglandin F1α-D9, and 6,15-diketo-13,14-dihydro prostaglandin F1α-D9 of 0.100 mg/mL in methanol was diluted using a mixture of water/methanol (50/50, v/v) to a concentration of 10.0 ng/mL of 6-keto prostaglandin F1α-D4, 250 ng/mL of 2,3-dinor-6-keto prostaglandin F1α-D9, and 10,000 ng/mL of 6,15-diketo-13,14-dihydro prostaglandin F1α-D9. The concentrations were calculated taking into consideration purity and salt factors, where applicable. The internal standard concentrations were adjusted to the respective purity, the degree of labeling and the content of unlabeled drug.

2.4. Sample preparation

To an aliquot of 500 μL of plasma, 20 μL of internal standard solution and 10 μL of phosphoric acid (8.5% in water) were added and mixed. The sample was incubated for approximately 15 min at 5 °C; 500 μL of water was added and after mixing, two aliquots of 400 μL of the mixture were transferred to a 96-solid phase extraction well plate which was conditioned with 450 μL of methanol and 450 μL of water prior to its use. A slight vacuum was applied and, after loading the sample, the cavity was washed with 450 μL of water containing 0.5% formic acid. Subsequently, the analytes were eluted using 100 μL methanol containing 0.1% ammonia solution (24.5%). An aliquot of 100 μL of water containing 0.5% formic acid was added to the sample and vortex mixed. An aliquot of 50 μL of the extracted sample was used for analysis by LC–MS/MS. During the analysis the samples were stored at room temperature in the autosampler tray.

2.5. Liquid chromatography and column switching

HPLC was performed using a binary pump 1200 (Agilent Technologies Inc., Santa Clara, CA, USA) and a HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland). For the column switching,

Table 1
SRM transitions for 6-keto prostaglandin F1α, 2,3–dinor-6-keto prostaglandin F1α, 6,15–diketo-13,14-dihydro prostaglandin F1α, and their internal standards.

Analyte	Parent ion mass [mu]	Product ion mass [mu]	Scan time [s]	Collision energy [V]
2,3-Dinor-6-keto PG	341.1	135	0.03	25
2,3-Dinor-6-keto PG-D9	350.1	135	0.03	25
6-Keto PG	369.1	245	0.03	25
6-Keto PG-D4	373.1	167	0.03	25
6,15-Diketo-13,14-dihydro PG	369.101	267	0.03	25
6,15-Diketo-13,14-dihydro PG-D9	378.1	232	0.03	25
6,15-Diketo-13,14-dihydro PG-D9	378.1	276	0.03	25

PG: prostaglandin.

a binary pump 1200 (Agilent Technologies Inc., Santa Clara, CA, USA) was used. The binary mobile phase for analytical separation consisted of water containing 0.2% ammonia solution (24.5%) (solvent A) and acetonitrile (solvent B). The linear gradient was increased after 0.5 min from 2.0% B to 20.0% B until 5.1 min and further increased in 0.2 min to 95.0% B. The separation was performed on a ReproSil Gold 120 C18, 2 mm × 50 mm, 3 μm, column (Dr. Maisch HPLC GmbH, Ammerbuch, Germany) at a flow rate of 0.300 mL/min. For the trapping pump, water containing 0.1% formic acid as the mobile phase was used (solvent C). The extracted plasma samples were injected onto a small pre-column (YMC Hydrosphere C18, 2.1 mm × 10 mm, 5 μm; YMC Co. Ltd., Kyoto, Japan) using the mobile phase C at 1500 μL/min. After 0.5 min, the analytes were transferred to the analytical column in back-flush mode.

2.6. Mass spectrometry

Assay validation was performed on a TSQ Vantage triple stage quadrupole instrument (Thermo Fisher Scientific, San Jose, CA, USA) in the negative ion mode using the electrospray ion source. The temperature of the ion transfer capillary was set to 350 °C, while that of the vaporizer was adjusted to 350 °C, and the instrument was operated at unit mass resolution. Sheath gas pressure was set to 40 psi and the auxiliary gas pressure at 10 psi. Data were acquired in selected reaction monitoring (SRM) mode using the transitions as shown in Table 1.

2.7. Validation of the assay

The assay was validated for the following variables: specificity, linearity, inter-batch and intra-batch precision and accuracy of the analytes (six replicates on three different days), analyte recovery and the effect of sample dilution. In addition, short-term temperature stability (20 h at −25 °C) and long-term temperature stability (96 days at −25 °C) of the analytes in human plasma was performed as well as the post-preparative stability and the stability of the analytes during three freeze-and-thaw cycles. For the determination of the specificity of the analytical method for the three analytes, human blank plasma samples from six different subjects were analyzed. Assay validation was successfully performed in accordance to the FDA guidance for Industry, Bioanalytical Method Validation [18], but not all results will be discussed below. Intra-batch and inter-batch (three different batches) accuracy and precision were determined by analyzing six aliquots of four different QC samples covering the calibration range. Recovery from plasma was determined by the comparison of analyte peak area (response) of the low (QC_L) and the high (QC_H) quality control sample with the corresponding peak area of standard solutions that represent 100% recovery (STD_L and STD_H). The determination was performed in triplicate for each QC concentration. The variability of the matrix effect was investigated using 6 different matrix lots which were fortified with the analytes at the low QC concentration.

The stability of the analytes in human plasma was determined in triplicate at low and high QC concentration after three freeze-and-thaw cycles. The samples were kept at −25 ± 5 °C for 24 h before they were thawed unassisted at room temperature for about 45 min for the first cycle and were frozen again for 12 h. The cycle was repeated two times and the samples were analyzed after the third cycle.

2.8. Investigations on the formation of 6-keto prostaglandin F1α in the presence of arachidonic acid

The possible conversion of AA to prostacyclin and subsequently to 6-keto prostaglandin F1α was investigated in an *in vitro* experiment. Fresh whole blood was spiked with AA obtaining concentrations of approximately 1000 and 10,000 ng/mL. Three aliquots at each concentration were fortified with indomethacin (5.00 μL of 0.04 M solution added to 1.00 mL whole blood) and three other aliquots were not fortified with indomethacin. All samples were stored at room temperature and aliquots were taken for analysis at 5 and 30 min. The whole blood samples were centrifuged for 30 min at 1300 rpm and 4 °C to generate plasma. The supernatant (plasma) was processed with SPE together with blank samples and a calibration curve. Aliquots of 50 μL were injected onto the HPLC–MS/MS system.

3. Results and discussion

3.1. Validation results

Chromatograms obtained by SRM of the respective transitions are shown in Figs. 2–4. In each of the chromatograms the respective transitions of a blank plasma extract (A), the lowest calibration standard (B), the highest calibration standard (C), and the used
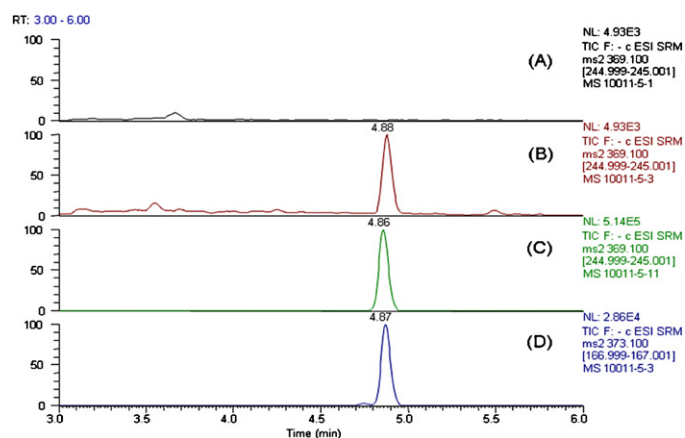

Fig. 2. LC–MS/MS chromatogram of 6-keto prostaglandin F1α in human plasma, blank (A), lowest calibration standard (B), highest calibration standard (C), and internal standards (D).
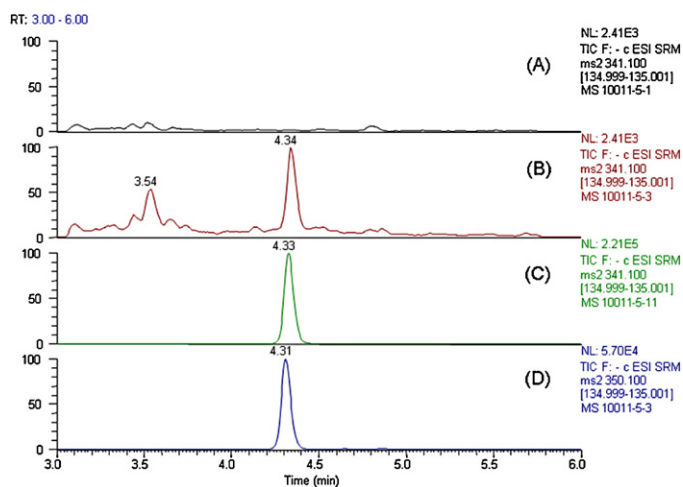
Fig. 3. LC–MS/MS chromatogram of 2,3-dinor-6-keto prostaglandin F1α in human plasma, blank (A), lowest calibration standard (B), highest calibration standard (C), and internal standards (D).

Fig. 4. LC–MS/MS chromatogram of 6,15-diketo-13,14-dihydro prostaglandin F1α in human plasma, blank (A), lowest calibration standard (B), highest calibration standard (C), and internal standards (D).

internal standards (ISTD) (D) are shown. The validation of the assay was performed in the range of 50.0–5000 pg/mL for 6-keto prostaglandin F1α and 6,15-diketo-13,14-dihydro prostaglandin F1α, and in the range of 100–10,000 pg/mL for 2,3-dinor-6-keto prostaglandin F1α. Quantification was done using the ratio of the peak areas of the analyte and the respective internal standard.

The calibration curves consisted of eight calibration points and were linear with least-square regression for the three compounds with a factor of determination (R^2) greater than 0.9918 for 6-keto prostaglandin F1α, greater than 0.9971 for 2,3-dinor-6-keto prostaglandin F1α, and greater than 0.9886 for 6,15-diketo-13,14-dihydro prostaglandin F1α. The fitting of data was performed with a weighting factor of $1/x^2$ using a linear regression with the method of least squares.

The relative standard deviation and the relative errors are summarized in Table 2. The inter-batch precision was better than 12.7%, 9.2%, and 9.4% for 6-keto prostaglandin F1α, 2,3-dinor-6-keto prostaglandin F1α, and 6,15-diketo-13,14-dihydro prostaglandin F1α, respectively. The inter-batch accuracy was between 97.3% and 100.8%, 97.5% and 103.0%, and 92.0% and 100.0% for 6-keto
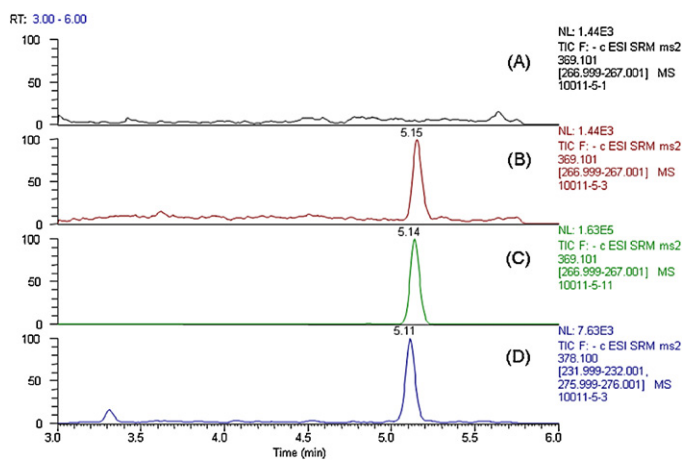
prostaglandin F1α, 2,3-dinor-6-keto prostaglandin F1α, and 6,15-diketo-13,14-dihydro prostaglandin F1α, respectively.

The mean recovery was 90.1% for 6-keto prostaglandin F1α, 90.3% for 2,3-dinor-6-keto prostaglandin F1α, and 85.4% for 6,15-diketo-13,14-dihydro prostaglandin F1α. For the analytes and the internal standards, an overall-recovery, combining the recovery and matrix effect, of around 60% was observed. The variability of the matrix effect in six different lots was in the range of 3.0% for 6-keto prostaglandin F1α, and 5.5% for 2,3-dinor-6-keto prostaglandin F1α, and 10.1% for 6,15-diketo-13,14-dihydro prostaglandin F1α for a duplicate analysis of the low QC spiked in the six different matrix lots.

The calculated mean concentrations from samples covering three freeze-and-thaw cycles were compared to the nominal values (Table 3). For the short-term temperature stability testing three aliquots of the low and high QC samples were analyzed after approximately 20 h of storage at room temperature and the calculated concentrations were compared to the respective nominal values (Table 3). Post-preparative stability was determined by analyzing three aliquots of the low and high QC samples after

Table 2
Validation results of 6-keto prostaglandin F1α, 2,3-dinor-6-keto prostaglandin F1α, and 6,15-diketo-13,14-dihydro prostaglandin F1α.

| Analyte	Level	QC_LOQ	QC_L	QC_M	QC_H
6-Keto prostaglandin F1α	Nominal concentration [pg/mL]	50.0	150	500	3750
	Mean [pg/mL]	50.4	150	503	3650
	SD (inter) [pg/mL]	6.40	8.00	18.1	90.3
	N	18	18	18	18
	RSD (inter) [%]	12.7	5.3	3.6	2.5
	Accuracy [%]	100.8	100.0	100.6	97.3
2,3-Dinor-6-keto prostaglandin F1α	Nominal concentration [pg/mL]	100	300	1000	7500
	Mean [pg/mL]	103	306	1000	7310
	SD (inter) [pg/mL]	9.52	16.9	42.6	235
	N	18	18	18	18
	RSD (inter) [%]	9.2	5.5	4.3	3.2
	Accuracy [%]	103.0	102.0	100.0	97.5
6,15-Diketo-13,14-dihydro prostaglandin F1α	Nominal concentration [pg/mL]	50	150	500	3750
	Mean [pg/mL]	50	138	487	3560
	SD (inter) [pg/mL]	4.70	11.2	31.8	204
	N	18	18	18	18
	RSD (inter) [%]	9.4	8.1	6.5	5.7
	Accuracy [%]	100.0	92.0	97.4	94.9

N: number of QC samples; SD: standard deviation; RSD: relative standard deviation.

Table 3
Stability results for 6-keto prostaglandin F1α, 2,3-dinor-6-keto prostaglandin F1α, and 6,15-diketo-13,14-dihydro prostaglandin F1α.

	Level	QC_L	Bias [%]	RSD [%]	QC_H	Bias [%]	RSD [%]
6-Keto prostaglandin F1α	Freeze/thaw stability	157	4.7	2.0	3860	2.9	0.9
	Short-term stability, 20 h at room temperature	152	1.3	5.5	3810	1.6	2.3
	Post-preparative stability, 50 h at room temperature	151	0.7	4.3	3690	−1.6	0.6
	Long-term stability, 96 days at −25 °C	143	−4.7	1.1	3600	4.0	2.2
2,3-Dinor-6-keto prostaglandin F1α	Freeze/thaw stability	328	9.3	0.8	7920	5.6	2.9
	Short-term stability, 20 h at room temperature	306	2.0	6.4	7930	5.7	0.6
	Post-preparative stability, 50 h at room temperature	280	−6.7	1.1	7170	−4.4	2.5
	Long-term stability, 96 days at −25 °C	281	−6.3	2.4	7420	−1.1	2.4
6,15-Diketo-13,14-dihydro prostaglandin F1α	Freeze/thaw stability	148	−1.3	7	3570	−4.8	2.7
	Short-term stability, 20 h at room temperature	140	−6.7	8.4	3610	−3.7	6.3
	Post-preparative stability, 50 h at room temperature	160	6.7	3.4	4000	6.7	3.5
	Long-term stability, 96 days at −25 °C	144	4.0	1.8	3390	−9.6	4.0

Bias [%] = 100 × (measured value − nominal value)/nominal value.
RSD: relative standard deviation.
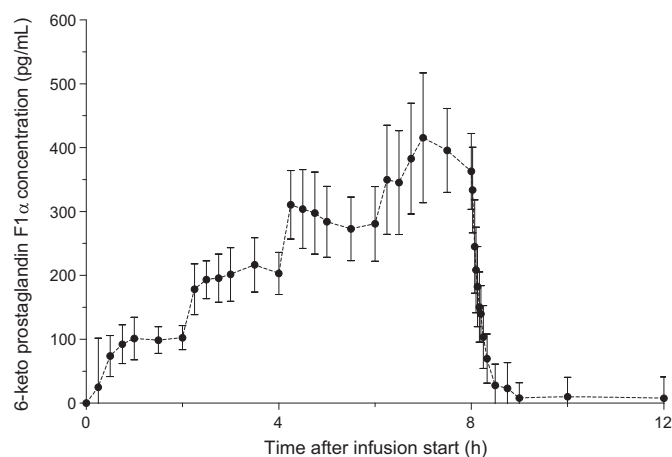
Fig. 5. Arithmetic mean plasma concentration and standard error *versus* time profile on linear scale for 6-keto prostaglandin F1α after infusions of 2, 4, 6, and 8 ng/kg/min epoprostenol sodium for injection (*n* = 18) each for a period of 2 h.

a resident time of 50 h in the autosampler tray at room temperature. Long-term temperature stability was demonstrated at a storage temperature of −25 °C for 96 days. The concentrations were compared to nominal values (Table 3). The results indicated that the analytes were stable in human plasma (K3-EDTA) after at least three freeze-and-thaw cycles and for 20 h of storage at room temperature. A post-extraction stability of 50 h storage in the autosampler tray at room temperature was also demonstrated.

3.2. Investigations on the formation of 6-keto prostaglandin F1α in the presence of arachidonic acid

High concentrations of free AA in whole blood (10,000 ng/mL) were needed to detect any formation of 6-keto prostaglandin F1α. Concentrations of 6-keto prostaglandin F1α were in any case below the lower limit of quantification (LLOQ) and could only be estimated. If no indomethacin was added to whole blood samples the obtained signal for 6-keto prostaglandin F1α was increased compared to the sample which contained indomethacin, but even then the signal was five times lower than the signal for 6-keto prostaglandin F1α of a LLOQ sample. The *in vitro* experiment was performed to mimic a post collection blood sample and even if AA concentrations were quite high, formation to 6-keto prostaglandin F1α was observed to a negligible extent only.

3.3. Application of the method to clinical study samples

The described method was applied to the quantification of 6-keto prostaglandin F1α, 2,3-dinor-6-keto prostaglandin F1α, and 6,15-diketo-13,14-dihydro prostaglandin F1α in plasma samples which were collected during the conduct of a pharmacokinetic study in about 18 healthy subjects. The mean concentration-time profile of 6-keto prostaglandin F1α following administration of epoprostenol is shown in Fig. 5. Incurred sample re-analysis (ISR) was successfully performed during the conduct of a clinical study. Approximately 3000 human plasma samples were analyzed and the ISR success rate was more than 90% using approximately 5% of the total number for re-analysis.

4. Conclusion

An HPLC column switching method with mass spectrometric detection for the simultaneous determination of 6-keto prostaglandin F1α, 2,3-dinor-6-keto prostaglandin F1α, and 6,15-diketo-13,14-dihydro prostaglandin F1α in human plasma was developed. The method was validated and successfully evaluated using samples collected from human subjects during the course of a clinical study.

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