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Journal of Chromatography B, 667 (1995) 137–147

JOURNAL OF
CHROMATOGRAPHY B:
BIOMEDICAL APPLICATIONS

Determination of BAY x 7195, a novel leukotriene D_4 antagonist, in human plasma by high-performance liquid chromatography with post-column photo derivatisation and fluorescence detection

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First received 2 September 1994; revised manuscript received 30 December 1994; accepted 30 December 1994

Abstract

Methods to determine plasma concentrations of the leukotriene D_4 antagonist BAY x 7195 by HPLC with post-column photo derivatisation and fluorescence detection are described. Following dilution and centrifugation plasma supernatant is injected onto the HPLC system allowing the selective determination of the drug with a limit of quantitation (LOQ) of 10 μg/l (method A). Sensitivity was further enhanced to a LOQ of 0.6 μg/l by employing solid-phase extraction whereby the analyte concentration in the injection solution was increased (method B). Data on recovery, accuracy and precision of both methods throughout the working range are presented. BAY x 7195 is stable in plasma after repeated freeze–thaw cycles and upon storage at −20°C for at least 13 months. Method A was applied to a clinical study with oral administration of 250 mg BAY x 7195 where ca. 1% of the maximum plasma concentrations still could be accurately and precisely quantified. Method B was employed to determine the drug in plasma after administration of 1 mg as aerosol.

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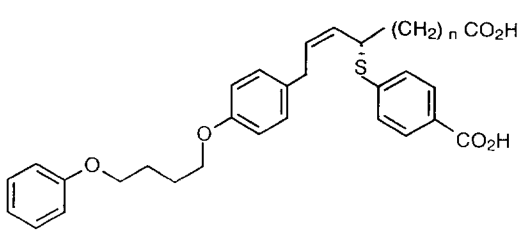
1. Introduction

BAY x 7195 [(*S*)-4-(4-carboxyphenylthio)-7-[4-(4-phenoxybutoxy)-phenyl]-5-(Z)-heptenoic acid] (Fig. 1) is a novel selective leukotriene (LT) D_4 receptor antagonist which was shown to be effective against LTD_4- and antigen-induced bronchoconstriction in preclinical studies in vitro and in vivo. The drug was effective in the Konzett–Rössler guinea-pig model after oral as well as local (i.e. as aerosol) administration [1,2]. Based on these findings the clinical evaluation of this compound is currently ongoing. It has recently been shown that also in man the bronchoconstriction caused by inhalation of LTD_4 was antagonized by oral administration of a 250-mg BAY x 7195 tablet [3].

In order to assess plasma concentrations of the drug in clinical studies, a sufficiently sensitive and specific analytical assay was required. Based on the chemical structure and physico-chemical properties of BAY x 7195 the first approach taken was a liquid chromatographic separation on RP-18 stationary phase followed by UV detection utilizing the absorption maximum at 224 nm. This technique allowed a limit of quantitation (LOQ) of 2.5 μg/l, however, a tedious and time-consuming sample preparation (combi-

0378-4347/95/$09.50
SSDI 0378-4347(95)00011-9



Compound	n =
BAY x 7195	2
BAY x 1308	3

Fig. 1. Structure of BAY x 7195 (I) and BAY x 1308 (II).

nation of solid-phase and repeated liquid–liquid extraction) was required to avoid interferences in the chromatogram [4].

In a second approach, fluorescence detection was attempted in order to gain sensitivity and specificity. As BAY x 7195 is only weakly fluorescent, 4-bromomethyl-6,7-dimethoxy coumarin, a readily available fluorescence tag for carboxylic acids [5] was reacted with the drug to yield the bis-ester derivative. However, when the pure derivative was injected onto the HPLC column coupled to a fluorescence detector, the increase in sensitivity as compared to UV detection was negligible. Moreover, when the derivatization agent was reacted with blank plasma extract, a multitude of interfering peaks was obtained that would likely have required multidimensional HPLC in order to separate the analyte [4]. Another reagent, 9-(hydroxymethyl)anthracene [6], failed to react substantially with BAY x 7195 [4]. Therefore the derivatization approach was not pursued further.

Another technique previously employed for bioanalysis of drugs to convert non-fluorescent into fluorescent species is post-column photoderivatisation (see e.g. Ref. [7]). Here the column effluent is irradiated by a mercury lamp (Beam Boost unit) and the analyte is decomposed to yield one or more fluorescent products. As BAY x 7195 is known to be light sensitive [4], the compound was a likely candidate for application of the Beam Boost.

After showing that one or more fluorescent species are actually formed in the post-column photoreactor, the primary objective of the present work was to develop and validate an assay that would require minimum sample pretreatment. With Beam Boost and fluorescence detection this was possible by simple centrifugation of diluted plasma and injection of the supernatant. A further refinement, however, became necessary when BAY x 7195 had to be quantified in plasma after doses as low as 1 mg administered locally as aerosol. Therefore optimisation of detection sensitivity was the secondary objective. A second method with improved limit of quantitation and its application to a study of 1-mg BAY x 7195 aerosol in man is presented.

2. Experimental

2.1. Chemicals

BAY x 7195 (I) and BAY x 1308 [(*R*,*S*)-5-(4-carboxyphenylthio) - 8 - [4 - (4 - phenoxybutoxy)-phenyl]-6-(Z)-octenoic acid] (II, internal standard, I.S., Fig. 1) were used as certified reference compounds (Bayer AG, Wuppertal, Germany) for quantitative analysis. The glucuronide metabolite of I was provided by Dr. M. Boberg (Institute of Pharmacokinetics, Bayer AG, Wuppertal). The bis(6,7-dimethoxybenzo-2-oxo-2H-pyron-4yl)methoxy ester of I was prepared by reaction with 4-bromomethyl-6,7-dimethoxy coumarin according to the procedure described in Ref. [5]. The identity of the product was confirmed by ^{1}H NMR. HPLC solvents were obtained in gradient-grade quality from E. Merck (Darmstadt, Germany). Phosphoric acid suprapur and acetone p.a. were also purchased from E. Merck. Water was purified by the Milli Q system (Millipore Waters, Eschborn, Germany). Solid-phase extraction (SPE) was performed on Sep-Pak RP18 500-mg cartridges

(Millipore Waters). Blank plasma was either obtained from fasted healthy volunteers in-house or purchased from the German Red Cross (DRK, Hagen, Germany).

2.2. Sample preparation

Sample preparation and instrumental analysis were carried out under yellow light (L 40/62, Osram, Germany).

Method A: In a stoppered test tube 4 ml of the I.S. working solution 1 [200 μg/l II in acetonitrile–0.1% phosphoric acid (6:4, v/v)] were added to 1 ml of plasma containing I. The mixture was placed in an ultrasonic bath (Bandelin, Berlin, Germany) for 30 s and the precipitate was removed by centrifugation at 2000 *g* (10 min). A 50-μl aliquot of the supernatant was injected onto the HPLC system.

Method B: Glassware was cleaned with acetonitrile and methanol prior to use to avoid chromatographic interferences. A vacuum manifold (Baker, Groß-Gerau, Germany) was used for pretreating, washing and drying of the SPE cartridges. In a disposable glass tube 1 ml of plasma was diluted with 1 ml of 5% phosphoric acid and 4 ml of the I.S. working solution 2 [0.46 μg/l II in acetonitrile–water (6:4, v/v)]. The mixture (6 ml) was manually shaken and applied to the SPE cartridge which had been pretreated with methanol (3 × 2.5 ml) and 0.025% phosphoric acid (2 × 2.5 ml). Cartridges were washed (8 × 2.5 ml) with methanol–0.025% phosphoric acid (7:3, v/v) and the SPE sorbent beds were air-dried for 5 min. Cartridges were then removed from the manifold and 1.5 ml of acetonitrile was passed through by gravity flow. The effluent was evaporated at 40°C in a Vortex evaporator (Haake-Buchler, Saddle Brook, USA) and the residue was reconstituted in 250 μl of acetonitrile–water (6:4, v/v) in an ultrasonic bath. A 50-μl aliquot was injected onto the HPLC system.

2.3. Instrumentation and operating conditions

A modular HP 1050 instrument (Hewlett-Packard, Waldbronn, Germany) was used equipped with a fluorescence detector HP 1046 A (excitation at 230 nm, emission at 310 nm). Prior to entering the fluorescence detector the column effluent was passed through a post-column photoreactor (Beam Boost, coil 5 m × 0.3 mm I.D., 254 nm lamp; ICT, Frankfurt, Germany). A Lichrosphere RP-18 column (250 × 4 mm I.D., 5 μm) preceded by a guard column (4 × 4 mm I.D.; LichroCART, E. Merck) was used for separation. The operating temperature was 45°C.

The mobile phase consisted of 0.1% phosphoric acid (a) and acetonitrile (b) (method A) and 0.025% phosphoric acid (a) and acetonitrile (b) (method B), respectively. The flow-rate was 1 ml min^{-1}. The gradient employed for elution is given in Table 1. Compounds I and II were eluted at ca. 12.1 and 13.0 min (method A) and 11.5 and 12.5 min (method B), respectively.

Table 1
Gradient elution conditions for determination of I in plasma

Method A			Method B		
Time (min)	%a	%b	Time (min)	%a	%b
0.0	45	55	0.0	45	55
7.0	35	65	4.5	35	65
13.0	35	65	13.0	35	65
13.1	0	100	13.1	0	100
16.5	0	100	20.0	0	100
16.6	45	55	20.1	45	55

In each time interval the gradients were linear.

2.4. Calibration and quality control

Calibration (CAL) samples were obtained by spiking aliquots of acetone working solutions into blank plasma to give thirteen concentrations in the range 10–6400 μg/l (method A). For method B eight CAL concentrations (0.4–16.0 μg/l) were used. Quality control (QC) samples were prepared at three levels covering the range of concentrations expected in unknown samples. QC samples usually contained I in concentrations of 50, 500 and 5000 μg/l (method A) and 2, 5 and 12 μg/l (method B). Two replicates of each QC sample were analysed together with calibration and unknown samples in one sequence.

CAL samples were freshly spiked prior to a sequence. QC samples were stored together with samples of the respective study at −20°C. CAL, QC and unknown samples were processed further as described in section 2.2.

Calibration curves were obtained by plotting concentration vs. peak-area ratio (I/II) and fitting the equation $y = a + bx + cx^2$ to the data points (Concalc software, Institute of Clinical Pharmacology International, Bayer AG, Wuppertal). The calibration curve was divided into two separate ranges [10–480 μg/l (8 data points) and 480–6400 μg/l (6 data points), method A].

Method B employed a linear regression line (peak-height ratio, 0.4–16.0 μg/l) for calibration.

2.5. Assay validation

Light stability was investigated in plasma, whole blood and acetonitrile–water (6:4, v/v), spiked with I. These solutions were exposed to yellow light and sunlight, respectively, up to a period of 6 days. Concentrations of I were determined in aliquots at given times with I.S. being added immediately prior to injection onto the HPLC system. Long-term stability upon storage at −20°C was investigated by repeated analysis of blank plasma spiked with I (50 and 500 μg/l). Six spiked samples at concentrations of 10, 20, 30, 40, 400, 2000 and 5600 μg/l (method A) and 0.6, 0.8, 4 and 14 μg/l (method B), respectively, were analysed within one day to assess intra-day variability. This procedure was repeated on three consecutive days to calculate inter-day precision and accuracy. Absence of matrix interferences was confirmed by analysis of blank plasma. Quality control samples (see section 2.4) served to evaluate inter-day precision and accuracy of the assay during routine analysis.

2.6. Application

Compound I was determined in plasma samples obtained in clinical pharmacological studies:

Study A: 12 healthy male volunteers (18–45 years of age) received one 250-mg tablet per day for seven consecutive days. Blood samples for pharmacokinetic studies were obtained on day 7 before and up to 48 h after administration.

Study B: 5 healthy male volunteers (18–45 years of age) received a single dose of 1 mg of I as a powder aerosol. Blood was sampled before and up to 12 h after administration.

Within 30 min after sampling plasma was prepared by centrifugation (2000 *g*, 20 min at 5°C) and stored at −20°C until analysis.

The trials were conducted according to the German Medicines Act, the Helsinki Declaration and Good Clinical Practice rules following endorsement by a local Ethics committee. All subjects gave written informed consent of participation.

3. Results

3.1. General aspects

Compound I and the internal standard (I.S.) II were readily separated from plasma constituents employing a RP-18 column with spherical particles and gradient elution with phosphoric acid and acetonitrile. A linear gradient was employed as outlined in Table 1. The effluent was passed through the Beam Boost unit, equipped with a 5-m PTFE coil and subsequently a fluorescence detector. The reaction time of the analyte in the photoreactor was 30 s. As the product(s) of the photo-decomposition was (were) not isolated and characterised, the optimum wavelength pair was determined under stopped-flow conditions with Beam Boost on and the fluorescence detector in the scan mode. Increasing the length of the reaction coil to 10 or 20 m led to increases in peak area of 12 and 13%, respectively. However, this did not translate into greater detection sensitivity as a corresponding increase in retention time (+ 3 and + 11%) and enhanced band-broadening (+ 3 and + 5%) was observed as well.

Interference from matrix components was negligible. Therefore plasma was simply diluted, the precipitate was removed and the supernatant was injected (method A). This method was applied to clinical studies with the tablet formulation.

The local administration of a 1-mg dose of I aerosol, however, necessitated further optimisation of the method sensitivity. To achieve the stated objective the analyte was extracted from plasma with a RP-18 solid-phase cartridge. Matrix interferences were removed by washing with methanol–phosphoric acid followed by elution of analyte and I.S. with acetonitrile (method B). Evaporation and reconstitution in the mobile phase afforded a 20-fold increase in the analyte concentration in the injection solvent as compared to method A.

In method B sensitivity was also enhanced due to a slightly steeper gradient leading to narrower elution bands. As impurities in the phosphoric acid contributed significantly to the baseline noise its concentration in the mobile phase was reduced 4-fold in method B.

3.2. Validation

Stability

The light stability of I was investigated in various matrices (Fig. 2). Exposure-time dependent decomposition was only observed if the compound was irradiated with sunlight in organic solution, where a decay half life of 256 h was estimated. Plasma and whole blood obviously contain sufficient amounts of UV absorbing constituents to prevent photolysis of the drug. Therefore, no special light-protection measures are warranted during sampling or centrifugation of blood. However, for the handling of stock and standard solutions of I with possible long-term light exposure, yellow light is recommended, as under these conditions stability for at least 6 days was confirmed.

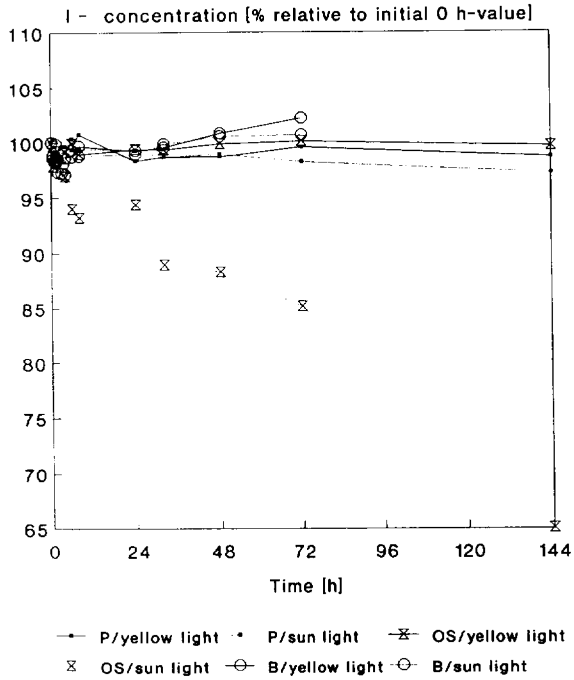


Fig. 2. Stability of I in whole blood (B), plasma (P) and organic solvent (OS) upon exposure to yellow light and sunlight for up to 6 days.

Stability upon storage at −20°C was analysed in plasma spiked with the drug. Measured concentrations were within ± 10% of the nominal value after 13 months of storage indicating that I is stable. Likewise, concentrations assayed after three freeze–thaw cycles were within the experimental error with reference to the nominal values.

The glucuronide of I has been identified as a relevant metabolite in human plasma [4]. Of particular concern was its stability during work-up because hydrolysis would have led to an overestimation of concentrations of I. However, it was found that under the generally acidic conditions during extraction hydrolysis of the conjugate was negligible (< 0.5%).

Further validation parameters of method A

Precipitation of proteins with acetonitrile–phosphoric acid and centrifugation yielded an almost quantitative recovery of 93 ± 6% (10–6400 μg/l, $n = 13$) of I in the supernatant. Recovery was constant throughout the investigated range of concentrations.

Fig. 3 shows typical chromatograms for blank plasma and plasma obtained 0.5 h after oral administration of the drug. The described assay was specific for I as signals interfering with the analyte or I.S. were absent. Only few detector signals could be attributed to matrix constituents in spite of the fact that no further sample clean-up was performed.

Calibration curves were obtained by plotting concentration vs. peak-area ratio (I/II) and fitting the equation $y = a + bx + cx^2$ to the data points. Due to the wide calibration range (10–6400 μg/l) and saturation of detector response which is typically observed with fluorescence detection, a linear fit was not appropriate. Relative residuals were minimised by employing two separate calibration curves for high (480–6400 μg/l) and low (10–480 μg/l) concentrations. Fig. 4 shows two calibration curves as well as the back calculated concentrations and absolute and relative residuals. Compared to other calibration functions (linear, linear with two slopes, logarithmic or quadratic with one range 10–6400 μg/l) the chosen calibration function afforded the optimum fit of the curve to the data points.

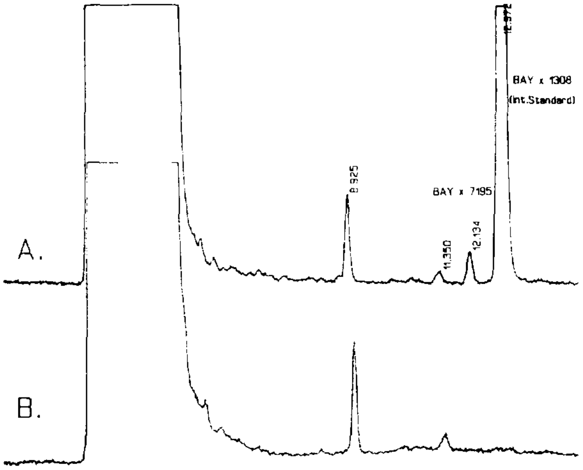


Fig. 3. Chromatograms of plasma of volunteer receiving a 250-mg I tablet. Before (B) and 0.5 h after (A) administration with added internal standard (method A). The signal for I (A) corresponds to a concentration of 21.7 μg/l plasma.

It was found that by virtue of the straightforward sample preparation an I.S. was not absolutely required to obtain valid results. However, relative residuals were minimised when calibration was performed with the I.S.

The results of a formal validation experiment are presented in Table 2. Inter-day precision was < 6% throughout the entire working range. Accuracy was also satisfactory (96–110%). Based on these results the limit of quantitation (LOQ) was 10 μg/l. The limit of detection (LOD, signal-to-noise ratio 3:1) was estimated at 5 μg/l.

Data on the long-term performance of the assay covering analysis of 1130 unknown samples and a period of ca. 8 months are given in Fig. 5. Quality control (QC) samples run concurrently with study samples confirmed the values for precision that were determined during the 3-day validation run. In QC samples containing I at 50–5000 μg/l, inter-day precision ranged from 4.1 to 5.0% and accuracy was between 96 and 105% ($n = 26$ per QC concentration).

HPLC columns were used for at least 750 consecutive injections without significant deterioration of separation efficiency or peak shape.

Further validation of method B

Introduction of the solid-phase extraction step yielded the following results for recovery of drug and I.S. from spiked plasma (Table 3). Recovery of the I.S. was investigated at the working concentration.

Fig. 6 shows representative chromatograms of blank plasma and plasma sampled 5 min after administration of I aerosol. There was a small constant matrix interference coeluting with I, which could not be chromatographically separated by using different mobile or stationary (spherical C_{18} material of various manufactures) phases. Compared to Fig. 3 (method A) there was an evident increase in matrix-derived signals which corresponds to a 20-fold increase in con-

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Plot of CALIB. FUNCTION

Method of evaluation       :  relative area / quadratic calib./ unweighted
                              smallest calib. point  480 ug/l ,  largest calib. point 6400 ug/l
No. calibration samples    :  6 ,   7  more not used for calibration

Analyte                    :  X7195

Calibration function       :   y  =  a  +  bx  +  cx²


        a  = -1.674687E-02          b  =  1.251951E-03          c  = -2.66153E-08
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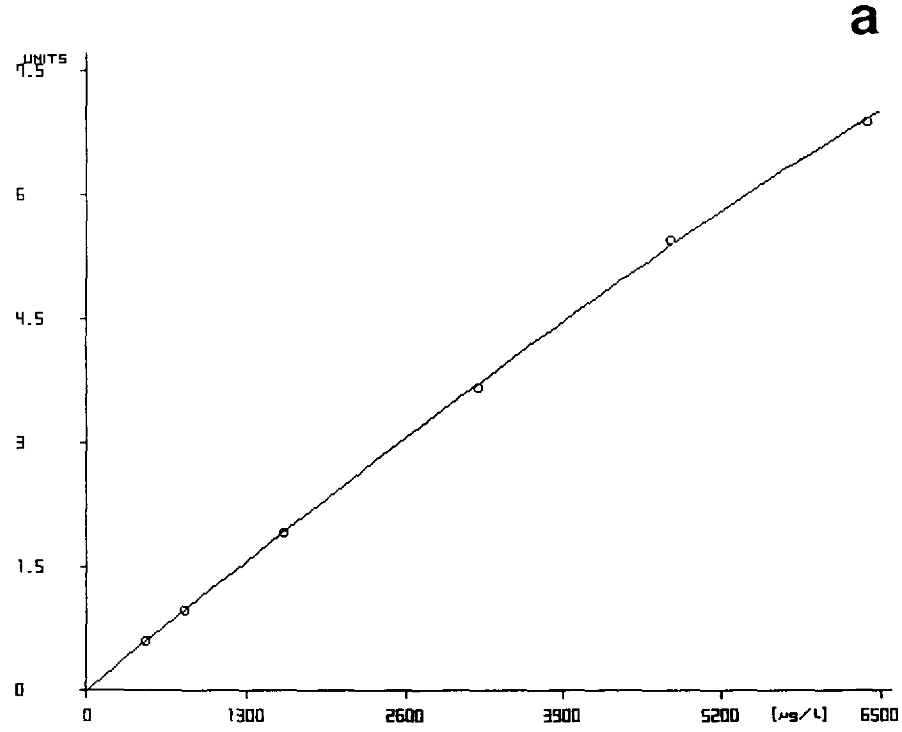

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Printing of Calibration Data

Method of evaluation       :  relative area / quadratic calib./ unweighted
                              smallest calib. point  480 ug/l ,  largest calib. point 6400 ug/l
No. calibration samples    :  6 ,   7  more not used for calibration

Analyte                    :  X7195

  Run No.  |  Rel. area    | Index value | Calculated | Absolute    | Relative    | Ratio
           |               |             |            | difference  | difference  | area/height
           |               |  [ ug/l  ]  | [ ug/l  ]  | [ ug/l  ]   |  [ %  ]     |
-----------|---------------|-------------|------------|-------------|-------------|-----------------
   1666    |      0.5972   |   480.000   |   495.614  |   +15.614   |    +3.25    |   0.0939
   1630    |      0.9600   |   800.000   |   793.579  |    -6.421   |    -0.80    |   0.0940
   1674    |      1.9117   |  1600.000   |  1594.422  |    -5.578   |    -0.35    |   0.0932
   1638    |      3.6748   |  3200.000   |  3161.104  |   -38.896   |    -1.22    |   0.0931
   1647    |      5.4395   |  4800.000   |  4860.417  |   +60.417   |    +1.26    |   0.0931
   1625    |      6.8827   |  6400.000   |  6374.932  |   -25.068   |    -0.39    |   0.0872
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Fig. 4.

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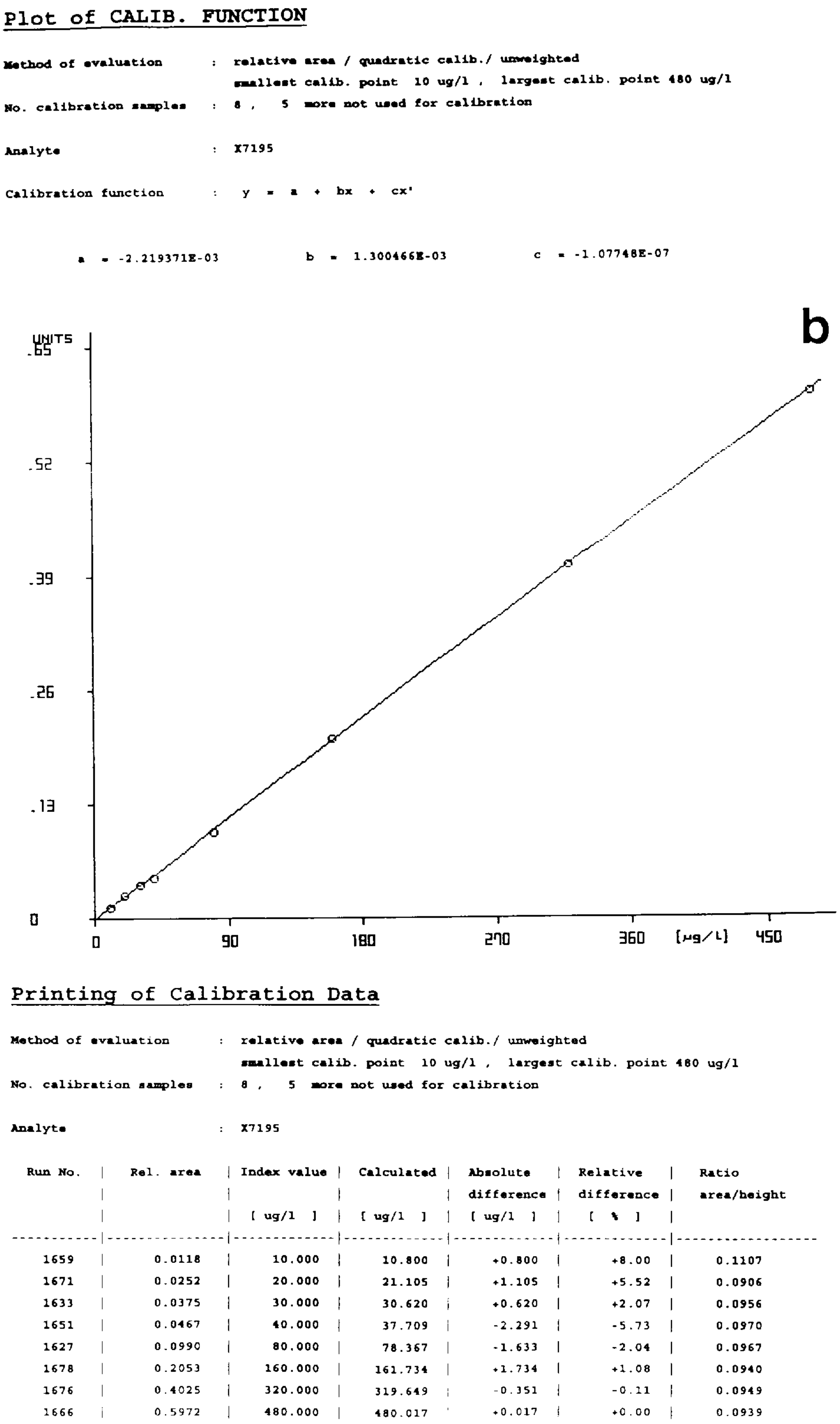

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Plot of CALIB. FUNCTION

Method of evaluation       :  relative area / quadratic calib./ unweighted
                              smallest calib. point  10 ug/l ,  largest calib. point 480 ug/l
No. calibration samples    :  8 ,   5  more not used for calibration

Analyte                    :  X7195

Calibration function       :   y  =  a  +  bx  +  cx'


      a  = -2.219371E-03          b  =  1.300466E-03           c  = -1.07748E-07
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Printing of Calibration Data

Method of evaluation       :  relative area / quadratic calib./ unweighted
                              smallest calib. point  10 ug/l ,  largest calib. point 480 ug/l
No. calibration samples    :  8 ,   5  more not used for calibration

Analyte                    :  X7195

  Run No.  |   Rel. area   | Index value | Calculated | Absolute    | Relative    |  Ratio
           |               |             |            | difference  | difference  |  area/height
           |               |  [ ug/l  ]  | [ ug/l  ]  | [ ug/l  ]   |   [ % ]     |
-----------|---------------|-------------|------------|-------------|-------------|-----------------
    1659   |      0.0118   |     10.000  |    10.800  |     +0.800  |     +8.00   |    0.1107
    1671   |      0.0252   |     20.000  |    21.105  |     +1.105  |     +5.52   |    0.0906
    1633   |      0.0375   |     30.000  |    30.620  |     +0.620  |     +2.07   |    0.0956
    1651   |      0.0467   |     40.000  |    37.709  |     -2.291  |     -5.73   |    0.0970
    1627   |      0.0990   |     80.000  |    78.367  |     -1.633  |     -2.04   |    0.0967
    1678   |      0.2053   |    160.000  |   161.734  |     +1.734  |     +1.08   |    0.0940
    1676   |      0.4025   |    320.000  |   319.649  |     -0.351  |     -0.11   |    0.0949
    1666   |      0.5972   |    480.000  |   480.017  '     +0.017  |     +0.00   |    0.0939
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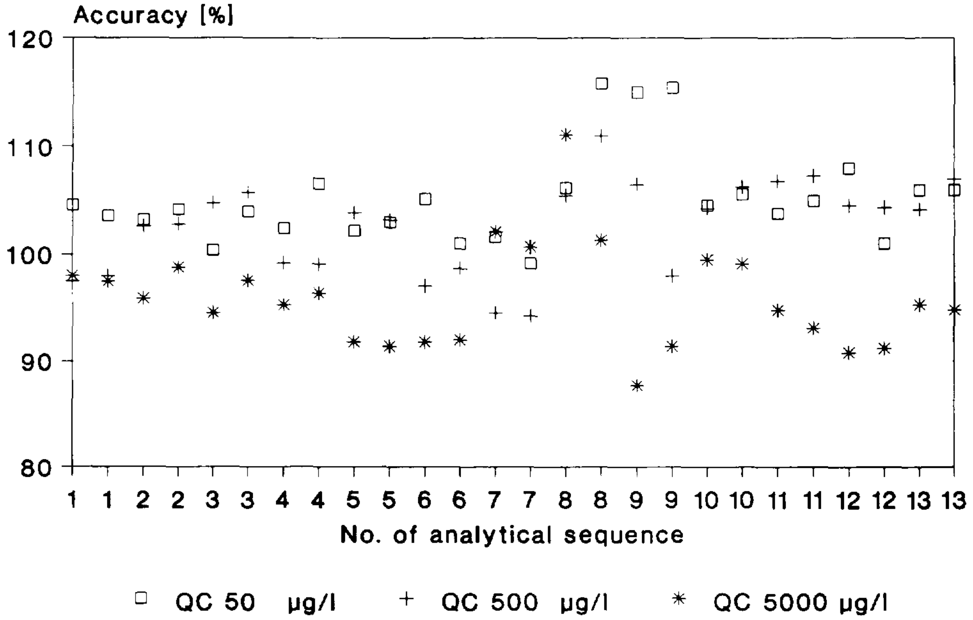
Fig. 4. Calibration curves for (a) high (480–6400 μg/l) and (b) low (10–480 μg/l) concentrations and back-calculated concentrations and residuals.



in each analytical sequence 2 replicates of every QC - Sample were analyzed

Fig. 5. Quality control chart of I in plasma (method A), covering the assay performance within 8 months. Accuracy was calculated with reference to the nominal (spiked) concentration.

centration in the injection solvent. Due to the interfering peak an accuracy bias limited the LOQ to 0.6 μg/l (Table 2). Precision at the LOQ was not limiting (8.6%) and was well within the tolerated 20% range. Throughout the working range precision was $<7\%$ and accuracy ranged from 93 to 105%. A concentration of 0.2 μg/l was equivalent to the LOD.

A linear calibration (I.S. method) was employed, the calibration range being narrower (0.4–16 μg/l) compared to method A.

3.3. Application

The described assays were applied to plasma samples obtained after administration of I as tablet and as aerosol, respectively (Fig. 7).

After oral administration concentrations above the LOQ were measured up to 10 h. Concentrations reached a maximum after ca. 2 h (mean t_{max}) and decreased in a biphasic fashion. The terminal elimination half-life was 5–7 h.

Even at t_{max} plasma concentrations were below the LOQ of method A after administration of 1 mg of I aerosol. The concentration–time profile was similar to that of the tablet, although up to 3 h only the first rapid distribution/elimination phase (apparent half-life of 1 h) could be observed. Terminal half-life was not estimated due to concentrations being below the LOQ after this small dose. With a mean t_{max} value of 1.3 h absorption tended to be faster compared to the tablet formulation.

4. Discussion and conclusions

Following post-column irradiation at 254 nm compound I is decomposed to species amenable to fluorescence detection. Due to the combined selectivities of photodecomposition and fluorescence detection diluted plasma can be injected onto the HPLC system without prior clean-up (method A). Sensitivity was sufficient to assess the pharmacokinetics of the drug after oral administration.

Table 2
Intra- and inter-day precision and accuracy of determination of I in human plasma

	Nominal concentration (μg/l)										
	Method A calibration range 10–6400 μg/l							Method B calibration range 0.4–16 μg/l			
	10	20	30	40	400	2000	5600	0.6	0.8	4	14
Concentration found (μg/l) (arithm. mean value)											
Day 1 ($n = 6$)	10.67	20.78	29.96	39.64	395.25	1926.94	5387.60	0.64	0.71	4.00	14.07
Day 2 ($n = 6$)	11.37	20.84	30.66	40.15	396.00	1969.76	5388.73	0.68	0.72	4.41	13.23
Day 3 ($n = 6$)	10.85	20.35	32.54	42.77	398.42	1947.80	5328.95	0.67	0.80	4.21	14.56
Inter-day ($n = 18$)	10.96	20.66	31.05	40.85	396.56	1948.16	5368.43	0.66	0.74	4.21	13.95
Accuracy (%) (arithm. mean value)											
Day 1 ($n = 6$)	106.7	103.9	99.9	99.1	98.8	96.3	96.2	107.1	88.4	100.0	100.5
Day 2 ($n = 6$)	113.7	104.2	102.2	100.4	99.0	98.5	96.2	112.7	89.7	110.3	94.5
Day 3 ($n = 6$)	108.5	101.8	108.5	106.9	99.6	97.4	95.2	111.3	99.7	105.3	104.0
Inter-day ($n = 18$)	109.6	103.3	103.5	102.1	99.1	97.4	95.9	110.4	92.6	105.2	99.7
Precision (%) (arithm. mean value)											
Day 1 ($n = 6$)	5.3	2.0	2.8	1.9	0.8	0.7	0.7	3.4	2.9	2.8	4.3
Day 2 ($n = 6$)	4.5	2.3	0.6	1.9	0.7	0.5	0.6	9.5	5.5	2.7	2.3
Day 3 ($n = 6$)	5.7	2.7	3.0	2.3	0.8	0.9	1.3	11.3	3.8	3.5	2.8
Inter-day ($n = 18$)	5.6	2.4	4.3	4.0	0.8	1.1	1.0	8.6	6.9	5.0	5.1

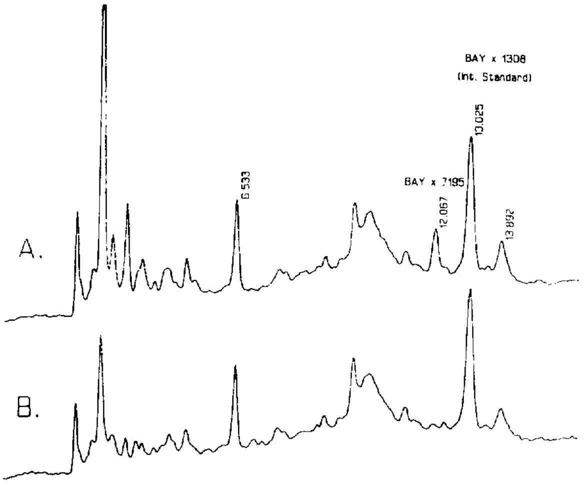


Fig. 6. Chromatograms of plasma of volunteer receiving 1 mg of I aerosol. Before (B) and 5 min after (A) administration with added internal standard (method B). The signal for I (A) corresponds to a concentration of 1.2 μg/l plasma.

Table 3
Recovery of I and II from plasma (method B)

Nominal concentration (μg/l)	n	Recovery (mean ± C.V.) (%)
Analyte I		
2	6	77.2 ± 3.4
6	12	88.2 ± 5.2
12	6	90.4 ± 6.8
Total	24	86.0 ± 8.0
I.S. II		
2	12	88.4 ± 4.4

Recovery of the analyte was constant and almost quantitative in the concentration range 10–6400 μg/l (method A). However, the data presented on recovery of I at a concentration of 2 μg/l (method B) are indicative of a tendency to decreased recovery at low concentrations. This may be due to adsorption of I to surfaces. Nevertheless, this affects calibration and un-

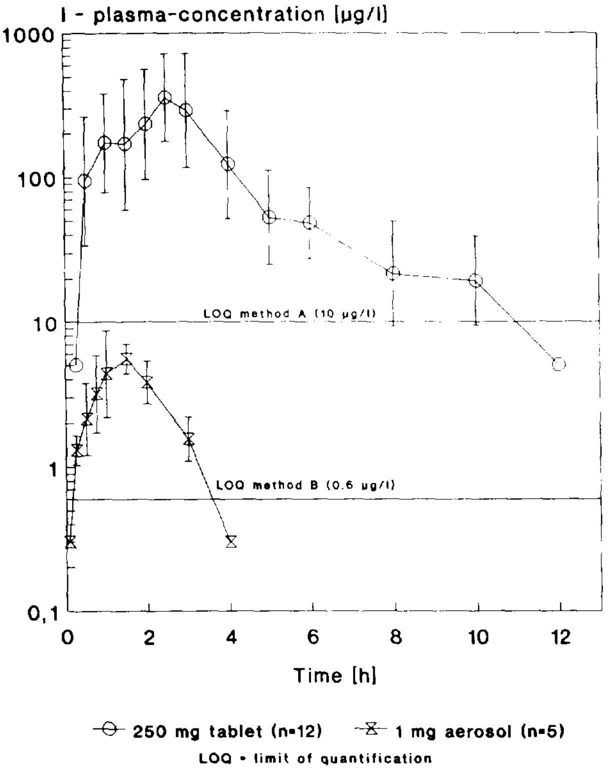


Fig. 7. Plasma concentration–time profiles of I in healthy volunteers receiving 1 mg of I aerosol and 250-mg I tablet, respectively [geometric mean and standard deviation, $n = 5$ (aerosol) and 12 (tablet)].

known samples alike and therefore will not cause an accuracy bias.

The criteria for assay validation were fulfilled when the external standard method was employed. However, with an I.S. the quality of fit of the calibration curve was improved. This may be due to subtle variations occurring in the post-column reactor.

The LOQ of 10 µg/l (method A) was further improved by introduction of a solid-phase extraction step. This gave rise to a small matrix interference coeluting with the analyte. Nevertheless the ensuing 20-fold increase in concentration of the analyte in the injection solvent translated into a comparable improvement of the LOQ (0.6 µg/l for method B). With this enhancement in sensitivity it became possible to determine plasma concentrations after a 1-mg dose administered by inhalation.

Alternative HPLC methods utilizing UV detection and fluorescence derivatization were investigated before developing the described assay. Compared to these approaches post-column photoderivatisation/fluorescence detection appeared to be superior in terms of sensitivity, selectivity and ease of sample pretreatment. It may be regarded as a minor drawback that the nature of the fluorescent species is not known unless isolation and structure elucidation is performed. The optimisation of an assay employing the Beam Boost therefore has to proceed empirically.

Acknowledgements

The excellent technical assistance of Mr. M. Kaiser is gratefully acknowledged. The author wishes to thank Dr. G. Wensing, the principal investigator of clinical-pharmacological studies with I and Dr. P. Norman who synthesized the fluorescent derivatives of I.

References

[1] T.S. Abram, H. Böshagen, J.E. Butler, N.J. Cuthbert, H.P. Francis, P.J. Gardiner, W. Hartwig, H.C. Kluender, P. Norman, H. Meier, U. Rosentreter, K.H. Schlemmer, S.R. Tudhope and W.A. Taylor, *Bioorganic Med. Chem. Lett.*, 3 (1993) 1517.
[2] T.S. Abram, N.J. Cuthbert, H.P. Francis, P.J. Gardiner, P. Norman and W.A. Taylor, *Am. Rev. Respir. Dis.*, 147 (Suppl.) (1993) A 179.
[3] G. Wensing, R. Heinig, M. Priesnitz and J. Kuhlmann, *Eur. J. Clin. Pharmacol.*, 227 (1994) 47.
[4] Data on file, Bayer AG, Wuppertal, Germany.
[5] R. Farinotti, P. Siard, J. Bourson, S. Kirkiacharian, B. Valeur and G. Mahuzier, *J. Chromatogr.*, 269 (1983) 81.
[6] H. Lingeman, A. Hulshoff, W.J.M. Underberg and F.B.J.M. Offermann, *J. Chromatogr.*, 290 (1984) 215.
[7] H.G. Schäfer, *J. Chromatogr.*, 616 (1993) 87.