Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 972–982
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Adaptation and performance of an immuno-PCR assay for the quantification of Aviscumine in patient plasma samples

Michael Adler[a,*], Martin Langer[b], Klaus Witthohn[b], Karin Wilhelm-Ogunbiyi[b], Patrick Schöffski[c], Pierre Fumoleau[d], Christof M. Niemeyer[e]

[a] *Chimera Biotec GmbH, Emil-Figge-Str. 76 A, D-44227 Dortmund, Germany*
[b] *VISCUM AG, Technologiepark, Friedrich-Ebert-Str., D-51429 Bergisch Gladbach, Germany*
[c] *Catholic University Leuven, Universitair Ziekenhuis Gasthuisberg, Division of Clinical Oncology, Herestraat 49, B-3000 Leuven, Belgium*
[d] *Department of Medical Oncology, Centre René Gauducheau, Centre Regional de Lutte Contre le Cancer Nantes-Atlantique, Nantes, France*
[e] *Universität Dortmund, Fachbereich Chemie, Lehrstuhl für Biologisch-Chemische Mikrostrukturtechnik, Otto-Hahn-Str. 6, D-44227 Dortmund, Germany*

Received 22 December 2004; received in revised form 27 April 2005; accepted 30 April 2005
Available online 27 June 2005

Abstract

An immuno-polymerase chain reaction (IPCR) assay is used to evaluate the kinetic behaviour of the novel anti-cancer drug Aviscumine in plasma samples taken from 41 patients during a 3-year clinical trial.

The ultrasensitive IPCR assay employed the amplification of a detection-antibody linked marker-DNA and an internal competitor DNA for standardization, thus enabling the detection of the antigen in concentrations far below the detection limit of conventional enzyme-linked immuno-sorbent assay (ELISA). The quantification of Aviscumine was carried out using external calibration curves obtained from individual patient plasma samples, collected previous to the administration of Aviscumine, which were spiked with known amounts of the reference substance Aviscumine. Additional controls were measured containing standardized human serum spiked with Aviscumine to assure the continuous general reproducibility of the assay as well as to estimate differences between individual patients.

Average recovery was found to be $95 \pm 19\%$ and the average deviation in precision of the assay was determined to be $9 \pm 5\%$. Data for the quantification of Aviscumine were obtained from all patient samples investigated with the exception of a single patient. The collected data provided the basis for the valid routine quantification of patient samples for the calculation of the pharmacokinetic behaviour of Aviscumine in patient plasma.

Keywords: Immuno-PCR; ELISA; Assay sensitivity; Pharmacokinetics; Quantification; Aviscumine

1. Introduction

The development of conventional immuno-assays, such as enzyme-linked immuno-sorbent assay (ELISA), is often hampered by various problems concerning the robustness, accuracy and the sensitivity of the respective method [1]. The clinical investigation of novel, highly potent drugs, which often are applied in only low dosages, requires the establishment of very sensitive and robust assays which allow for the detection and quantification of the respective drug, even in complex biological matrices, such as human plasma- or sera-samples.

Abbreviations: B_{PC}, B_{PC13}, B_{NC}, control samples of spiked standardized human plasma with PC = 40 ng/mL, PC13 = 13 pg/mL and NC = no Aviscumine, respectively; C_{max}, Aviscumine concentration found at $t = 1$ h; ELISA, enzyme-linked immuno-sorbent assay; ELOSA, enzyme-linked oligonucleotide-sorbent assay; IPCR, immuno-PCR; I_M, absolute fluorescence signal intensity obtained for the marker-DNA in IPCR; I_N, normalized signal intensity; P #*n*d*m*, patient number *n*, dose *m*; S_e, S_i, S_M, S_C, S_T, standard deviation calculated for ELOSA ($_e$), IPCR ($_i$), signals obtained from marker-DNA ($_M$), competitor DNA ($_C$) and total error ($_T$), respectively; t0, patient plasma sample negative control "zero plasma", obtained previous to application of Aviscumine; V_H, V_M, V_L, t0-sample spiked with Aviscumine of high ($_H$), medium ($_M$) or low ($_L$) concentration, respectively

* Corresponding author. Fax: +49 231 9742 844.
E-mail address: adler@chimera-biotec.com (M. Adler).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.04.051

The immuno-PCR (IPCR) method, first described by Sano et al. in 1992 [2], combines the well-established ELISA methodology with the signal amplification power of the PCR. A number of research applications describe the advantages of the method [3–5], that is, in particular, its high sensitivity and good quantification capabilities due to the great linearity and compatibility with established ELISA protocols [6].

IPCR was recently adapted to the detection of Aviscumine in samples of human plasma [7]. Aviscumine (rViscumin) is a heterodimeric 57 kDa recombinant mistletoe lectin (rML) [8,9], heterologously expressed in *Escherichia coli*-based on the genetic information of plant-derived lectin, which belongs to the type II ribosome inactivating protein (RIP)-family. Aviscumine has proven its activity as a cytostatic drug in several studies employing animal models [10–12] and is currently manufactured under GMP (good manufacturing practice) conditions. In animal model studies low amounts of about 3–500 ng/kg body weight were applied to mice in order to investigate the anti-tumor activity of Aviscumine [10–12]. The resulting plasma concentrations of these doses of Aviscumine were significantly below the limit of detection of a conventional ELISA of approx. 3.5 pmol/L (200 pg/mL) [7,13]. Hence, a quantitative IPCR assay was established to circumvent these problems, allowing the detection of concentrations as low as 100 fg/mL Aviscumine in standardized human serum samples [7].

The present study describes modifications of the original IPCR assay to allow for the quantification of a large number of human plasma samples obtained during the clinical trial EORTC 16002/VIS009V1.01 and to apply this method for the generation of pharmacokinetic data in this clinical trial study [14]. The work presented here elucidates specific details of the robustness, recovery and precision of the IPCR assay during the 3-year clinical trial. The results clearly emphasize the suitability of this method for routine analyses.

2. Materials and methods

2.1. Patient plasma samples

Object of the analysis were frozen citrate plasma samples of the clinical trial EORTC 16002/VIS009V1.01, obtained from different patients (No. 1–41, in the following referred to as P #1–41) [14]. The study was conducted in accordance with the Good Clinical Practice Guidelines as issued by the International Conference on Harmonisation and the Declaration of Helsinki. Subsequent to collection, the samples were immediately frozen below −20 °C on dry ice and maintained at this temperature until analysis by IPCR. The assay was designed such that each set of samples from an individual patient contained a “zero-plasma”-sample collected 15 min prior to Aviscumine administration (t0) and seven samples taken at different times subsequent to the application of Aviscumine after 1, 1.25, 1.5, 2, 4, 8 and 24 h. Aviscumine was given twice weekly (days 1 and 4) as a 1 h i.v. infusion through a central line. The samples analysed were from “dose 1” (d1), the immediate start of the patient treatment cycle and “dose 11” (d11), a late step of the treatment cycle (after 39 days). In the case of some patients, only the d1 samples of the first infusion were available for analysis and/or some of the samples were not included in the time series to be measured by IPCR. The Aviscumine dose level was increasing during the study. For the first 9 patients (P #1–9), each patient received a separate dose level Aviscumine (P #1: 10 ng/kg, P #2: 20 ng/kg, P #3: 40 ng/kg, P #4: 100 ng/kg, P #5: 200 ng/kg, P #6: 400 ng/kg, P #7: 800 ng/kg, P #8: 1600 ng/kg, P #9: 2400 ng/kg). The following patients P #10–41 were subgrouped to five cohorts (4 patients P #10–13: 3200 ng/kg, 6 patients P #14–19: 4000 ng/kg, 10 patients P #20–29: 4800 ng/kg, 7 patients P #30–36: 5600 ng/kg, 5 patients P #37–41: 6400 ng/kg) (for more details, see also [14] and Figs. 5 and 6).

2.2. Test substance

Aviscumine (INN); 2 μg/mL, reference substance batch-no.: 970303 (VISCUM AG, Bergisch Gladbach, Germany) was used to prepare external standard curves for quantification purposes. Control calibration samples were prepared from standardized virus-inactivated human sera BISEKO (Biotest, Langen, Germany) as the biological matrix. Polyclonal rabbit-anti-mistletoe-lectin antibody (VISCUM AG) was used as capture antibody and for the synthesis of the custom-made antibody–DNA conjugates as reagents for IPCR (CHIMERA, Dortmund, Germany).

2.3. Preparation of calibration curves and spiking controls

As a consequence of individual differences between patient samples and sample handling (see Section 3.1) calibration curves and spiked controls were prepared from the zero sample “t0” (see Section 2.1) of each individual patient/dose course and these standards were frozen and stored at −20 °C. To simulate the possible standing time of samples during clinical sample preparation previous to freezing at −20 °C, a 25 min timeframe for sample handling and bench-time on ice was maintained during the preparation of calibration curves and spiked control samples. The concentrations of Aviscumine within the calibration curves and spiked samples are listed in Table 1.

2.4. Assay layout and sample preparation

Each individual IPCR assay contained a calibration curve and three spiked controls termed V_H, V_M and V_L, containing a “high”, “medium” and “low” concentration

Table 1
Quantification parameters

Patient	Regression model	Concentration Aviscumine for calibration curves	Average corellation coefficient r^2	Concentration Aviscumine (V_H, V_M, V_L) and average recovery	
(a) #1–8	Harris	1000, 600, 300, 100, 60, 30, 10 pg/mL	0.99	800 pg/mL	90 ± 7%
				200 pg/mL	95 ± 15%
				20 pg/mL	52 ± 22%
(b) #9–11	Harris	10, 6, 3 ng/mL; 1000, 600, 300, 100, 60, 30, 10 pg/mL	0.99	800 pg/mL	86 ± 16%
				200 pg/mL	108 ± 21%
				20 pg/mL	107 ± 24%
(c) #12–41	Linear	6.1, 5.1, 4.1, 3.1, 2.1, 1.1, 0.1 ng/mL	0.97	4.8 ng/mL	91 ± 6%
				2.4 ng/mL	91 ± 10%
				0.8 ng/mL	112 ± 17%

Average recovery of high, medium and low concentrated samples spiked with Aviscumine in patient plasma (V_H, V_M, V_L), standard deviations and correlation coefficient were calculated for all patients using the respective regression model.

of Aviscumine (see Table 1), obtained from zero dose (t0) plasma. In addition, a positive control "B_{PC}" of 40 ng/mL Aviscumine in BISEKO and a negative control of BISEKO containing no Aviscumine "B_{NC}" were also included. In some cases, a third positive control of 13 pg/mL Aviscumine in BISEKO "B_{13PC}" was measured as well. Similar to patient samples, the BISEKO controls were frozen at −20 °C as well.

For P #1–8, a calibration curve in "t0" plasma with a concentration range from 10 to 1000 pg/mL Aviscumine (see Table 1a) was prepared, similar to a calibration curve in BISEKO, prepared as previously reported [7]. During the course of the clinical trial study, the calibration curve was adapted to higher concentrations of Aviscumine in the case of patients P #9–11 (see row b in Table 1 and Fig. 5) to adjust the method to the increased amounts of Aviscumine administered. To avoid further changes in the methodology and allow for a standardized protocol, all subsequent quantifications (P #12–41) were carried out using the same linear quantification curve covering a concentration range of 100–6100 pg/mL Aviscumine (Table 1c and Fig. 4). Therefore, patient plasma samples of P #12–41 from $t = 1$ to 2 h were routinely diluted in "t0" plasma previous to the IPCR to keep the signal intensities within the calibration curve. In particular, these samples from P #12–18 were diluted 1:3 and samples from P #19–41 were diluted 1:8 with individual "t0" patient plasma. For control purposes, each individual sample was also measured undiluted. If the undiluted sample yielded a signal within the range of the calibration curve, this signal was used for quantification. Otherwise, the signal from the diluted sample was used.

All calibration samples, spiked controls, BISEKO controls and patient plasma samples were additionally diluted 1:3 in a detergent-containing sample dilution buffer (CHIMERA) immediately prior to analysis by IPCR. The IPCR assays were carried out in duplicate. For the total amount of 2 × 30 μL IPCR assay volume, 44 μL sample dilution buffer were added to 22 μL sera/plasma-sample.

All patient samples and controls were thawed in a thermomixer, and subsequent to removing 22 μL of the thawed samples, the remaining samples were refrozen with liquid nitrogen for long-term storage at −80 °C.

2.5. Immuno-PCR

IPCR reagents were custom made by CHIMERA. Taq DNA polymerase from Biomaster (Cologne, Germany) was used for DNA-amplification and IPCR was carried out as a one-step assay according to the protocol previously described [7]. A schematic drawing of the assay is shown in Fig. 1. In brief, top yield modules were coated overnight at 4 °C using 30 μL/well of the polyclonal rabbit-anti-lectine antibody T2 (15 μg/mL). The modules were washed and blocked with 240 μL/well CHIMERA blocking buffer overnight. Following another washing step, the modules were incubated for 25 min and orbital shaking at room temperature with 30 μL/well of the diluted samples containing Aviscumine. Subsequently, the modules were incubated for 25 min/RT and orbital shaking with 30 μL/well of a 1:300 dilution of the Aviscumine-specific antibody–DNA conjugate also synthesized from T2. The marker-DNA (I in Fig. 1) of the IPCR reagent was amplified (primus 96 PCR cycler, MWG, Ebersberg, Germany) in the presence of a constant concentration of an internal competitor DNA (II in Fig. 1) which is co-amplified with the marker-DNA during PCR. The competitor-DNA is an integral component of CHIMERA's IPCR quantification mix for the Aviscumine assay. Both DNA products were labeled during PCR by incorporation of a hapten-labeled nucleotide. Subsequent to PCR amplification, the amount of the two PCR-products, i.e. the marker-DNA (I) and the competitor-DNA (II), was determined using an enzyme-linked oligonucleotide-sorbent assay (ELOSA). In this assay, labeled PCR amplicons are quantified by hybridisation with solid-phase bound capture oligonucleotides and subsequent labeling with an antibody–enzyme conjugate und fluorescence detection of

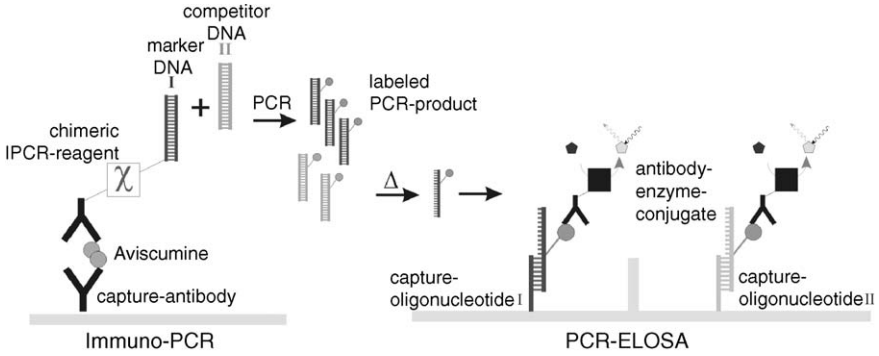

Fig. 1. Schematic drawing of the IPCR detection of Aviscumine on microtiter plates coated with an anti-Aviscumine capture-antibody. The immobilized antigen is tagged with a tailor-made IPCR conjugate, containing an Aviscumine-specific antibody and the marker-DNA (I). Following to PCR amplification of marker-DNA (I) and competitor-DNA (II), the PCR-products, labeled with digoxigenin are allowed to hybridize to microplate-bound capture oligonucleotides, specific for either marker-(I) or competitor-DNA (II). For quantification, anti-digoxigenin alkaline-phosphatase conjugate and a fluorogenic substrate were used [4].

the fluorogenic substrate AttoPhos (Roche), using a VICTOR microplate multilabelcounter (Perkin-Elmer, Boston, US) [7]. This detection of PCR-amplificate was also carried out in duplicate.

2.6. Data analysis

The raw data of the eight ELOSA signals obtained for each sample was processed as previously described [7]. In brief, the average fluorescence signal intensities of the four ELOSA samples obtained from the duplicate IPCR samples were calculated separately for the marker (I_M) and the competitor DNA (I_C):

$$I_M = \frac{I_M^{e1i1} + I_M^{e2i1} + I_M^{e2i2} + I_M^{e2i2}}{4}$$

$$I_C = \frac{I_C^{e1i1} + I_C^{e2i1} + I_C^{e1i2} + I_C^{e2i2}}{4}$$

Wherein, for instance, "I_M^{e1i1}" is the ELOSA fluorescence signal intensity no. "1" of the IPCR sample no. "1" of the marker-DNA, and e.g. "I_C^{e2i1}" is the ELOSA fluorescence signal intensity no. 2 of the IPCR sample no. 1 of the competitor-DNA, and so on.

Subsequently, the normalized ratio "I_N" was calculated according to:

$$I_N = \frac{I_M/I_C}{\overline{I}_{M-NC}/\overline{I}_{C-NC}}$$

wherein $\overline{I}_{M-NC}$ and $\overline{I}_{C-NC}$ are the average I_M values calculated for a triplicate analysis of the negative control "t0". Additionally, a normalization of the signals obtained only by marker-DNA without internal competitor, calculated by dividing I_M by $\overline{I}_{M-NC}$ was carried out for evaluating the effect of the internal competitor. A comparison of I_M, $I_M/\overline{I}_{M-NC}$ and I_N-values is given in Fig. 4B.

The calculated signals, given in arbitrary units [a.u.], were plotted against the spiking concentration. In addition to linear regression, a Harris regression model was used to obtain the calibration curve of the first samples (P #1–11):

$$Y = \frac{1}{a + bX^c}$$

Wherein X is the calculated concentration (C_c) and Y are the I_N values obtained by IPCR/PCR–ELOSA.

For P #1–4 a method, error was calculated as the standard deviation between at least two independent IPCR assays carried out during initial method adaptation. Starting with P #5, the experimental layout was standardized and the following parameters were used for monitoring of the precision of the assay.

The standard deviation of the double determination for PCR–ELOSA (S_e) was calculated from I^{e1i1} and I^{e2i1} as well as from I^{e1i2} and I^{e2i2}, respectively.

In addition, the average standard deviation of the IPCR double determinations (S_i) was calculated by the comparison of the two average values of the ELOSA double determination $I^{i1} = ((I^{e1i1} + I^{e2i1})/2)$ and $I^{i2} = ((I^{e1i2} + I^{e2i2})/2)$ obtained for each sample.

While the errors of PCR–ELOSA and IPCR double determinations were calculated separately to monitor potential error sources and/or outliers, the total error of the quantification of the marker (S_{TM}) resp. competitor (S_{TC}) DNA was calculated using I_M^{e1i1}, I_M^{e2i1}, I_M^{e1i2}, I_M^{e2i2} for S_M and I_C^{e1i1}, I_C^{e2i1}, I_C^{e1i2}, I_C^{e2i2} for S_C.

The total error of the marker (S_M) and the competitor (S_C) quantification were combined according to:

$$S_T = \sqrt{S_{TM}^2 + S_{TC}^2}$$

In addition to the BISEKO control samples (B_{PC}, B_{13PC}, B_{NC}), three individual spiked samples V_H, V_M and V_L were prepared for each patient dose (see Table 1) using the individual "t0" negative control plasma. These samples were analyzed using the individual calibration curve of the patients

and the percent recovery rate R of the spiked concentration was calculated according to:

$$R(\%) = \frac{C_C \times 100}{C_S}$$

wherein C_C is the calculated concentration of the spiked sample (pg/mL) and C_S is the spiked concentration of the spiked sample (pg/mL).

3. Results and discussion

The IPCR method was used during the complete clinical trial EORTC 16002/VIS009V1.01 for the quantification of Aviscumine in samples of altogether 41 patients in 64 experiments (498 samples). Aviscumine was administered intravenously over a period of 1 h at various concentrations in the dose–escalation phase 1 trial.

3.1. Initial adaptations of the IPCR method

In the initial phase of the study, the original IPCR assay [7], developed for the detection of Aviscumine in standardized human serum (BISEKO), required further optimization to generate valid data from patient plasma samples. This experimental adaptation of the assay was carried out using sample materials from P #1–4. The general principle of the IPCR assay is outlined in Fig. 1. Anti-mistletoe–lectin antibody, which specifically binds to Aviscumine, was used as the capture antibody. Subsequent to the incubation of the Aviscumine-containing samples an Aviscumine-specific antibody–DNA conjugate was added. The marker-DNA of this conjugate (I in Fig. 1) was amplified in the presence of a constant concentration of an internal competitor-DNA (II in Fig. 1). During PCR DNA II is co-amplified with the marker-DNA I and digoxigenin labels are incorporated. After PCR, the concentration of both products of the templates I and II was determined by an enzyme-linked oligonucleotide-sorbent assay (ELOSA). Therein, the hybridisation of the digoxigenin-labeled PCR-amplicons with solid-phase bound capture oligonucleotides was quantified using an anti-digoxigenin antibody–alkaline phosphatase conjugate and fluorescence detection [7].

During the comparison of calibration curves derived from freshly prepared Aviscumine-spiking samples of either BISEKO or frozen patient plasma, it was evident that the sample handling and the freezing/thawing process significantly decreased the signal-to-noise ratio for Aviscumine. Thorough investigation of individual sample-handling steps indicated that freezing/thawing procedures needed to be carried out in a highly standardized manner (Fig. 2) Moreover, it was found that exposure of the plasma samples to room temperature should be minimized (see Fig. 2), while short incubation times ($t < 30$min) on ice had no influence on assay's performance. For estimation of assay robustness during sample handling, control samples from two individuals were compared with 30, 10 and 0 min standing time in combination with freezing at −20 °C and liquid nitrogen, each as a negative control without Aviscumine and spiked with a uniform concentration of the antigen, respectively. Following normalization to negative controls, an average standard deviation of $9.1 \pm 4.6\%$ was observed between the two individuals compared to an average $5.1 \pm 0.8\%$ between different standing times on ice. Average spike recovery for was found at $95 \pm 3\%$ for plasma with 30 min bench-time on ice and subsequently frozen at −20 °C. With a standing time of 30 min/ice, the difference between freezing at −20 °C and liquid nitrogen was found at $3.7 \pm 1.8\%$.

Eventually, the simulation of various freezing procedures with spiked model samples indicated that these variations can be compensated for by using a frozen set of calibration samples as well as by the establishment of a strictly reproducible freezing/thawing procedure for the plasma samples and control samples. The most important conclusion of these investigation was that control samples for quantification curve/spiking need to be treated identical to the patient's

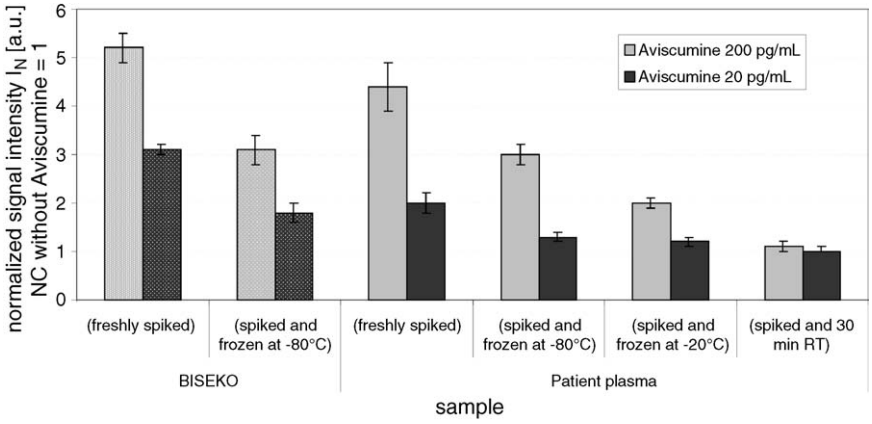

Fig. 2. Comparison of different sample handling protocols previous to IPCR quantification. Samples of BISEKO standardized human serum as well as patient citrate plasma were spiked with Aviscumine and either immediately quantificated or frozen/thawed and subsequently analyzed. The patient plasma samples were taken from an individual single patient. The frozen samples were stored overnight at the given temperature.

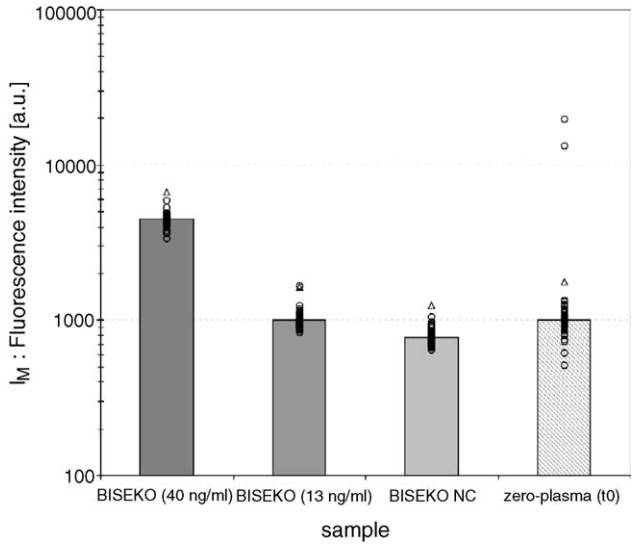

Fig. 3. Overview of the I_M values obtained for control samples containing 40 ng/ml (positive control), 13 pg/ml or no Aviscumine (negative control). Dots indicate the fluorescence signals obtained in the separate experiments and bars show the average signal. *Left:* Two positive and one negative control of spiked standardized human serum samples (BISEKO). Note the high homogeneity of the values with a total average standard deviation of 9.8% for all values of B_{NC}. *Right:* In contrast to the BISEKO samples, the samples taken from the patient's negative plasma previous to application of Aviscumine (zero plasma, t0) show a larger diversity. Here, an average standard deviation of 18.5% and two outliers (P #11D1 and 15D1) were observed. For one patient (P10D11, triangular dots), high values for both, BISEKO and patient plasma were observed, indicating a systematic deviation in this case.

plasma samples. These precautions effectively minimized temperature-related deviations in between different experiments.

Compared to a stable signal obtained for BISEKO positive and negative controls (B_{PC}, B_{NC}) these initial studies also revealed significant fluctuations between the various "t0" individual human plasma samples (Fig. 3). As discussed below under "assay specificity" and robustness", these variations, likely, reflect changes in the plasma composition of individual patients leading to a low degree of non-specific binding of the anti-mistletoe lectin antibodies, which are usually not detectable by conventional ELISA but are now becoming visible due to the high sensitivity of IPCR. Hence, it was necessary to prepare individual calibration curves for each patient under investigation from the respective "t0" plasma sample.

The individual differences between the patients are obvious from the various calibration curves obtained from the individual patients, shown in Fig. 4. Employment of these individual calibration curves and normalization against the "t0" samples permitted us to compensate for the observed fluctuations of background signal intensities of the patient samples. Moreover, normalization using the internal competitor for the calculation of I_N values further increased the quality of the signals (Fig. 4B), and therefore, enabled us to carry out a valid quantification of the Aviscumine concentration.

While an overall good similarity of the calibration curves was observed, the presence of individual differences between the patients (for instance, see P #18 or P #26 in Fig. 4A) underline the necessity of individual calibration curves for an optimized quantification in this system. Patient P #15 is omitted in the figure due to the very high signal intensities (see also Fig. 3).

3.2. Quantification parameters

According to the method established for spiked BISEKO samples [7], a linear regression of the log concentration against the log of normalized signal intensity ($\log I_N$) was carried out for Aviscumine quantification. Using this method, the average correlation coefficient of the regression for P #1–8 was only 0.959 ± 0.02. Data evaluation with different regression methods revealed that uniformly an even better average correlation coefficient of 0.985 ± 0.01 could be obtained using a Harris model instead of several simple non-linear regression methods.

Following the observation that the concentration range of the first calibration curve (Table 1(a)) was not sufficient for the highest Aviscumine concentrations of 2400 ng/kg in P #9 and 3200 ng/kg in P #10, respectively, the calibration curve was modified by (i) expanding the concentration range to up to 10.000 pg/mL for P #9–11 (Table 1(b)) and (ii) by placing a novel set of calibration samples into the observed linear range of the expanded calibration curve (Table 1(c) and Fig. 4), thus eliminating the need for non-linear regression models for the remaining patients P #12–41.

This standardized calibration curve with a linear concentration range between 100–6100 pg/mL Aviscumine in regular intervals of 1000 pg between the samples was chosen according to the lower detection limit of 100 pg/mL as revealed by assay recovery in spiked plasma samples (V_L, see also "assay sensitivity", below). A very good linear correlation between spiked Aviscumine concentration and signal intensity was found in this interval for all patients with an average correlation coefficient of 0.97 ± 0.02.

3.3. Assay precision

During the course of the clinical study, it was established that the average standard deviation of the double determination of the ELOSA (S_e) was: $2.9 \pm 1.2\%$, while the average value of the standard deviation for the IPCR double determination (S_i) was: $4.7 \pm 2\%$.

The combined total error S_T of marker- and competitor-DNA signals for an IPCR double determination did not exceeded a limit of 50%, as determined as threshold for a valid assay. A singular exception observed for patient #6, dose 1, $t = 1.5$ h was identified as an outlier in the double determination of the IPCR by Henning-test [15]. For quantification, this outlier was ignored and the concentration was calculated from the remaining six ELOSA values.

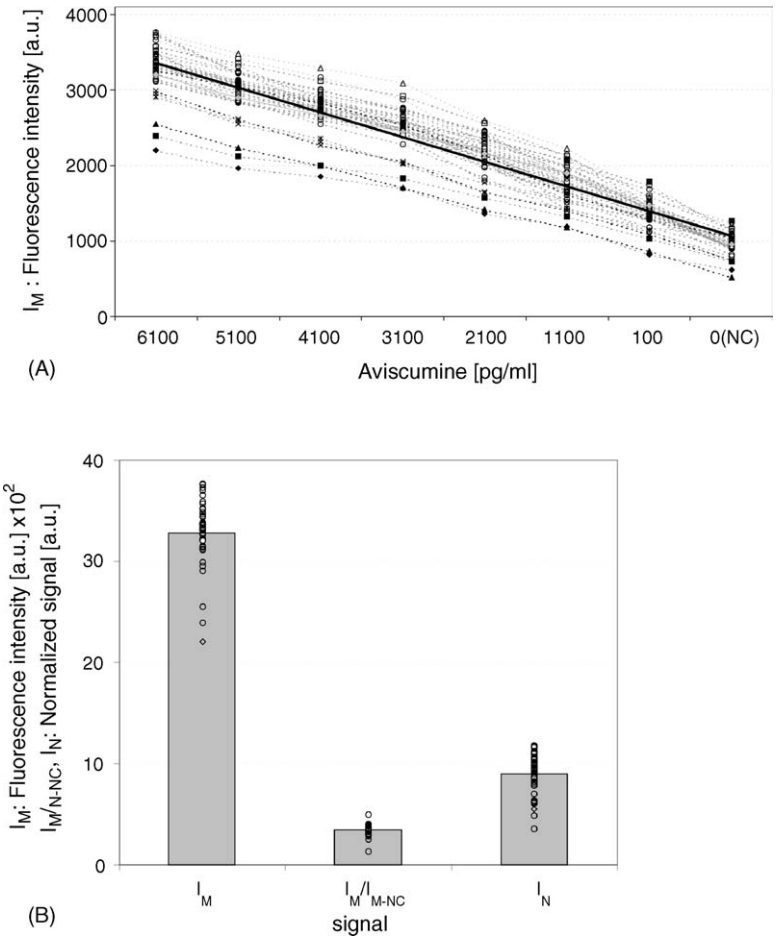

Fig. 4. (A) Overview of the absolute fluorescence intensities (I_M) obtained from the calibration curves for the marker-DNA for patients P #12–24 (see Table 1(c)) and linear regression of the average signal intensity (thick black line, $r^2 = 0.99$). Note that while the majority of the curves are similar in slope, larger differences occur between individual patients. Some patients show uniformly lower (e.g. P #18D1 and D11: black diamonds and squares; P #26D1: black triangles) or higher (e.g. P #14D1: white triangles, P #17D1: white squares) signals. For all patients, a good linear correlation between spiked Aviscumine concentration and signal intensity with an average correlation coefficient for linear regression at 0.97 was found. (B) Effect of the normalization using the"t0" plasma from the individual patients, exemplified by the signals obtained for 6100 pg/mL Aviscumine. Dots are indicating individual patient signals and bars indicate average values. While the absolute fluorescence signals (I_M, note the difference in scale) are largely scattering, the normalization with the t0-signals (I_M/I_{M-NC}, middle) leads to more homogenous distribution, however, with low intensity values. The signal strength is increased if one calculates the ratio of these values and the values measured for the internal competitor DNA, thus leading to the normalized ratio values I_N (right).

The average value of S_T was found at to be $9 \pm 5\%$. The S_T values obtained for the individual samples are shown in Fig. 5. Uniformly, the signals are well separated and display only a small and very homogenous error. No exceptional accumulation of high errors and/or increase/decrease in total error S_T was observed.

3.4. Assay specificity and robustness

A comparison of the average absolute signal intensities I_M obtained for patient negative control samples "t0" with the standardized B_{NC} revealed that the patient plasma negative controls yield a much higher average standard deviation of 18.5% than that observed for standardized human serum BISEKO (9.8% for B_{NC}, see also Fig. 3). Moreover, the control assays with BISEKO revealed a very homogenous performance during the complete time of the 3-year study. Only for three measurements (accompanying patients P #3d1, P #4d1, P #10d11 and P #11d1), higher values for B_{NC} of larger than 20% above the average B_{NC} were observed, whereby the value for P #10d11 was identified as a true outlier according to the Henning-test [15]. This particular sample also revealed unusual high signal intensities significantly above the average signal intensities of all the other samples. Except of this single outlier, the other values revealed a high homogeneity. This, again, underlines the robustness of the IPCR assay for quantifying the standardized control serum samples.

The inter-subject variability, i.e. variations occurring through the use of t0 samples as the matrix for the standard curve was determined as insignificant for quantification. In the entire study, samples from dose 1 and 11 were available from 22 individual patients. For the calculation of intra-patient variations, the signal intensities were measured for the zero plasma sample (t0, taken previous to the

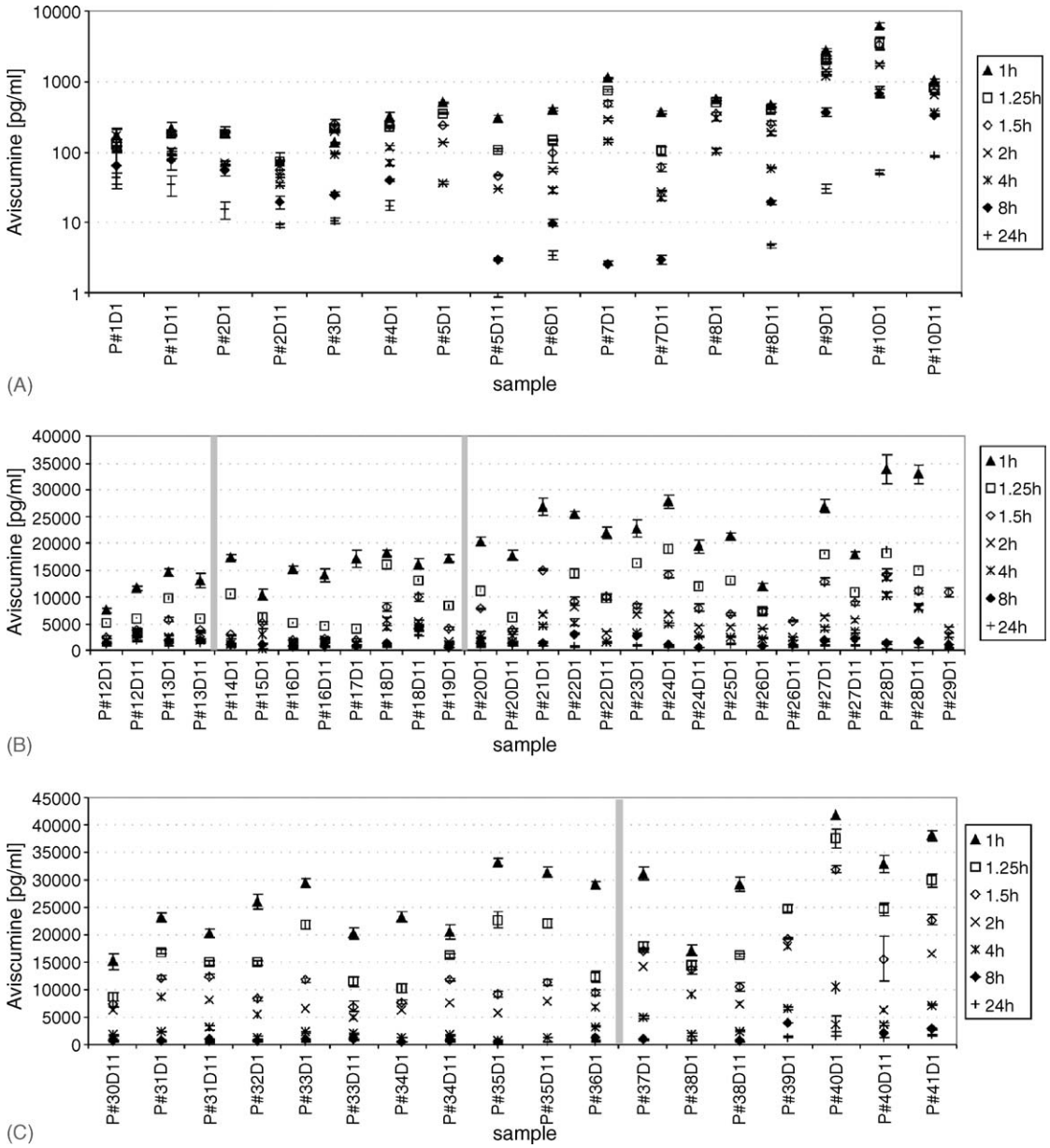

Fig. 5. Results of the quantification of Aviscumine in patient plasma samples, obtained by IPCR-quantification. (A) Patients P #1–10: Each patient received an increasing dose of Aviscumine (P #1: 10 ng/kg, P #2: 20 ng/kg, P #3: 40 ng/kg, P #4: 100 ng/kg, P #5: 200 ng/kg, P #6: 400 ng/kg, P #7: 800 ng/kg, P #8: 1600 ng/kg, P #9: 2400 ng/kg, P #10: 3200 ng/kg); (B) patients P #12–13: 3200 ng/kg, P #14–19: 4000 ng/kg, P20–29: 4800 ng/kg; (C) patients P #30–36: 5600 ng/kg, P #37–41: 6400 ng/kg). For several patients, two dose groups (dose 1: first treatment, "D1" and dose 11: eleventh treatment after day 39, "D11") were studied. The error bar represents the total error S_T, calculated as described in the text.

administration of Aviscumine) of both, dose 1 or 11. The data revealed an average error of about 3.3%, including the single outlier of patient #10.

Using the patient-specific individual calibration curves (Fig. 4), the initial differences between patient plasma samples were compensated during quantification. The high robustness of the Aviscumine IPCR of the patient plasma samples was also evident from the overall very good recovery rates observed in the spiked samples (see Table 1). In some patient samples, however, absolute I_M values 20% above (P #7, d1 and 11, P #10 d11, P #12, d1 and 11, P #14, d1) or below (P #7, d1 and 11, P #12, d1 and 11, P #14, d1) the average absolute signal intensities I_M were observed. Nonetheless, the corresponding calibration curves (see Fig. 4) permitted a quantification using the normalized I_N values without any aberration from the standard operating procedure. Notably, the hemolysis during citrate plasma preparation, which occurred in a few plasma samples, was also without any negative effects for IPCR quantification.

Exceptional high "t0" background signals were observed for P #11, dose 1 and, to a lesser extent, also for P #15, dose 1. There, the absolute fluorescence intensity I_M was higher than

10,000 a.u., as compared to an average signal of ca. 1,500 a.u. for all other "t0" negative control samples. These two data points were clearly identified as outliers. In both cases, the corresponding BISEKO controls B_{PC} and B_{NC} did not give untypical results, and therefore, we concluded that the results were due to an interfering patient plasma component. While quantification could be carried out for P #15 dose 1 with an increased average S_T error of 21 ± 8%, no quantification was possible for P #11. However, in retrospective comparison of the patients following the study, P #11 also revealed atypical fatigue grade 3 and died 17 days after this event [14], so that the anomalous plasma background of this patient in IPCR could possibly be linked to these conditions.

In conclusion, an overall high robustness and a good tolerance of the method against patient-specific variations was observed, with only a single exception out of 41 patients analyzed. Even in the case of exceptionally high or low background signals, the normalization to individual "t0" samples enabled us to carry out the valid quantification of Aviscumine concentration in the plasma.

3.5. Recovery and detection limit

With an average recovery R of 95 ± 19% for all spiked patient plasma samples (V_H, V_M, V_L) in the investigation (excluding P #11), the spike recovery values of the quantifications for each patient were found to be within a valid range. Generally, a stable and high recovery was found for the samples spiked with medium and high concentrations of Aviscumine (V_H and V_M) with an average R of 92 ± 9.8%. During the standardized routine protocol for P #12–41, a very narrow range of variations for the range of R was found with only a single R (P #15) larger than 9% than the average values of V_H and V_M. Comparison of the average recovery for each patient, the maxima were 83% (P #18D11) and 106% (P #12D1), deviating less than 15% from an average 98 ± 6%. These results additionally emphasize the small difference from an ideal R_R of 100%, and thus, confirm the valid quantification of the patient plasma samples.

Depending on the regression model used and the setup of the calibration curve, the average recovery of the samples spiked with low concentrations of Aviscumine (V_L) was found to be systematically lower in the case of the Harris regression and higher in the case of the linear regression models (see Table 1). While the average recovery of the lowest doses (V_L = 20 pg/mL) was found to be 107 ± 24% for P #9–10 using Harris regression, only 52 ± 22% average recovery of 20 pg/mL Aviscumine was found for P #1–8, using the Harris regression model. Because of these variations for the lowest concentrated samples, the quantification limit of Aviscumine detection in patient plasma by the IPCR assay was set at 100 pg/mL. This value, together with the upper detection limit, was further confirmed by a set of additional spiking samples and the recalculation of the spiked concentrations using various calibration curves. In particular, for 100 pg/ml Aviscumine (lowest concentration of the calibration curve), an average recovery of 105 ± 9.6% was determined, and for 6100 pg/ml (highest concentration of the calibration curve), an average recovery of 106.5 ± 2.1% was found using a linear regression.

3.6. Assay sensitivity

The lower limit of quantification was found to be limited by the low recovery rates of the V_L sample measured for P #1–11. As described above, the quantification limit was set to approx. 100 pg/mL Aviscumine in patient plasma. While lower concentrations of Aviscumine could clearly be detected, quantification was not carried out using the standardized protocol.

As summarized in Table 1(c), the spiking of patient plasma samples with defined amounts of Aviscumine, led to linear correlation between signals and spiked concentrations in the concentration range between 100–10,000 pg/mL.

While the non-linear quantification range of the IPCR significantly exceeded this window, a linear correlation between the signal and Aviscumine concentration was preferred for facilitating the standardization of the routine assay. During the course of the clinical trial study, increased amounts of Aviscumine were administered to the patients, thus, leading to higher plasma concentrations. In these cases, to avoid further adaptations of the calibration curves, samples from t = 1 h (C_{max}) to t = 2 h were routinely measured as both undiluted as well as diluted in "t0" patient plasma (see Section 2.4). The concentrations calculated from the diluted samples and the corresponding calibration curve are shown in Table 2(a). Comparison of these data to that calculated from the undiluted samples (shown in Table 2(b).) verifies that the values are not corrupted by the dilution step. Further confirmation of the valid concentrations by quantifying diluted samples and using linear calibration curves was achieved by the measurement of spiked samples with concentrations of 48, 24, 9 and 6 ng/mL Aviscumine. The first two samples were diluted 1:8, the latter ones 1:3. For the first two samples, a spike recovery of 97.5 ± 4.6 and 90.2 ± 5.2%, and for the latter two samples, a spike recovery of 95.4 ± 3.2 and 89.0 ± 7.6 was obtained.

The similar concentration measured for diluted and undiluted samples of several patients, as well as the spiking experiments clearly demonstrated that the dilution of higher concentrated samples did not affect the accuracy of the IPCR quantification.

The narrow range of Aviscumine concentrations ranging from 100 to 6100 pg/mL in the quantification curve was intentionally chosen to have a maximum correlation with the concentration of Aviscumine expected in patient plasma, and thus, to allow for the determination of the Aviscumine pharmacokinetics. Indeed, the majority of antigen concentrations during the timeframe monitored after administration of Aviscumine was found in the concentration range between 6100 and 100 pg/mL (see Fig. 5).

It should be emphasized, that the conventional corresponding ELISA, using the same antibodies, is approximately

Table 2
Recovery rates of Aviscumine concentrations higher than the concentration range covered by the calibration curve

Aviscumine sample $t = 1$ h	P #12–19 (dose: 3200–4000 ng/kg Aviscumine)	P #20–29 (dose: 4800 ng/kg Aviscumine)	P #30–41 (dose: 5600–6400 ng/kg Aviscumine
(a) Average concentration as calculated by diluted sample (ng/mL)	12.6	23.3	35.9
(b) Average concentration as calculated by undiluted sample (ng/mL)	14.8	23.8	26.7
Comparison of "a" and "b" (in percent "b")	117	102	74

The values were calculated from average values of $t = 1$ h (C_{max}) for selected patients by either (a) dilution of the sample in "t0" (1:3 f or P #12–18, 1:8 for P #19–41) or (b) by using the calibration curve and undiluted control samples.

1000–10,000-fold less sensitive for Aviscumine [7], and hence, did not permit the valid quantification of Aviscumine concentrations in this range.

3.7. Quantification results

Based on the results of the recovery measurements, the Aviscumine concentrations within the patient plasma samples were calculated using the recorded calibration curves (see Table 1 and Fig. 4) and the measured I_N values. An overview of the Aviscumine concentrations is shown in Fig. 5. Individual data suitable for kinetic calculation from the decrease in Aviscumine concentration during time was recorded for each patient (except P #11), no exceptional outlier were observed. The overall increase of the Aviscumine concentration during the study is clearly visible from the increase of the C_{max} (concentration measured after $t = 1$ h subsequent to administration) for different patient dose levels, shown in Fig. 6. In addition, the IPCR assay provided quantitative data which indicated the fast decrease of Aviscumine concentration in the plasma during the timeframe monitored ($t = 1$–24 h). Notably, only 13 out of 490 valid data points were below the quantification limit. The lower concentrations measured for late time points of the treatment cycle were also well accessible by the IPCR assay.

Based on these results, pharmacokinetic data were calculated and very recently reported by Schöffski et al. [14]. Briefly, the data revealed a uniformly short half-life of Aviscumine of 13 min as well as a linear kinetics on dose levels ≥1600 ng/kg. Fig. 6 shows the linear correlation between average C_{max} values found in two different dose groups (D1 and D11) and plasma concentration of Aviscumine measured by IPCR. Starting with P #9 (2400 ng/kg), a linear regression of the average concentrations revealed a correlation coefficient of 0.98.

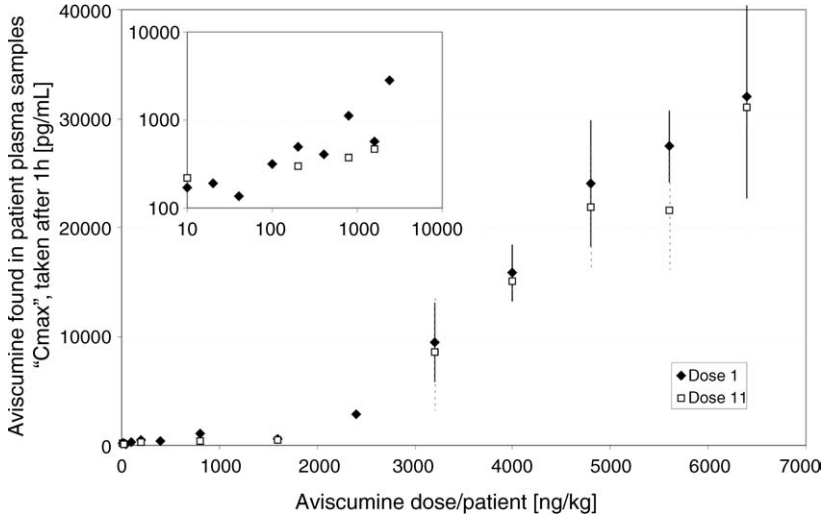

Fig. 6. Correlation between Aviscumine dose levels and concentrations in plasma as quantified by IPCR in the sample taken 1h after administration. For 10–2400 ng/kg Aviscumine, only 1 patient (P #1–9, respectively) was studied for each dose. Starting with 3200 ng/kg, cohorts ranging between 4 and 10 individuals (3200 ng/kg: P #10–P #13, 4000 ng/kg: P #14–19, 4800 ng/kg: P #20–29, 5600 ng #/kg; P #30–36 and 6400 ng/kg: P #37–41) were treated with the same dose. The figure shows average concentrations for these patients as calculated from samples of the first (dose 1, black diamonds) and a later treatment cycle (dose 11 after day 39, white squares). The inset shows the magnification of the 10–2400 ng/kg concentration range. Note the linear relation between the administered dose and concentration measured in plasma for both curves [14].

4. Conclusions

This work describes the development and application of an IPCR assay for the routine analysis of a large number of plasma samples in the context of a clinical trial study for the development of the cytostatic drug Aviscumine. To adjust a previously reported IPCR assay [7], some minor alterations were carried out which included the adaptation of the calibration curves and the handling of the plasma samples. The optimized IPCR assay allowed the quantification of the Aviscumine directly from plasma samples without any cumbersome and time-intensive work-up and purification steps. The IPCR assay proved to be very sensitive, highly robust and well suited for quantification of Aviscumine concentrations in the studied range of 100 to approx. 40,000 pg/mL within the patient plasma samples. The assay revealed a very good recovery and precision, thus providing valuable data for the generation of pharmacokinetic parameters. For instance, the IPCR assay revealed a linear relationship between the administered dose and the maximum Aviscumine concentration C_{max} found in the blood plasma taken 1 h after application of the drug (Fig. 6). Moreover, the Aviscumine half-life in plasma could be calculated [14], which is significant for further evaluation of this novel drug.

To the best of our knowledge, this is the first application of the IPCR method in a routine clinical study. The large amount of statistical data collected during this work underlines the enormous potential of the IPCR as an ELISA-enhancing detection tool. We have already demonstrated that the larger amount of time, necessary to perform an IPCR assay rather than a conventional ELISA, can be dramatically reduced by using real-time PCR technology and that this instrumental modification also further increases the sensitivity and robustness of the IPCR [16]. Hence, we anticipate that the IPCR method will not only enhance the performance of many clinical assays already established on the basis of ELISA, but will also open up entirely new fields in diagnostics and quality control.

References

[1] C.P. Price, D.J. Newman (Eds.), Principles and Practise of Immunoassay, second ed., Macmillan Reference Ltd., London, 1997.
[2] T. Sano, C.L. Smith, C.R. Cantor, Science 258 (1992) 120–122.
[3] C.M. Niemeyer, M. Adler, B. Pignataro, S. Lenhert, S. Gao, L. Chi, H. Fuchs, D. Blohm, Nucleic Acids Res. 27 (1999) 4553–4561.
[4] C.M. Niemeyer, M. Adler, D. Blohm, Anal. Biochem. 246 (1997) 140–145.
[5] S. Schiavo, A. El-Shafey, N. Jordan, C. Orazine, Y. Wang, X. Zhou, N.H. Chiu, I.S. Krull, PharmaGenomics (January 2004) 36–44.
[6] M. Adler, in: G.S. Makowski (Ed.), Advances in Clinical Chemistry, vol. 39, Elsevier, Oxford, 2005 (chapter 9) (in press).
[7] M. Adler, M. Langer, K. Witthohn, J. Eck, D. Blohm, C.M. Niemeyer, Biochem. Biophys. Res. Commun. 300 (2003) 757–763.
[8] J. Eck, M. Langer, B. Mockel, A. Baur, M. Rothe, H. Zinke, H. Lentzen, Eur. J. Biochem. 264 (1999) 775–784.
[9] J. Eck, M. Langer, B. Mockel, K. Witthohn, H. Zinke, H. Lentzen, Eur. J. Biochem. 265 (1999) 788–797.
[10] U. Schumacher, S. Feldhaus, U. Mengs, Cancer Lett. 150 (2000) 171–175.
[11] B. Schaffrath, U. Mengs, T. Schwarz, R.D. Hilgers, J. Beuth, B. Mockel, H. Lentzen, B. Gerstmayer, Anticancer Res. 21 (2001) 3981–3987.
[12] U. Elsasser-Beile, T. Ruhnau, N. Freudenberg, U. Wetterauer, U. Mengs, Cancer 91 (2001) 998–1004.
[13] M. Adler, C.M. Niemeyer, K. Witthohn, M. Langer, D. Blohm, Biotechnology 2000 Congress & Exhibition Poster, Berlin, 2000 (Abstracts) (available in Biotechnology 2000, Book of Abstracts, vol. 3, 96–97, 2000; and Biol. Chem., vol. 381, Special Supplement, S178, 2000).
[14] P. Schöffski, S. Riggert, P. Fumoleau, M. Campone, O. Bolte, S. Marreaud, d. Lacombe, B. Baron, H. Zwierzina, K. Wilhelm-Ogunbiyi, H. Lentzen, C. Twelves, Ann. Oncol. 15 (2004) 1816–1824.
[15] S. Kromidas, Handbuch der Validierung in der Analytik, Wiley-VCH, Weinheim, 2000.
[16] M. Adler, R. Wacker, C.M. Niemeyer, Biochem. Biophys. Res. Commun. 308 (2003) 240–250.