*International Journal of Pharmaceutics*, 82 (1992) 157–164

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IJP 02706

**Research Papers**

# Comparative assay of amoxicillin by high-performance liquid chromatography and microbiological methods for pharmacokinetic studies in calves

Pierre Leroy [a], Catherine Gavriloff [a], Alain Nicolas [a], Philippe Archimbault [b] and Germaine Ambroggi [b]

[a] *Laboratoire de Chimie Analytique, URA CNRS 597, Faculté des Sciences Pharmaceutiques et Biologiques, B.P. 403, 54001 Nancy Cedex (France) and* [b] *Virbac S.A., B.P. 27, 06511 Carros Cedex (France)*

(Received 20 September 1991)
(Accepted 15 November 1991)

*Key words*: Amoxicillin; HPLC; Microbiological assay; Pharmacokinetics; Statistical comparison

## Summary

An HPLC method and a microbiological assay developed for measuring amoxicillin in calf plasma were used in pharmacokinetic studies of the β-lactam antibiotic following intramuscular administration of the test compound. Collected samples were concomitantly assayed by the two methods. The HPLC technique included protein precipitation of plasma samples with trichloroacetic acid, isocratic elution on a reversed-phase column (LiChrospher RP 18 end-capped, 5 μm) and fluorimetric detection after post-column derivatization with fluorescamine. The microbiological agar gel diffusion method used *Sarcina lutea* ATCC 9341 as the test organism, paper disks being impregnated with plasma followed by 1 h diffusion and 16 h incubation. Both methods show full selectivity with regard to endogenous compounds and a coadministered antibiotic (i.e. colistin). A similar limit of quantitation (0.1 μg of amoxicillin per ml of plasma) was attained with good reproducibility (relative standard deviation values were, respectively, 1.4 and 7.3% for HPLC and microbiological techniques). Linearity was in the range of 0.1–5 μg $ml^{-1}$ (HPLC) and 0.1–1.6 μg $ml^{-1}$ (microbiological assay). Results obtained by HPLC and the microbiological assay were compared by a statistical evaluation test which showed a good correlation between the two methods.

## Introduction

There has recently been a move to replace biological assays of antibiotics by chemical methods and in particular by high-performance liquid chromatography (HPLC). It has been shown in several studies that comparable results can be obtained by HPLC and bioassays for the measurement of various classes of antibiotics, e.g., β-lactams (Kedzierewicz et al., 1989), tetracyclines (Ray and Harris, 1989), peptides (Pavli and Sokolic, 1990), glycopeptides (Georgopoulos et al., 1989) and aminoglycosides (Sekkat et al., 1989), manufacturing process and quality control in pure product batches (Ray and Harris, 1989; Sekkat et al., 1989; Pavli and Sokolic, 1990), stability under various storage conditions (Kedzierewicz et al., 1989; Ray and Harris, 1989; Pavli

*Correspondence:* A. Nicolas, Laboratoire de Chimie Analytique, URA CNRS 597, Faculté des Sciences Pharmaceutiques et Biologiques, B.P. 403, 54001 Nancy Cedex, France.

and Sokolic, 1990), and monitoring in biological fluids (Georgopoulos et al., 1989). The final choice of the technique depends upon criteria such as sensitivity, accuracy, reproducibility and selectivity vs degradation products, metabolites and other related antibiotics. Other aspects of performance can also be compared: simplicity, rapidity, cost and automation in relation to the workload. All these factors in the replacement of microbiological assays by chromatographic techniques have been extensively discussed (Thomas, 1987; Vanderhaeghe, 1989; Horton, 1990). In this report, we have developed a simple and automated HPLC method for the measurement of amoxicillin in calf plasma samples following intramuscular injection and have compared its efficiency to a classic microbiological assay.

## Materials and Methods

*Reagents, chemicals and standards*

All chemicals and solvents used were of analytical reagent grade. Amoxicillin trihydrate, in compliance with the requirements of the European Pharmacopoeia, was obtained from Virbac S.A. Laboratories (Carros, France). For the HPLC assay, a stock solution was prepared each day at a concentration of 0.50 mg $ml^{-1}$ in 0.01 M phosphate buffer pH 7.2; it was diluted in the same buffer to obtain solutions at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 $\mu g$ $ml^{-1}$ to determine the calibration curves. For the microbiological assay, a stock solution was prepared at a concentration of 1.6 mg $ml^{-1}$, diluted to 32 $\mu g$ $ml^{-1}$ with double-distilled water and further diluted with blank pooled plasma to give final concentrations of 0.1, 0.2, 0.4, 0.8 and 1.6 $\mu g$ $ml^{-1}$. In both assays, diluted solutions were stored at 5°C until analysis.

The pharmaceutical form was an oily suspension of amoxicillin (100 mg $ml^{-1}$) which also contained colistin sulfate.

Fluorescamine was purchased from Sigma (St Louis, MO, U.S.A.); a 0.2 mg $ml^{-1}$ solution was prepared in anhydrous acetonitrile, stored at −20°C for up to 1 month and degassed for 5 min in an ultrasonic bath before use.

*Dose administration and sample collection*

Six Abondance-Montbeliard cross-bred calves weighing between 71 and 89 kg and aged 8–9 weeks were selected for this study. They were housed in stables and fed with a milk replacer twice daily. Administration of the oily suspension of amoxicillin to animals was by intramuscular injection in the neck. They received a 0.1 ml injection per kg every 24 h for 3 days at a single site. Blood samples from the jugular vein were collected into EDTA $K_3$ Vacutainer tubes (Becton Dickinson, Grenoble, France) at the following intervals: 0.5, 1, 2, 3, 4, 5, 7, 9, 12 and 24 h after the first injection; 1, 2, 3, 4 and 24 h after the second injection and 0.5, 1, 2, 3, 5, 7, 9, 12, 24 and 36 h after the third injection. They were immediately centrifuged and plasma samples were stored at −80°C until analysis was performed.

*Preparation of plasma samples for HPLC assay*

The following operations were performed using 5-ml screw-capped glass tubes: 0.1 ml of either the amoxicillin standard solutions (for calibration curves) or $10^{-2}$ M phosphate buffer pH 7.2 (for blank and unknown samples) and 0.5 ml of a 10% (w/v) trichloroacetic acid solution were added to 1 ml of plasma. After vortex-mixing for 15 s, samples were immediately centrifuged at $1800 \times g$ for 10 min at 5°C. 500 $\mu l$ of supernatant was mixed with an equal volume of a 0.5 M $Na_2HPO_4$ solution adjusted to pH 6.5 with citric acid. The resulting solutions could be kept at room temperature for up to 4 h before injection onto the HPLC system. Solid-phase extraction was performed on Sep-pak C-18 cartridges (Millipore, Milford, MA, U.S.A.).

*Chromatographic equipment and operating conditions*

The HPLC system consisted of a double reciprocal piston pump with a pulse damper (model Spectroflow 400, Applied Biosystems, Foster City, CA, U.S.A.), an autosampler (model SP 8775, Spectra-physics, Les Ulis, France) equipped with a 50 $\mu l$ sample loop, and a column oven (model Croco-Cil, Spectra-Physics, Les Ulis, France). An analytical column (125 × 4 mm i.d.), prefilled with LiChrospher RP 18 end-capped (5 $\mu m$) and pro-

tected by a guard column (4 × 4 mm i.d.) prepacked with the same stationary phase, was eluted with methanol/$10^{-2}$ M phosphate buffer pH 7.2 (7.5:92.5, v/v) at a flow rate of 1.5 ml $min^{-1}$. The guard and analytical columns were heated at 35°C during analysis. The outlet of the analytical column was connected to a three-way post-column reagent delivery pump (model Hitachi 655 A-13, Merck-Clévenot, Nogent-sur-Marne, France). A PTFE knitted open tubular (KNOT) reactor (3 m × 0.5 mm i.d., Supelco, St-Germain-en-Laye, France) was used to perform the post-column reaction at ambient temperature. The fluorescamine solution was introduced into the effluent at a flow rate of 0.2 ml $min^{-1}$. The derivative was monitored by fluorimetry (model Spectroflow 980, Applied Biosystems) with excitation at 385 nm and emission selected by a long-pass filter at 415 nm. Some experiments were carried out with direct UV detection at 225 nm (model LMC 313, Merck-Clévenot). Chromatograms were recorded and peak-area were measured with a data station (Winner, Spectra-Physics).

*Microbiological assay*

The stock suspension of the test organism *Sarcina lutea* ATCC 9341 was diluted in isotonic sodium chloride solution so that the transmittance at 550 nm in a 1 cm cell averaged 75%. After further 1:5 dilution in isotonic sodium chloride solution, 0.5 ml of the inoculum was mixed with 125 ml of culture medium No. 11 from Difco. 18 ml of the harvested suspension was distributed into Petri dishes (120 × 120 × 17 mm). Paper disks (6 mm diameter) were impregnated with 20 µl of unknown plasma samples, blank plasma or pooled plasma spiked with amoxicillin standard solution, dried for 30 min and put on the gelose surface. After 1 h diffusion at room temperature and 16 h incubation at 37°C, inhibition zones were measured with a precision of 0.5 mm.

*Statistical comparison of HPLC and microbiological results*

Amoxicillin concentrations determined by HPLC and microbiological methods were compared by calculating the regression line. To confirm that there was no significant deviation from unit slope and zero intercept, the ellipse which has a probability of 99% to include the real values $A$ and $B$ (respectively, the slope and intercept of the regression line) was calculated. The corresponding equation was as follows (CETEMA, 1986):

$$N(B-b)^2 + 2(A-a)(B-b)\Sigma x_i + (A-a)^2\Sigma x_i^2 = 2s^2F_p \quad (1)$$

where $N$ denotes the number of couples of values $(x_i, y_i)$ in the data set, $s^2$ is the estimation of the variance, $F_p$ represents the value of the variable $F$ of Snedecor with $\nu_1 = 2$ and $\nu_2 = N - 2$ degrees of freedom to obtain the probability: $F < F_p$ equal to $P$, $a$ is the value of the slope of the calculated regression line ($y$-axis), $b$ is the value of the intercept of the calculated regression line ($x$-axis) and $A$ and $B$ are real variables.

## Results and Discussion

*Optimization and validation of HPLC assay*

The HPLC analysis of amoxicillin in biological fluids requires a simple handling procedure combined with selective and sensitive detection to permit a quantitation limit of 0.1 µg $ml^{-1}$. This lowest concentration value of the linearity range meets the requirements of the present pharmacokinetic studies. Amoxicillin has amphoteric properties ($pK_a$ = 2.4 and 7.2) (Tsuji et al., 1979), thus precluding liquid-liquid extraction. Therefore, previously reported procedures of plasma preparation to measure amoxicillin by HPLC used either solid-phase extraction (SPE) (Lee and Brooks, 1984) or protein precipitation with acidic solutions (Carlqvist and Westerlund, 1985).

An SPE technique combined with UV detection had a quantitation limit of 0.5 µg $ml^{-1}$ in plasma (Lee and Brooks, 1984). Other detection modes had therefore to be considered in order to lower this value. Amperometry has been tried out as an alternative; based upon the electro-oxida-

tion of the phenolic group of amoxicillin, the high potential applied to the glassy carbon electrode generates many interferences from biological matrices (Brooks et al., 1981). Post-column coulometric oxidation of amoxicillin followed by fluorimetric detection has been recently proposed as a highly sensitive technique (Mascher and Kikuta, 1990). *o*-Phthaldialdehyde has also been used as a post-column fluorigenic reagent for $\beta$-lactam antibiotics with a primary amino group, but did not result in rapid condensation at room temperature in an alkaline medium (Rogers et al., 1983). Thus, fluorescamine is still the major fluorigenic reagent involved for derivatization of amoxicillin (Lee et al., 1979; Carlqvist and Westerlund, 1985) and various cephalosporins (Blanchin et al., 1987). Such a detection mode incorporated in a two-column switching system was claimed to afford a quantitation limit of 50 ng $ml^{-1}$ in plasma (Carlqvist and Westerlund, 1985).

The aim of our initial tests was to choose between UV and fluorimetric detection. Since protein precipitation and final UV detection could not measure amoxicillin in plasma at concentrations below 0.5 $\mu$g $ml^{-1}$ due to a lack of selectivity, SPE with a C-18 cartridge was investigated as previously described (Lee and Brooks, 1984). Amoxicillin recovery decreased from 92.5 to 85.0% in the concentration range 1–0.2 $\mu$g $ml^{-1}$, indicating the poor accuracy of the method and the need of an internal standard for its improvement.

The procedure finally adopted combined protein precipitation as a single purification step of plasma samples and post-column derivatization with fluorescamine. The results obtained for both selectivity and sensitivity were very satisfactory without the requirement of a column switching system as described earlier (Carlqvist and Westerlund, 1985).

Trichloroacetic acid, rather than acetonitrile or ethanol, was chosen to precipitate proteins, because of the lower dilution factor and better amoxicillin recovery (higher than 90% with trichloroacetic acid and less than 60% with organic solvents). However, amoxicillin is unstable in acidic media. The pH must be adjusted close to its isoelectric point (4.8) in order to prevent analyte loss in supernatants before HPLC analysis and to enable full automation with an autosampler injector (Carlqvist and Westerlund, 1985). The isocratic elution of amoxicillin on a hydrophobic reversed-phase column has been optimized in the following manner: buffering at pH 7.2 resulted in rapid condensation of the analyte with fluorescamine without further post-column base addition, and greater durability of the silica-based column; no ion pairing agent was necessary to increase the capacity factor of the analyte and the methanol content was such as to give a short retention time for amoxicillin (less than 10 min). Furthermore, no late eluting peak appeared; this permitted a high frequency in sample analysis (5–6 per h).

Derivatization and detection conditions were similar to those previously elaborated (Carlqvist and Westerlund, 1985). The peak of amoxicillin showed no significant broadening compared with direct UV detection when a slow flow rate for fluorescamine addition was used with an open tubular reactor in a knitted configuration.

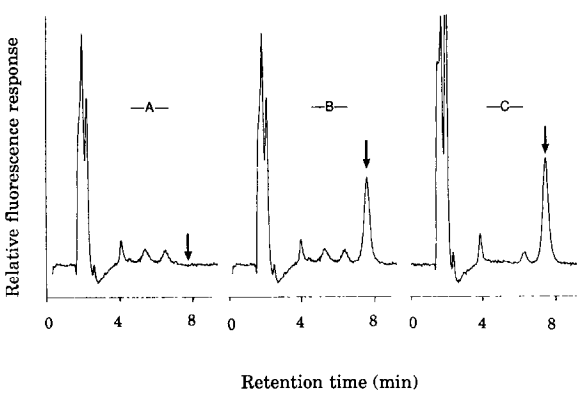


Fig. 1. Typical chromatograms obtained from calf plasma samples after protein precipitation with trichloroacetic acid. The column (LiChrospher RP 18 end-capped (5 $\mu$M) 125×4 mm i.d.) was eluted at 35°C with methanol-10 mM phosphate buffer pH 7.2 (7.5:92.5, v/v) at a flow rate of 1.5 ml $min^{-1}$. Fluorimetric detection ($\lambda_{ex}$ = 385 nm; $\lambda_{em}$ > 415 nm) followed post-column derivatization with fluorescamine (0.2 mg $ml^{-1}$ in acetonitrile; 0.2 ml $min^{-1}$ inflow). (A) Blank; (B) spiked with 1 $\mu$g $ml^{-1}$ of amoxicillin; (C) 8 h after the first intramuscular administration of amoxicillin suspension to calves.

Selectivity of the technique was tested on a coadministered antibiotic, colistin, and various β-lactam antibiotics (ampicillin, cephalexin, cephadrin and cloxacillin): no chromatographic interference occurred. The limit of detection was examined in spiked plasma and reached 30 ng $ml^{-1}$ for a signal-to-noise ratio of 3. This value was improved by a factor of 5 by lowering the excitation wavelength from 385 to 265 nm, since the deuterium source of the detector used in this study supplied higher energy at this value. This fact could be of a real interest for further studies needing measurements at lower concentrations than in the present work, such as in investigation of amoxicillin residues in edible tissues. Typical chromatograms are shown in Fig. 1 and major validation patterns of the HPLC assay are detailed in Tables 1 and 2. Excellent interassay reproducibility was achieved for each concentration value tested (ranging from 0.45 to 1.43%) and interday variability did not exceed 5%.

*Development and validation of microbiological assay*

No extraction procedure of amoxicillin or purification step of plasma samples was necessary for the microbiological assay. Direct impregnation of paper disks by the biological fluid and placing on the gelose surface once dry do not lead to interference due to the initial matrix (no inhibition zone resulted with a blank plasma). Furthermore, full selectivity was achieved even at concentrations greater than 400 IU $ml^{-1}$ of colistin. Quantitation took place when dilutions of standard solution were prepared in drug-free plasma. Since linearity was only observed over a small concentration range (0.1–1.6 μg $ml^{-1}$), unknown samples with concentrations exceeding the maximum value must be diluted in drug-free plasma before performing another analysis. Validation patterns of this bioassay are detailed in Tables 2 and 3.

*Comparison of plasma amoxicillin concentrations found by HPLC and microbiological assays*

To our knowledge, no comparative study of HPLC and microbiological methods to measure β-lactam antibiotics in biological fluids has been reported previously. In earlier papers on this subject, the techniques developed have been applied to quality or stability control of pharmaceutical dosage forms (Kedzierewicz et al., 1989).

TABLE 1

*Accuracy, reproducibility and recovery results of the HPLC assay of amoxicillin in calf plasma*

| Concentration of amoxicillin in plasma (μg $ml^{-1}$) | | Relative standard deviation (%) | Recovery [a] (%) |
|---|---|---|---|
| Theoretical | Experimental [a] | | |
| 0.00 | 0.00 | – | – |
| 0.10 | 0.13 ± 0.00 | 1.43 | 90.9 ± 1.3 |
| 0.20 | 0.20 ± 0.00 | 1.01 | 92.3 ± 0.9 |
| 0.50 | 0.50 ± 0.00 | 0.45 | 99.6 ± 0.45 |
| 1.00 | 0.99 ± 0.01 | 0.96 | 98.5 ± 0.95 |
| 2.00 | 1.96 ± 0.01 | 0.61 | 100.1 ± 0.61 |
| 5.00 | 5.02 ± 0.04 | 0.91 | 94.0 ± 0.85 |

[a] Mean ($n = 8$) ± S.D.

TABLE 2

*Accuracy and reproducibility obtained for the microbiological assay of amoxicillin in calf plasma*

| Concentrations of amoxicillin (μg $ml^{-1}$) in plasma | | Relative standard deviation (%) |
|---|---|---|
| Theoretical | Experimental [a] | |
| 0.00 | 0.00 | – |
| 0.10 | 0.10 ± 0.01 | 7.26 |
| 0.20 | 0.19 ± 0.02 | 7.90 |
| 0.40 | 0.40 ± 0.02 | 4.40 |
| 0.80 | 0.86 ± 0.06 | 6.84 |
| 1.60 | 1.54 ± 0.15 | 9.84 |

[a] Mean ($n = 10$) ± S.D.

TABLE 3

*Regression equations and correlation coefficients of calibration graphs obtained for the HPLC and the microbiological assays of amoxicillin in spiked plasma samples*

| Assay | Equation of regression line | Correlation coefficient | Linearity range [a] |
|---|---|---|---|
| Microbiological | $y$ [b] = 12.36 log $x$ [a] + 23.87 | 0.999 | 0.1–1.6 |
| HPLC | $y$ [c] = 1160581 $x$ [a] − 31198 | 1.000 | 0.1–5.0 |

[a] Amoxicillin concentrations in μg $ml^{-1}$.
[b] Diameter of inhibition zone (in mm).
[c] Peak area (in arbitrary units).

Plasma concentrations of amoxicillin over time resulting from microbiological and HPLC assays in the pharmacokinetic studies in the calf are represented in Fig. 2. Fig. 3 depicts the correlation of these results; there is a linear correlation between the two methods over the plasma concentration range found. The fitted curve can be expressed by the equation $y = 0.965x + 0.023$ ($n = 144$; $r = 0.989$) where $x$ are the bioassay values and $y$ the HPLC values. In many comparative studies in this field, only a correlation coefficient has been calculated to represent the random error. However, a full comparison between two methods needs to confirm that there is (i) no proportional difference between the methods, i.e. the slope is equal to unity; and (ii) no constant error between the methods, i.e. the intercept is equal to zero. If both conditions are fulfilled, differences observed between the two methods result only from the variability of measurements. A simple way of testing that there is no significant deviation from unit slope and zero intercept is to calculate the ellipse according to Eqn 1. Results of the corresponding curve calculated are shown in Fig. 4. The hypotheses are confirmed since the point of the coordinates (slope = 1, intercept = 0) is localized inside the ellipse.

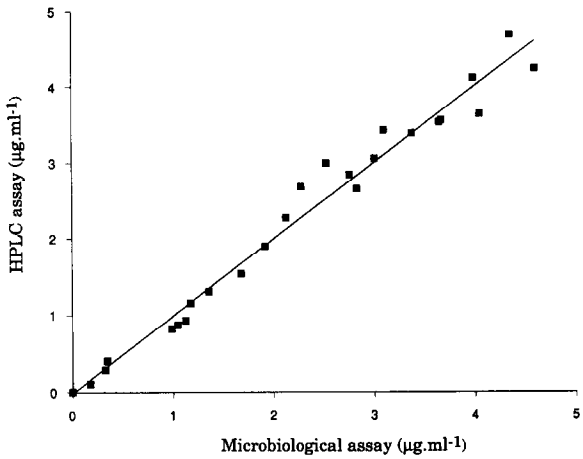


Fig. 3. Comparison of amoxicillin concentrations in calf plasma in the pharmacokinetic studies between the microbiological and HPLC methods. Data are expressed in $\mu g\ ml^{-1}$. The equation of the regression line is $y = 0.965x + 0.023$ ($n = 144$; $r = 0.989$).

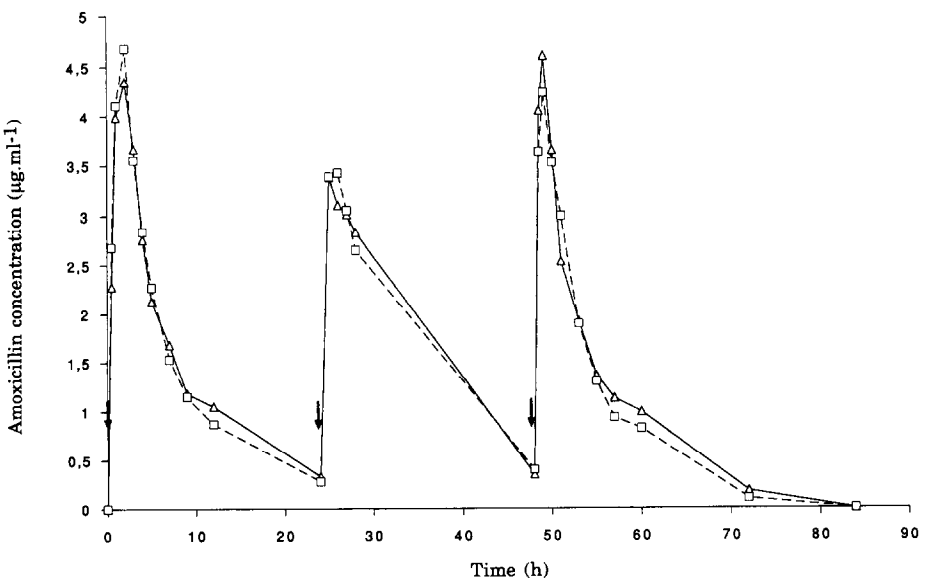


Fig. 2. Concentrations of amoxicillin in calf plasma determined by microbiological (△) and HPLC (□) assays in the pharmacokinetic studies. Data at each time interval represent the mean values of measurements taken from six different calves treated in the same conditions. For drug administration and plasma sampling conditions, see Materials and Methods. Arrows indicate times of the successive intramuscular injections.

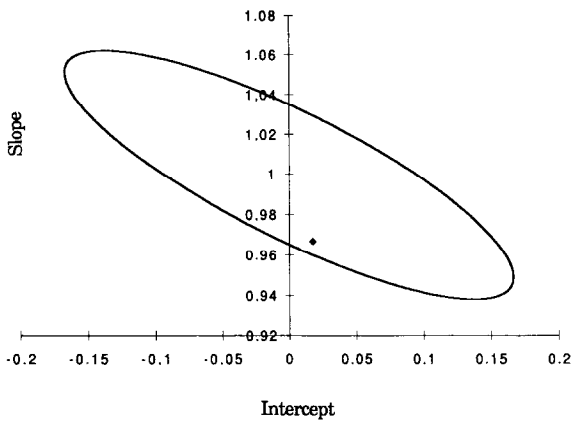


Fig. 4. Confidence ellipse with probability $P = 99\%$ to confirm the hypothesis that slope ($y$-axis) and intercept ($x$-axis) of the regression line are not significantly different from 1 to 0, respectively. (♦) Represents the point of $x$ and $y$ coordinates equal to real values of the regression line shown in Fig. 3.

Other parameters may further develop the comparison of the two methods. The HPLC method displays a better linearity range (0.1–5 $\mu$g ml$^{-1}$) and interassay reproducibility (RSD of 0.45–1.43%) than the microbiological technique with respective values of 0.1–1.6 $\mu$g ml$^{-1}$ and 4.40–9.84%. In the bioassay, the overall preparation and incubation period is approx. 18 h and data are available within 2 days. Furthermore, other $\beta$-lactam antibiotics, regardless of possible differences in sensitivity to *S. lutea,* may interfere with the microbiological assay since it is not specific for amoxicillin. The HPLC method, however, rapidly determines amoxicillin in calf plasma since the analysis results are ready within about 30 min. With the exception of protein precipitation, all steps in analysis and data evaluation are fully automated.

The final choice between the two methods is nevertheless difficult to make, since both assays meet the requirements concerning selectivity and the limit of quantitation. The versatility of each technique will, in the future, be an important point to evaluate. The efficiency of the described HPLC assay has already demonstrated in our laboratory through measuring different cephalosporins, e.g. cephalexin, which requires only a short development time and very few modifications in the operating conditions.

## Acknowledgements

The authors are grateful to Florence Sar for valuable aid in the preparation of the manuscript and Etienne Klein for statistical calculations. This work was supported by Virbac S.A. Laboratories.

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