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Determination of penicillin in pharmaceutical formulations by flow injection analysis using an optimised immobilised penicillinase reactor and iodometric detection

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Abstract: An automated assay for the determination of penicillin in formulations suitable for use in pharmaceutical quality control is presented. The method is based on the classical iodometric penicillin assay which is incorporated in a flow injection analysis (FIA) system. The required hydrolysis is performed on-line by using an immobilised penicillinase reactor. Packed-bed and single-bead-string enzyme reactors are compared. It turns out that a packed-bed penicillinase reactor (10 cm × 1.5 mm i.d.) provides complete hydrolysis within short residence time, while only little back-pressure is generated. This enzyme reactor is stable for at least 9 months. Enzymatic hydrolysis of the β-lactam ring results in the formation of the corresponding penicilloic acid, which consumes iodine. The iodine consumption is determined colorimetrically by measuring the decrease of the absorbance of the blue coloured iodine/starch complex. The optimum reactor length and flow rate for the colourimetrical detection reaction are determined. The optimised method is applied to the assay of penicillin in formulations and the results are compared with the "true" results obtained with a reference method: a mercurimetric titration. The reliability of the flow injection method is evaluated quantitatively by determining the maximum total error (MTE). The reliability is shown to be highest when measuring at a 0.3-mM level. Eight formulations including capsules, tablets and injectables containing penicillin G, amoxicillin or flucloxacillin are assayed. The MTE does not exceed the 6% level and the most probable MTE is between 1.5 and 3.5%.

Keywords: *Flow injection analysis; penicillin; immobilised penicillinase reactor; iodometric detection; pharmaceutical formulations.*

Introduction

The antibacterial drug penicillin and various closely related (semi-)synthetic compounds are in widespread use nowadays. Many analytical procedures for the determination of these penicillins in pharmaceutical formulations, in fermentation broths and in biological fluids have been developed. These assays including titrimetric, spectrophotometric, chromatographic and microbiological methods, have been reviewed recently by Van Krimpen *et al.* [1].

Flow injection analysis (FIA) is characterized by a high sample throughput and can easily be automated. These features make the technique well suited for use in quality control. Surprisingly, among the large number of penicillin assays relatively few FIA methods have been reported [2–7]. All of them include enzymatic hydrolysis of the native penicillin to the corresponding penicilloic acid (Fig. 1) using immobilised [2–6] or dissolved penicillinase [7]. Immobilised enzymes are preferred because they can be reused and often possess a higher long-term stability. The joint use of FIA and immobilised enzymes is rapidly growing [8] and different types of reactors have been reported [9, 10]. Most reported FIA methods used direct determination of the enzymatically hydrolysed penicillin [2–6]. Direct detection was based on measurement of the reaction enthalpy [2] or on monitoring the pH-change due to the penicilloic acid formation [3–6]. However, care has to be taken with pH-measurements because the pH, despite being dependent on the concentration of the liberated acid, is also influenced by the nature and the strength of the applied buffer system. Indirect detection, e.g. colorimetric detection

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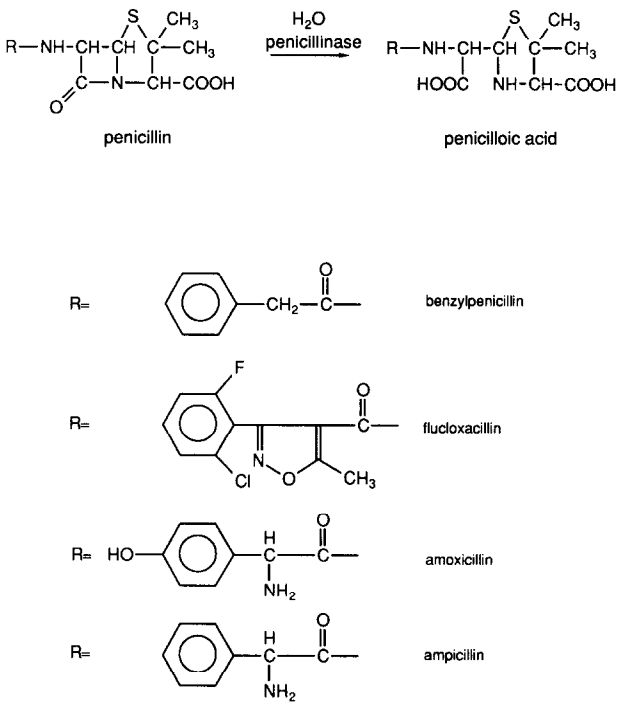

Figure 1
The enzymatic hydrolysis of penicillin and the structures of the investigated penicillins.

after performing a suitable colour reaction with the penicilloic acid, may improve the selectivity of the assay. Schneider [7] described an FIA system with colorimetric detection based on the formation of molybdenum blue by penicilloic acid in the presence of mercury(II) chloride. This reaction, however, is performed at 45°C leading to the formation of gas bubbles, which have to be removed prior to detection.

An alternative approach to determine penicilloic acid is the iodometric detection principle, which is based on the consumption of iodine by penicilloic acid. This principle was described in 1946 by Alicino [11] in an iodometric titration assay. Later on, iodometric detection was incorporated in a chromatographic method [12] and in (semi-)automated air-segmented flow analysis (ASFA) methods [13–16]. Until now it has not been applied in any FIA method. The main difference between ASFA and FIA is the absence of air-segmentation in the latter resulting in simplified flow-systems and enabling the use of immobilised enzyme reactors. Furthermore, due to controlled and reproducible timing in FIA, non-steady state measurements can be performed providing short residence times and hence, a high sample throughput.

The present FIA method involves on-line hydrolysis of penicillin with an immobilised enzyme reactor and iodometric detection of the produced penicilloic acid. The iodine consumption is colorimetrically detected by measuring the decrease of the absorbance of an iodine/starch complex. The system has been optimised using low back-pressure, high sensitivity and high sample throughput as the optimisation criteria. Packed-bed enzyme reactors (PBERs) and single-bead-string enzyme reactors (SBSERs) have been tested. The influence of flow rate and reactor length on peak height and peak width have been studied for the iodometric reaction. Finally, the optimised FIA method has been applied to the determination of penicillin (amoxicillin, ampicillin, benzylpenicillin or flucloxacillin) in various pharmaceutical formulations (tablets, capsules and injectables). The reliability of the proposed FIA method is discussed by evaluating the maximum total error (MTE) [17]. The MTE combines both accuracy (bias) and pre-

cision (repeatability) and, additionally, it takes into account the statistical uncertainty in the estimation of errors.

Experimental

Reagents and solutions

All chemicals used were analytical-reagent grade, Millipore-Q water was used for solution preparation. Reference standards (purity >99%) of benzylpenicillin (BP), amoxicillin (AMOX), ampicillin (AMPI) and flucloxacillin (FLU) were donated by Gist-brocades BV (Delft, The Netherlands). The corresponding penicilloic acids (BP-acid, AMOX-acid, AMPI-acid and FLU-acid) were prepared by alkaline hydrolysis: an appropriate amount of the parent penicillin was dissolved in 2 ml of 2 M sodium hydroxide, the solution was sonicated for 15 min and then 2 ml of 2 M nitric acid were added. Penicillin formulations were obtained from OPG (Utrecht, The Netherlands): injectables (BP), capsules (AMOX, AMPI and FLU) and tablets (AMOX). The specifications are summarised in Table 1.

A 0.2 M potassium phosphate buffer (pH 6.5) was used as carrier. The iodine stock solution contained 0.5 mM iodine in 0.5 mM potassium iodide. The starch stock solution (0.15% w/w) was prepared in boiling water. The iodine and starch solutions were stored at 4°C. A starch/iodine complex was prepared every day by mixing iodine stock solution, starch stock solution and carrier (1:1:3, v/v/v); this blue iodine/starch complex was used as reagent. Carrier and reagent solution were filtered (0.2 μm) just before use.

Stock solutions of reference standards or samples were prepared freshly in water (50 mM); working solutions were obtained by appropriate dilution of stock solutions with carrier.

Penicillinase P-0389 type I, produced by *Bacillus cereus*, and glutaraldehyde, 25% aqueous solution purified Grade I, were obtained from Sigma (St Louis, MO, USA). Controlled-pore glass (CPG) support with pore dia. of 50 nm and particle size of 125–177 μm and (3-aminopropyl)triethoxysilane were purchased from Pierce (Rockford, IL, USA). Glass beads (0.6 mm) were obtained from Euroglas (Delft, The Netherlands) and 1.0-mm glass beads from Tamson (Zoetermeer, The Netherlands).

Immobilisation procedure and enzyme reactors

After washing the support (3 g of 1.0 mm beads or 1.5 g of 0.6 mm beads or 500 mg of CPG) with 6% nitric acid, it was subsequently, aminosilanised and activated with glutaraldehyde according to customary procedures [18]. Then penicillinase was coupled to the glutaraldehyde-activated support. Usually enzyme coupling is performed in batch at low temperature (5°C) over several hours (one night). A simplified enzyme coupling procedure was used in this study. Teflon tubing (1.5 mm i.d. for 1.0-mm beads or for CPG; 0.9 mm i.d. for 0.6-mm beads; 0.5 mm i.d. for CPG) was filled with the glutaraldehyde-activated support. Then a penicillinase solution (500 U ml^{-1} in 0.1 M sodium phosphate buffer pH 7) was recirculated through the reactor at a flow rate of 0.1 ml min^{-1} for 4 h at room temp. After this coupling procedure the reactor was washed, sequentially with 10 ml of cold water, 10 ml of cold 1 M sodium chloride solution and 10 ml of 0.1 M sodium phosphate buffer pH 7 at a flow rate of 1.0 ml min^{-1}. The required reactor length was cut off and the remaining

Table 1
Specifications of the pharmaceutical formulations

Type*	Trade mark	Formulation	Labelled content	Average weight (n = 10)
A AMOX	clamoxyl	capsule	375† mg	434.9 mg
B AMOX	clamoxyl	tablet	375† mg	949.4 mg
C AMOX	flemoxin	capsule	500† mg	585.8 mg
D AMOX	flemoxin	capsule	500† mg	615.1 mg
E AMP	amfipen	capsule	250 mg	286.6 mg
F BP	penicillin-G	injectable	10^6 i.u.‡	629.7 mg
G BP	penicillin-G	injectable	10^7 i.u.‡	6548.9 mg
H FLU	floxapen	capsule	500 mg	571.6 mg

*Type of penicillin; for abbreviations see Experimental.
†Labelled as AMOX anhydrate. The formulations contained AMOX-trihydrate.
‡10^6 i.u. = 600 mg Na-benzylpenicillin.

Teflon tubing containing the immobilized enzyme was stored in the sodium phosphate buffer pH 7 at −15°C. The coupling efficiency was measured by determining the protein content (Lowry) in the enzyme solution before and after the enzyme immobilisation procedure. Two PBERs and two SBSERs were prepared. PBER1 (10 cm × 0.5 mm i.d.) and PBER2 (10 cm × 1.5 mm i.d.), both filled with penicillinase immobilised on CPG; SBSER1 (50 cm × 0.9 mm i.d.) and SBSER2 (50 cm × 1.5 mm i.d.) filled with penicillinase immobilised on 0.6-mm or 1.0-mm beads, respectively.

Apparatus

Flow system. The experimental setup of the flow system is shown in Fig. 2. The FiaStar Analyser (model 5020, Tecator, Högenäs, Sweden) was used to control the injection. The analyser was equipped with an injection valve provided with a fixed 30-μl loop, an autosampler (model 5007, Tecator) and two peristaltic pumps which were used to fill the sample loop and to rinse the needle of the autosampler. The carrier and reagent solutions were delivered by a separate, variable speed, peristaltic pump (model IPN8, Ismatic, Zürich, Switzerland). A colorimetric flow-through detector (model 5023, Tecator) provided with a control unit (model 5032, Tecator) was used. The enzyme reactor was a PBER-type or an SBSER-type as described above. A single-bead-string reactor (SBSR) was used for mixing and subsequent reaction of the produced penicilloic acid with iodine. Signals were recorded using a flat-bed recorder (model BD40, Kipp & Zonen, Delft, The Netherlands).

Conditions. Flow rates of 0.30 and 0.70 ml min^{-1} were used to deliver the carrier and the reagent, respectively. The maximum absorption wavelength (590 nm) was chosen as detection wavelength. Amounts of 30 μl of standard or sample solutions were injected into the carrier. A PBER of 10 cm × 1.5 mm i.d. was used for the enzymatic hydrolysis and an SBSR of 25 cm × 1.5 mm i.d. filled with 1.0-mm uncoated glass beads for the iodometric reaction. After use, the flow system, except for the PBER, was rinsed daily with thiosulphate solution and with water. The PBER was stored at −15°C. A blank determination was defined as the response (the iodine consumption) obtained after injection of a standard or sample solution) omitting the enzymatic hydrolysis. For that purpose, the PBER was replaced by a dummy reactor: a packed-bed reactor of equal dimensions to the enzyme reactor but filled with uncoated CPG.

Results and Discussion

Optimisation

Enzyme reactor. In the design of an immobilised enzyme reactor (IMER) for FIA applications two factors affect the performance of the reactor: the amount of product formation, i.e. the conversion, and the dispersion of the sample zone. High conversion will improve sensitivity, reproducibility and stability of the assay; therefore, the conversion is desired to be close to 100%. On the other hand, to obtain high conversion may require a relatively long residence time of the sample plug in the reactor, which leads to increasing dispersion. An optimal compromise should be found.

Usually, the enzymes are immobilised onto CPG and reactors are packed with the immobilised enzyme preparation. This PBER-type may generate relatively high backpressure which is undesirable in a usually low-pressure FIA system. The SBSR, introduced by Reijn *et al.* [19] is typically packed with relatively large beads; the bead diameter is about ⅔ of the inner diameter of the tube. Effective mixing, low dispersion and low backpressure make the SBSR favourable for use in

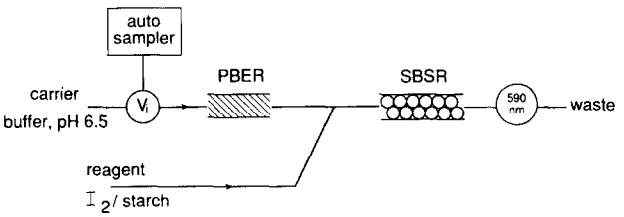

Figure 2
Flow diagram of the experimental setup; V_i: injector; PBER: packed-bed enzyme reactor; SBSR: single-bead-string reactor.

FIA systems. A drawback of the SBSR as an immobilised enzyme reactor may be the relatively low specific surface area of the glass beads compared to CPG. This drawback, however, can be omitted by using a longer reactor.

The enzyme load factor of glass support is defined by the amount of protein coupled per unit of surface area. The protein coupling was determined by measuring the protein content (Lowry) in the enzyme solution before and after the immobilisation procedure. The specific surface area of CPG is indicated by the manufacturer and the specific surface area of the 0.6 and 1.0-mm beads was calculated assuming spherical shapes of the beads. The results are summarised in Table 2. It is shown that per m^2, CPG is lowly loaded due to the very large surface area, while the glass beads are rather highly loaded. However, these calculations only account for the amount of penicillinase immobilised onto the support and no conclusions can be drawn with respect to the activity of the immobilised enzyme.

Four reactors which are specified in Table 3 were prepared. From the combined data of Table 2 and 3 the total enzyme protein content in the reactors can be calculated: 15, 123, 150 and 135 μg enzyme protein in the PBER1, PBER2, SBSER1 and SBSER2, respectively. Due to its relatively high back-pressure, PBER1 could not be used in FIA. The remaining three reactors were tested in the FIA-system and the amount of conversion was determined at different residence times. Solutions of BP and of BP-acid (0.3 mM each) were subsequently injected to determine the conversion. The conversion was calculated from the ratio of peak heights produced by BP and by BP-acid, respectively. The residence time of the sample plug in the enzyme reactor was varied by varying the flow rate of the carrier between 0.2 and 0.5 ml min^{-1}. The mean residence time was calculated from the ratio of flow rate and void volume. The void volume (see Table 3) of each reactor was determined colorimetrically. The conversion as a function of mean residence time is presented in Fig. 3. Obviously, 100% conversion can be achieved with all three of the enzyme reactors by adjusting the residence time. However, for PBER2 it takes only 30 s, while for SBSER1 and SBSER2 65 and 170 s are needed, respectively. The required time of 30 s in PBER2, corresponding to a flow rate of 0.3 ml min^{-1}, is very short and is accompanied by a favourable low dispersion. Finally, the sp. act., α, defined as the conversion (%) s^{-1} μg^{-1} immobilised protein times 100, was calculated to be 2.7 for PBER2 and 1.0 or 0.4 for SBSER1 and SBSER2, respectively. Obviously, the highest specific enzyme activity is

Table 2
Load factor of different glass supports

Glass support*	Surface area (m^2/g glass)	Protein-coupling (mg/g)	(%)	Load-factor (mg/m^2)
CPG	70	1.84	92	$2.6\ 10^{-2}$
0.6 mm beads	$3.5\ 10^{-3}$	0.45	68	128
1.0 mm beads	$1.4\ 10^{-3}$	0.12	38	86

*0.5, 1.5 and 3.0 g of CPG, 0.6-mm beads and 1.0-mm beads, respectively, were treated with 5 ml of 0.2 mg protein/ml solution.

Table 3
Specifications of the immobilised enzyme reactors

Type	Length (cm)	Dia. (mm)	Pack-factor (mg/cm)	Void-volume (μl)	Back-press* (bar)
PBER1	10	0.5	0.8	17	2.1
PBER2	10	1.5	6.7	157	0.22
SBSER1	50	0.9	6.6	238	0.12
SBSER2	50	1.5	22.5	531	0.02

*Back-pressure calculated according to Darcy's equation, assuming a volumetric flow rate of 0.5 ml min^{-1}: $P = L\ \eta\ \phi\ <v_o>\ 10^{-5}/\epsilon\ d_p^2$, where L = length (m), η = dynamic viscosity (Ns/m^2), ϕ = column resistance factor, $<v_0>$ = mean velocity (m/s), i.e. the velocity that was obtained in an empty tube at the same volumetric flow rate, ϵ = void fraction and d_p = the particle diameter (m). The term "$\eta\ \phi$" is assumed to equal 1 or 0.5 for the packed-bed reactor and the single-bead-string reactor, respectively [19].

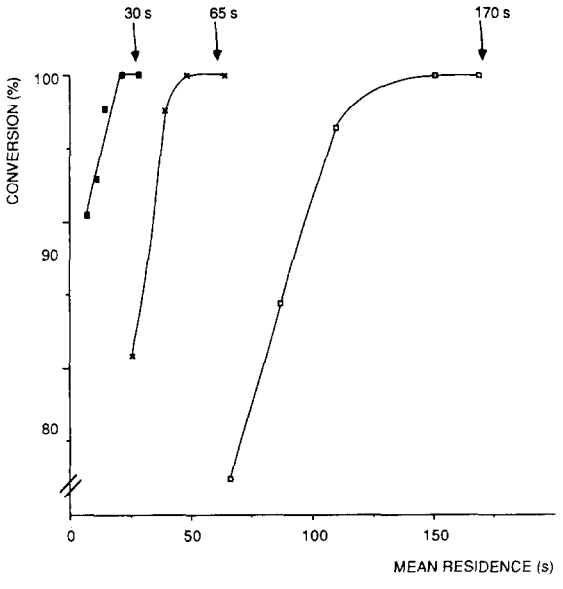

Figure 3
Fractional conversion vs mean residence time for three different enzyme reactors. (■) PBER2: 10 cm × 1.5 mm i.d.; (×) SBSER1: 50 cm × 0.9 mm i.d. and 0.6-mm beads; (□) SBSER2; 50 cm × 1.5 mm i.d. and 1.0-mm beads.

obtained with PBER2. The relatively high α of PBER2 cannot be explained by a relatively high absolute amount of penicillinase, since the investigated reactors contained approximately equal amounts of penicillinase. Therefore, it may be concluded that the high sp. act. should be attributed to a different loading factor and to different hydrodynamical conditions in both types of enzyme reactors. The favourable conditions in the PBER appear to lead to high turn-over, while penicillinase in the SBSERs might be partially deactivated. Gnanasekaran and Mottola [5] obtained satisfying results with a penicillinase SBSER. However, no comparison with a packed-bed reactor was made in that study and the experimental conditions differed, e.g. the surface of the glass beads was etched prior to immobilisation and penicillinase was immobilised onto the tube wall as well as onto the beads. Additionally, a much higher flow rate was used by which (a) the residence time was decreased leading to low dispersion and (b) the thickness of the stagnant layer was decreased leading to improved mass transfer between substrate or product and the active enzyme centres. No further investigations were made in our study to improve the specific enzyme activity of a SBSER-type since a wide dia. (1.5 mm) PBER-type was satisfactory. PBER2 was chosen for all further experiments.

The conversion was also determined for AMOX, AMPI and FLU and proved to be 100% for all of them. The calculated pressure drop at the applied carrier flow rate (0.3 ml min^{-1}) is 0.12 bar which is acceptable in a FIA system. The 100% conversion was retained for at least 9 months.

The iodometric detection reaction. A SBSR-type reactor (1.5 mm i.d. filled with uncoated 1.0-mm glass beads) was used for mixing and subsequent reaction of penicilloic acid with iodine. The influence of reactor length and flow rate on the response was examined using 30-μl injections of 0.3 mM BP-acid in a single-line FIA system. First, the reactor length was varied between 10 and 100 cm at a flow rate of 1 ml min^{-1}. Peak height and width (at 10% of the peak height) were measured. Generally, peak height depends on kinetics and on dispersion, while peak width only depends on dispersion. The results obtained are shown in Fig. 4. Although the peak height does not vary very much it decreases slightly at increasing reactor lengths, while the peak width increases with increasing reactor lengths. A reactor length of 25 cm was chosen; it provided a maximal peak height vs peak width ratio as shown in Fig. 5 for BP-acid and for AMOX-acid. Then, at 25-cm reactor length the flow rate was varied between 0.2 and 1.0 ml min^{-1}.

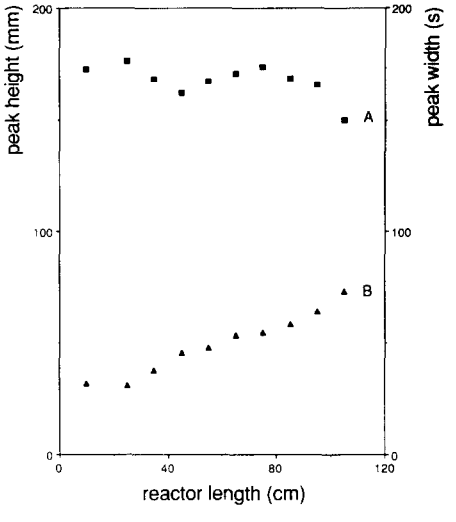

Figure 4
Dependence of the FIA signal on the reactor length. Benzylpenicilloic acid after reacting with the iodine/ starch complex in a single line FIA system using a single-bead-string reactor and a flow rate of 1.0 ml min^{-1}; (A): peak height and (B) peak width as a function of the reactor length.

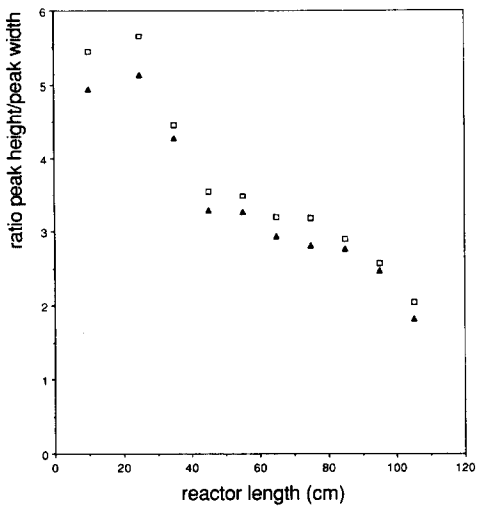

Figure 5
Dependence of the ratio of peak height and peak width on the reactor length. Benzylpenicilloic acid (□) or amoxicilloic acid (▲) after reacting with the iodine/starch complex in a single line FIA system using a single-bead-string reactor and a flow rate of 1.0 ml min^{-1}.

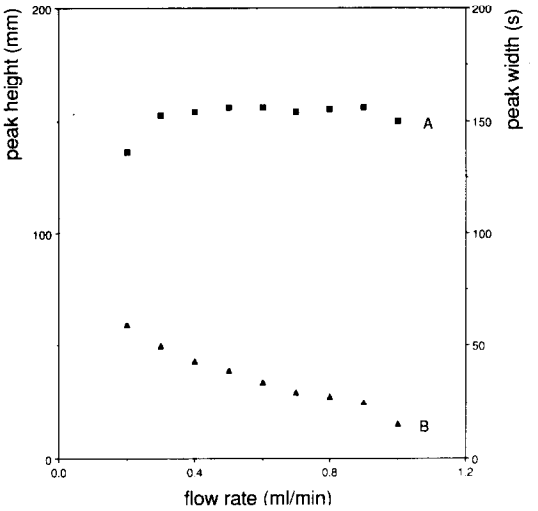

Figure 6
Dependence of the FIA signal on the flow rate. Benzylpenicilloic acid after reacting with the iodine/starch complex in a single line FIA system using a single-bead-string reactor of 25 cm; (A): peak height and (B) peak width as a function of the flow rate.

Peak height and peak width (at 10% of peak height) were measured and plotted vs the flow rate. The results are shown in Fig. 6. The peak height is almost constant; the peak width decreases with increasing flow rate due to decreasing dispersion at higher flow rates. A flow rate of 0.7 ml min^{-1} was chosen for the reagent stream in the final experimental setup which resulted in an overall flow rate of 1.0 ml min^{-1} in the iodometric reactor afer combining 0.3 ml min^{-1} from the carrier stream and 0.7 ml min^{-1} from the reagent stream. The optimised conditions in the final experimental setup provided a response time (maximal signal after sample injection) of 1 min and a wash-out time (return to baseline after sample injection) of 2 min. The resulting sampling frequency was 30 h^{-1} with a good reproducibility (RSD = 0.75%; n = 10).

Performance

Linearity and detection limit. The system was calibrated at the optimised conditions with standard solutions of BP, AMOX, AMPI and FLU. A calibration graph was recorded for all four investigated penicillins at concentrations ranging from 0.1 mM to 0.5 mM. The linear correlation coefficient was approx. 0.993 for all penicillins. The detection limit was determined experimentally and proved to be 0.025 mM at a signal-to-noise ratio of 3.

Discriminating power. The discriminating power of the present assay between the parent penicillin and the main degradation product, the penicilloic acid, was investigated. Usually, native penicillin will not consume iodine. Hence, the blank response, which was defined as the response obtained after replacing the enzyme reactor by a dummy reactor (a reactor with equal dimensions but filled with uncoated CPG) will indicate the presence of penicilloic acid. Blanks were measured for BP, AMOX, AMPI and FLU. Freshly prepared reference standard solutions of BP, AMPI or FLU as high as 5 mM, proved to reveal blank responses corresponding to only 0.025 to 0.05 mM acid. Hence, these reference standards contained 0.5–1.0% of the corresponding penicilloic acid which well agree with the declared purity of at least 99%. On the contrary, a concentration of only 0.5 mM AMOX resulted in a remarkable response corresponding to approx. 0.2 mM AMOX-acid. However, using the reference method (the mercurimetric titration according to the European Pharmacopoeia [20]) the AMOX-reference standard showed a purity of 99%. Hence, the high blank FIA-response is caused by consumption of iodine by native AMOX. This phenomenon has been described in the literature [21, 22] and is attributed to the presence of the *p*-hydroxy group in the side chain (Fig. 1), which may lead

to direct oxidation of AMOX by iodine or to direct addition of iodine. As a consequence, by performing a blank assay the presence of the corresponding penicilloic acid in the reference standard can be indicated provided that the native penicillin does not react with iodine. The blank response shown by AMOX will not interfere when the proposed assay is applied to AMOX formulations, since careful calibrations will always be performed.

Selectivity. The samples to be assayed are pharmaceutical formulations which may contain components which interfere with the analysis of the active compound. The influence of this sample matrix can result in an additional response. The existence of such matrix effects can be indicated easily by comparing the blank response (response without enzyme reactor) produced by a reference standard solution and the blank response produced by a sample solution of equal conc. No significant differences were observed, which demonstrates that the sample matrix does not contain substances that contribute to the iodine consumption and, hence, no interference exists in the pharmaceutical formulations investigated in this study.

Quantitative validation of the reliability

Initially, the present flow injection system was applied to the determination of AMOX in formulations. Accuracy and precision are the most suitable evaluation criteria for the reliability of an assay. Usually, these criteria are considered separately. The maximum total error, MTE, which has been recently introduced by Wolters and Kateman [17] includes both accuracy and precision. This measure gives the maximum difference between a measured value and the true value that can occur with a probability of 95%. Its estimate is presented by an upper and lower limit and a most probable value. In order to determine the MTE the following design of experiments was used. Four formulations of AMOX were used (A, B, C and D in Table 1). The average weight of one species of each formulation was determined (n = 10). The content of 10 separate species was accumulated and homogenised. Ten samples were taken from one homogenised batch and diluted to approx. 0.1, 0.2, 0.3, 0.4 or 0.5 mM, each conc. in duplicate, and analysed by the FIA method. The homogenised batch was also assayed according to a reference method, the mercurimetric titration [20], and the titration results were considered to be the "true" results. Four (A, B, C and D) homogenised batches resulted in 40 sample solutions. These solutions were randomised and analysed by 4 series of 10 samples. Each series was preceded and followed by a calibration series ranging from 0.1 to 0.5 mM. The FIA-output of two calibration series and one series of 10 samples is presented in Fig. 7. The calibration data were used to estimate the random error (RE) of the method. RE has been estimated from the multiple measurements of calibration samples used for one

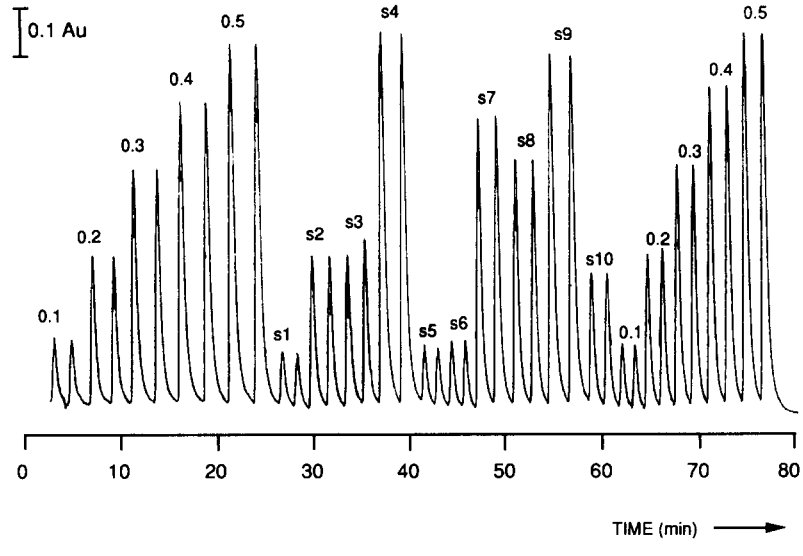

Figure 7
FIA-output of two calibration series (0.1, 0.2, 0.3, 0.4 and 0.5 mM amoxicillin) and one series of 10 samples amoxicillin (S1 t/m S10). Samples were randomised taken from four different pharmaceutical formulations.

calibration graph. The obtained REs for the four different calibration graphs were pooled. The FIA results obtained for the samples were compared with the "true" results. From the differences between test (FIA) and reference data the systematic error (SE) of the method was estimated. The maximum total error (MTE) was found by combining RE and SE. The most probable values for RE, SE and MTE, together with the corresponding upper and lower limits were calculated [17] for five conc. levels ranging from 0.1 to 0.5 mM. The

Table 4
Evaluation of the reliability of the FIA method for the assay of amoxicillin in pharmaceutical formulations. Conc. levels (mM), absolute errors (mM) and relative errors (%)

Concn.	SEmean*		SEmax		SEmin	
(mM)	(mM)	(%)	(mM)	(%)	(mM)	(%)
0.1	−0.0215	−21.5	−0.0176	−17.6	−0.0254	−25.4
0.2	−0.0087	−4.33	−0.0016	−0.79	−0.0157	−7.87
0.3	−0.0014	−0.47	0.0061	2.05	−0.0089	2.98
0.4	−0.0078	−1.94	−0.0019	−0.49	−0.0136	−3.40
0.5	−0.0302	−6.04	−0.0209	−4.18	−0.0395	−7.89
Concn.	**REmean***		**REmax**		**REmin**	
(mM)	(mM)	(%)	(mM)	(%)	(mM)	(%)
0.1	0.0056	5.62	0.0096	9.57	0.0040	3.98
0.2	0.0044	2.22	0.0086	4.28	0.0030	1.50
0.3	0.0038	1.25	0.0065	2.14	0.0027	0.89
0.4	0.0031	0.77	0.0053	1.32	0.0022	0.55
0.5	0.0044	0.87	0.0074	1.49	0.0031	0.62
Concn.	**MTEmean***		**MTEmax**		**MTEmin**	
(mM)	(mM)	(%)	(mM)	(%)	(mM)	(%)
0.1	0.0271	27.1	0.0350	35.0	0.0215	21.5
0.2	0.0131	6.55	0.0233	12.2	0.0047	2.29
0.3	0.0052	1.72	0.0154	5.12	0.0027	0.89
0.4	0.0109	2.72	0.0189	4.72	0.0041	1.03
0.5	0.0345	6.91	0.0469	9.38	0.0240	4.80

*Most probable value of the error.

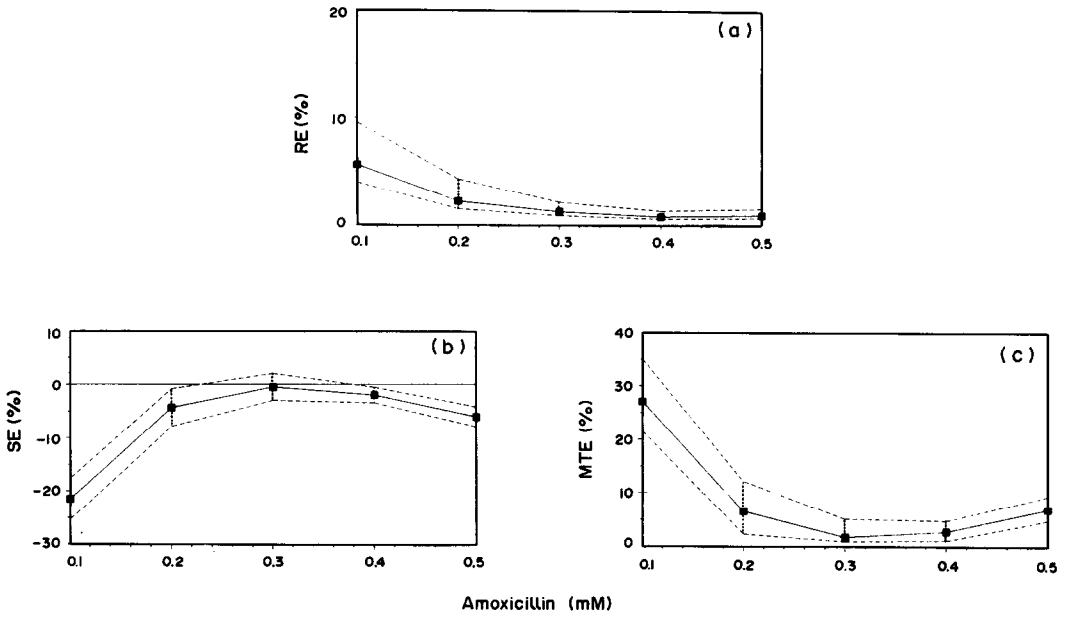

Figure 8
Most probable value (■) plus the upper and lower limits of the RE (8a), the SE (8b) and the MTE (8c). Amoxicillin ranging from 0.1 to 0.5 mM.

results are shown in Table 4 and the relative errors are plotted in Fig. 8. The RE decreases with increasing conc. (Fig. 8a); the absolute RE (conc. units) is fairly constant over the investigated range (Table 4). The SE shows significant negative values towards the ends of the conc. range (Table 4 and Fig. 8b). A possible cause of systematic error is the interfering effect of components in the sample matrix of pharmaceutical formulations. However, in the above described blank examinations such matrix effects were not observed. Therefore, the observed phenomenon cannot be attributed to interference of the sample matrix. Non-linearity of the calibration graph may also lead to a systematic error. The relatively low linear correlation coefficients which were found (approx. 0.993, see linearity) also indicate non-linearity. However, alternative calibration models have not been investigated in the present study but will be discussed in a separate study. Since the upper limit of the MTE at 0.3 mM is only 5% and the most probable value is only 2% (Table 4 and Fig. 8c) it was decided to dilute the samples to a level of 0.3 mM and to use only the 0.2–0.4 mM range for calibration.

Finally, the present FIA method was used for the determination of penicillin in eight formulations (see Table 1): AMOX (A, B, C, D) AMPI (E), BP (F, G) and FLU (H). The measurements were performed at a 0.3 mM level and the system was calibrated with the corresponding reference standard solutions (BP, AMOX, AMPI or FLU) of 0.2, 0.3 and 0.4 mM. For each formulation, 10 sample solutions (0.3 mM) were prepared and analysed. RE, SE and MTE, together with the upper and lower limits were calculated. The results are shown in Table 5 and the relative MTE (most probable MTE and its upper and lower limits) is shown in Fig. 9. Obviously, the upper limit of the maximum total error does not exceed 6% and the most probable value of the MTE is 3.5% or less. This estimated maximum difference between the true and

Table 5
Evaluation of the reliability of the FIA method for the assay of penicillin in 8 pharmaceutical formulations at the 0.3 mM level. The absolute errors (mM) and relative errors (%)

	SEmean		SEmax		SEmin	
Type*	(mM)	(%)	(mM)	(%)	(mM)	(%)
A	−0.0045	−1.67	0.0018	0.67	−0.0108	−4.01
B	−0.0055	−2.01	0.0001	0.01	−0.0110	−4.03
C	−0.0040	−1.27	0.0012	0.38	−0.0092	−2.92
D	0.0100	0.35	0.0074	2.61	−0.0054	−1.90
E	−0.0043	−1.54	0.0011	0.41	−0.0097	−3.49
F	0.0056	1.82	0.0130	4.24	−0.0019	−0.60
G	0.0055	1.78	0.0082	2.64	0.0028	0.91
H	0.0005	0.17	0.0042	1.47	−0.0032	−1.12
	REmean		REmax		REmin	
Type*	(mM)	(%)	(mM)	(%)	(mM)	(%)
A	0.0031	1.14	0.0056	2.08	0.0021	0.78
B	0.0029	1.05	0.0052	1.92	0.0020	0.72
C	0.0041	1.30	0.0075	2.37	0.0028	0.89
D	0.0028	1.00	0.0052	1.83	0.0019	0.69
E	0.0042	1.50	0.0077	2.74	0.0029	1.03
F	0.0030	0.99	0.0055	1.80	0.0021	0.68
G	0.0025	0.82	0.0046	1.50	0.0017	0.57
H	0.0039	1.35	0.0070	2.46	0.0026	0.93
	MTEmean		MTEmax		MTEmin	
Type*	(mM)	(%)	(mM)	(%)	(mM)	(%)
A	0.0076	2.80	0.0164	6.08	0.0021	0.78
B	0.0084	3.07	0.0163	5.96	0.0020	0.72
C	0.0081	2.57	0.0167	5.92	0.0028	0.89
D	0.0038	1.36	0.0126	4.44	0.0019	0.69
E	0.0085	3.04	0.0174	6.22	0.0029	1.03
F	0.0086	2.80	0.0186	6.03	0.0021	0.68
G	0.0080	2.60	0.0128	4.14	0.0046	1.48
H	0.0044	1.52	0.0113	3.94	0.0026	0.93

*See Table 1.

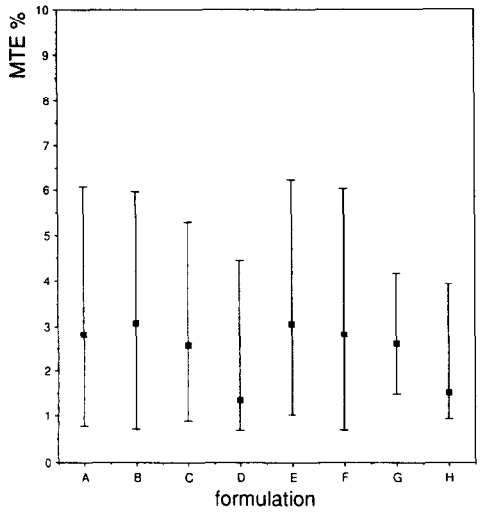

Figure 9
Most probable value (■) plus upper and lower limit of the MTE. Eight different pharmaceutical formulations, A–H (see Table 1) measured at 0.3 mM level.

measured value is satisfactory in quality control. It should be noted that the MTE includes both the accuracy (bias) as well as the precision (repeatability) of the method.

Conclusions

The present FIA method involves a rapid and automated assay for the determination of penicillin and has proven to be suitable for determining the penicillin content in various pharmaceutical formulations. The assay is based on the iodometric assay which was introduced originally as a iodometric titration method. Titrimetric methods are absolute assays which implies that calibration can be omitted and the availability of a pure and stable reference standard is not necessary. However, the iodometric titration of penicillins needs to be calibrated because the stoichiometry is not constant and depends on experimental conditions. Because of this drawback the titrimetric approach is less suitable and the flow-injection approach may be more favourable. The proposed FIA method provides a short time for analysis resulting in a sample throughput as high as 30 samples h^{-1}.

The success of an enzymatic FIA determination depends mainly on the performance of the enzyme reactor. The introduced simplified enzyme-coupling procedure, by recirculating the enzyme solution through the reactor at room temperature, appeared to be satisfactory. Furthermore, a relatively wide dia. packed-bed reactor has proven to be favourable for high conversion within only 30 s and for relatively low back-pressure (0.12 bar) at the optimum flow rate of 0.3 ml min^{-1}. Long term stability of the enzyme reactor was at least 9 months.

The linear range has proven to be small and the detection limit is relatively high. However, this limitation will not restrict the intended application of the present FIA method because only high levels have to be measured and a dilution to 0.3 mM (the optimum concentration to be measured) is easily controlled.

Blank determinations of standards and samples give insight into (a) the amount of degradation (formation of penicilloic acid) provided that the native penicillin does not consume iodine and (b) the presence of interfering components in the formulations. An advantage of the FIA approach, which was not applied in the present study but which might easily be incorporated, is the possibility of performing blank and real determinations, simultaneously, by placing a dummy reactor and an immobilized enzyme reactor in parallel. The injected sample plug is split and by taking a dummy and enzyme reactor of different lengths the penicillin response and the blank response can be measured sequentially within a single run.

The proposed method has proven to be suitable for the determination of AMOX, BP, AMPI or FLU in capsules, tablets or injectables. The most probable value of the MTE, which includes the random error and the systematic error, is 3.5% or less.

The iodine–starch reagent is not stable and has to be prepared freshly every day. Some adjustment of the dilution with carrier buffer was necessary, because the iodine content of the stock solution decreased slightly in the course of time. Besides, the starch stock solution has to be refreshed frequently. Therefore work is in progress to use an iodine generator followed by electrochemical detection [23]. Additionally, the non-linearity will be further investigated and the calibration model will be adjusted, if necessary, in a future study.

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