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Measurement of amoxicillin in plasma and gastric samples using high-performance liquid chromatography with fluorimetric detection

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Abstract

A rapid, selective and sensitive HPLC assay has been developed for the routine analysis of amoxicillin in rat plasma, gastric juice aspirate and gastric tissue which is applicable to low concentrations of amoxicillin (<1 μg mL^{-1}) or small sample volumes. Amoxicillin was converted, via an internal rearrangement, to form a fluorescent product which was subsequently recovered using liquid–liquid extraction. A Kromasil ODS 3 μm (150×3.2 mm I.D.) column was maintained at 40 °C and used with a mobile phase consisting of methanol–water (55:45, v/v). Fluorimetric detection was at an λ_{ex} of 365 nm and an λ_{em} of 445 nm. The limits of quantitation for amoxicillin were 0.1 μg mL^{-1} for gastric juice aspirate (500 μL), 0.5 μg mL^{-1} for plasma (50 μL) and 0.075 μg g^{-1} for gastric tissue (250 mg). The method was linear up to at least 15 μg mL^{-1} in gastric juice aspirate, up to 200 μg mL^{-1} in plasma and up to 100 μg g^{-1} in gastric tissue, with inter- and intra-day RSDs being less than 19%. The assay has been applied to the measurement of amoxicillin in rat plasma, gastric juice aspirate and gastric tissue for pharmacokinetic studies in individual rats.

Keywords: Amoxicillin

1. Introduction

Amoxicillin [D-(−)-α-amino-*p*-hydroxybenzylpenicillin; Fig. 1] is an orally absorbed, semi-synthetic broad-spectrum antimicrobial drug. It is now widely used in a standard eradication treatment of gastric *Helicobacter pylori* infections, where it is combined with a second antibiotic and an acid-suppressing agent [1,2].

In order to eradicate *H. pylori* effectively in vitro, amoxicillin has to reach a minimum inhibitory concentration (MIC) of 0.5 μg mL^{-1} at neutral pH [3]. Amoxicillin has been proven to be unstable in aqueous solutions with a pH below 2 [4–8], though it is considered to be stable in the pH range of 4 to 7. In a previous study of human gastric juice of pH 2.0, we have shown that the half-life of amoxicillin was 15.2 h and hence antimicrobial action is maintained at this pH [4]. The determination of amoxicillin levels in acidic gastric juice, and the extent to which it degrades in those conditions, is therefore crucial to ensure an effective pharmaco-therapeutic regimen. The exact transfer mechanisms that govern how amoxicillin reaches the gastric juice from the systemic circulation still remain to be elucidated, and research in this area has been hampered by the lack

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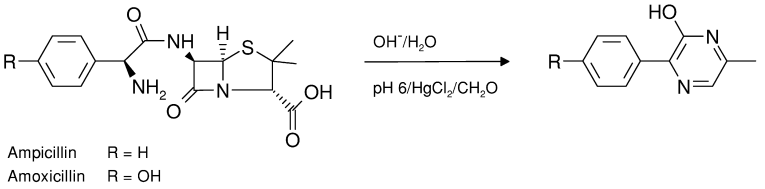

Fig. 1. The degradation of amoxicillin and the internal standard ampicillin into a fluorescent degradation product. R = OH for amoxicillin, and R = H for ampicillin (after Ref. [14]).

of a suitably sensitive assay to measure the low concentration of amoxicillin in rat gastric juice and tissue.

Since the concentration of amoxicillin in plasma is readily detectable (>1 μg mL^{-1}) the majority of the reports on the measurement of amoxicillin in plasma or serum require volumes of 0.2 mL or more [6,7,9–12] and these methods mainly use simple sample preparation procedures such as filtration. However, when the concentration of amoxicillin falls to less than 1 μg mL^{-1} or when a sample volume of less than 50 μL is available, interference from endogenous components can make quantitation difficult for HPLC methods with UV detection. We wished to measure amoxicillin in small volume plasma (50 μL or less) samples generated by single animal pharmacokinetic studies. In addition, we wanted to measure amoxicillin concentrations in gastric tissue and in gastric juice aspirates from rats and human volunteers following intravenous administration of the antibiotic in relation to its anti-*Helicobacter pylori* action. Previous studies have failed to accurately measure amoxicillin in gastric aspirate because of the very low concentrations which are present, normally less than 1.0 μg mL^{-1} [13].

The polar, amphoteric nature of amoxicillin makes it difficult to develop selective extraction methods to prevent interference from other components of the biological matrix. Co-extraction of interfering endogenous compounds is a severe limitation to current assay methods for amoxicillin and this is a particular problem with gastric juice samples which contain interfering components which are difficult to separate from amoxicillin either chromatographically or by sample preparation (unpublished observations). Fluorescence [6,7,10,11] or UV detection [9,14] has been combined with pre-column [10,11] or post-column derivatisation [6,7,14] in attempts to improve the specificity and sensitivity of the measurement of amoxicillin in biological matrices. However, we evaluated a number of published methods for the determination of amoxicillin in plasma and gastric samples, but the presence of one or more interfering compounds, particularly in the gastric juice matrix, prevented effective quantitation at concentrations of <1.0 μg mL^{-1} in most cases. One fluorescence method [15] of determination of ampicillin, a penicillin closely related in structure to amoxicillin, showed promise in terms of selectivity and sensitivity and was therefore investigated further.

In this paper we describe the development of a rapid, sensitive and selective HPLC assay suitable for the determination of amoxicillin pharmacokinetics in rat plasma, gastric juice aspirate and gastric tissue samples.

2. Experimental

2.1. Chemicals

Amoxicillin, ampicillin trihydrate, sodium hydroxide, mercury (bis)chloride and phosphate buffered saline were purchased from Sigma (Poole, UK). Omeprazole sodium was kindly donated (Astra Hässle, Mölndal, Sweden). Sodium chloride was purchased from Fluka (Poole, UK). Di-sodium hydrogenphosphate dihydrate was obtained from Fisons (Loughborough, UK). Hydrochloric acid, perchloric acid, formalin, ethylacetate and methanol were purchased from Fischer Scientific (Loughborough, UK). Pentagastrin was bought from Cambridge Laboratories (Newcastle upon Tyne, UK). Chemicals were of analytical grade or better. Water was obtained from an Elga Maxima water purification system (Elga, High Wycombe, UK). Blank rat plasma and blank rat stomach tissue were obtained from untreated rats.

2.2. Instrumentation

The HPLC system consisted of a Gilson 231XL automatic sample injector, Gilson 401 dilutor, Gilson 305 solvent pump, a computer running 715 software (Gilson Medical Electronics, Villiers le Bel, France), and a Perkin-Elmer LC-240 fluorescence detector, set at a λ_{ex} of 365 nm and a λ_{em} of 445 nm (Perkin-Elmer, Huntingdon, UK). A guard column (20×2.0 mm I.D.) packed with pellicular ODS material and a Kromasil ODS 3 μm (150×3.2 mm I.D.) (HiChrom, Reading, UK) analytical column were used. The column temperature was maintained at 40 °C using a model 7990 column oven from Jones Chromatography (Hengoed, UK). The mobile phase consisted of methanol–water (55:45, v/v). The mobile phase was filtered through a 0.45 μm nylon membrane filter (Whatman, Maidstone, UK) and degassed using helium. The flow-rate was 0.4 mL min^{-1} and the sample injection volume was 20 μL.

2.3. Calibration standards

Calibration samples of amoxicillin were prepared in blank plasma (50 μL), saline (500 μL) and phosphate buffered saline (200 μL). Saline was used to sample the stomach contents (see Section 2.6.) and was therefore also used as a blank for gastric juice aspirate. Stomach tissue calibration samples were prepared by adding blank stomach tissue (250 mg) to stock solutions of amoxicillin in saline. The plasma, gastric aspirate and stomach tissue calibrations were performed over amoxicillin concentration ranges suitable for the expected concentrations in the biological matrix: 0.10 to 15.0 μg mL^{-1}, 5.0 to 200 μg mL^{-1} and 1.0 to 100 μg g^{-1}, respectively, in gastric juice aspirate, plasma and gastric tissue.

2.4. Extraction procedure

To 50 μL of a plasma sample in an Eppendorf tube (1.5 mL capacity), 450 μL of water was added to increase the volume of the sample. The ampicillin internal standard solution (10 μL, 0.2 μg mL^{-1}) and 5 μL of an ice-cold 50% (w/v) perchloric acid solution were added. The solution was vortex-mixed briefly and centrifuged at 11 600 *g* for 10 min. The supernatant (450 μL) was transferred into a 2.0 mL amber Eppendorf vial, 50 μL sodium hydroxide (5 *M*) was added and the solution was vortex-mixed for 5 min. Hydrochloric acid (50 μL, 5 *M*) was added to neutralise the solution and 250 μL phosphate buffer (0.5 *M*) containing 0.1% (w/v) mercury (bis)chloride and 1.0% (v/v) formaldehyde was added. The solution was vortex-mixed, incubated at 50 °C for 40 min and cooled in water for 5 min. The solution was extracted by adding ethylacetate (800 μL), vortex-mixing briefly and centrifuging at 11 600 *g* for 5 min. The organic (top) layer (600 μL) was transferred into an amber Eppendorf tube (1.5 mL) and evaporated to dryness in vacuo for 40 min at 50 °C using the Jouan microcentrifuge evaporator (RC 10.22 sample concentrator/evaporator coupled to RCT 120 cold trap and an Edwards 5 vacuum pump). The residue was reconstituted by sequentially adding and mixing aliquots of methanol (100 μL) and water (100 μL) into the tube. The residue was subsequently transferred onto an Eppendorf filter device (Vectaspin micro, 0.45 μm pore size polypropylene filter inserts) (Whatman) and centrifuged at 11 600 *g* for 5 min to remove undissolved particulate matter. The filtrate was analysed for amoxicillin content by HPLC.

The rat stomach was snap-frozen in liquid nitrogen and transferred into a polyethylene sample bag. The frozen stomach was then rapidly crushed using a table-top vice to a homogenous liquefied mass and this was then re-frozen immediately in liquid nitrogen, transferred into a pre-weighed Eppendorf tube (1.5 mL capacity) and weighed. Phosphate buffered saline (200 μL) was added to obtain an homogenised stomach tissue sample for each stomach analysed. Gastric aspirate (500 μL) and homogenised stomach tissue samples were treated in an identical manner to plasma except that the initial addition of 450 μL of water was omitted.

2.5. Validation

The peak-area ratios of amoxicillin to the internal standard were calculated and used to construct calibration lines of peak-area ratio against drug concentration in plasma, gastric aspirate and stomach tissue by unweighted linear regression analysis. Slope, intercept and regression coefficient of the calibration lines were determined. Quality control

Table 1
Validation of the determination of amoxicillin in rat gastric juice aspirate, plasma and gastric tissue: intra- and inter-day precision and accuracy for the assay. Tissue concentrations are reported in μg g^{-1}

Spiked amoxicillin (μg mL^{-1} or μg g^{-1})	Intra-assay ($n=6$)			Inter-assay ($n=6$)		
	Measured (mean±STD) (μg mL^{-1} or μg g^{-1})	RSD (%)	Accuracy (%)	Measured (mean±STD) (μg mL^{-1} or μg g^{-1})	RSD (%)	Accuracy (%)
Gastric aspirate						
0.250	0.236±0.014	5.9	94.6	0.242±0.022	9.0	96.9
0.999	0.984±0.065	6.6	98.5	0.977±0.071	7.2	97.8
9.99	10.40±0.24	2.3	104.1	10.08±0.26	2.6	100.9
Plasma						
49.9	51.4±1.7	3.3	102.8	49.0±1.1	2.2	98.1
99.9	101.7±1.9	1.8	101.8	101.5±1.3	1.3	101.6
150	150±2.9	1.9	100.0	148±1.2	0.8	99.1
Gastric tissue						
1.00	1.05±0.06	6.2	104.8	1.05±0.19	18.3	105.2
5.99	6.26±0.30	4.8	104.5	5.83±0.27	4.7	97.3
49.9	51.1±3.1	6.1	102.2	47.9±4.2	8.8	95.8

samples of fixed concentration (see Table 1) were prepared to determine the intra- and inter-day precision and accuracy of the assay. The intra-day data were collected from the analysis of six batches of QC samples on the same day and inter-day data were collected from the analysis of the QC samples on six separate days. The limit of detection was defined by the concentration of amoxicillin in the sample matrix giving a signal-to-noise ratio of 3:1.

2.6. Amoxicillin pharmacokinetic study in rats

Male Wistar rats, weighing 230–370 g, were fasted 24 h prior to experimentation. The stomach of the anaesthetized rat was cannulated to sample the gastric juice produced by securing a cannula into the antral part of the stomach via the duodenum and pylorus The stomach was gently lavaged four to six times using this cannula with 1.5 mL aliquots of 0.9% saline until the aspirate was free of debris. A clean 1.5 mL aliquot of saline was introduced into the stomach and the abdominal wound was covered with moistened tissue. Gastric acid secretion was stimulated by administering i.v. boluses of 25 μg kg^{-1} pentagastrin every 15 min during the experiment

Amoxicillin was administered initially by a 11 mg kg^{-1} i.v. bolus followed by a continuous i.v. infusion (20 mg kg^{-1} h^{-1}) of amoxicillin. The animal remained under controlled anaesthesia during the experiment and blood plasma samples were taken at 15 min intervals for a total duration of 2 h. The complete gastric content was aspirated at the same time points, recording volume and pH. The stomach was removed and halved at the end of the experiment. Plasma, gastric juice aspirate and tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C pending analysis.

3. Results and discussion

3.1. Chromatography

Several attempts were made to develop an HPLC method for the determination of amoxicillin in low concentrations in gastric juice aspirate before the method reported here was developed. Reversed-phase HPLC methods using UV detection (for example, Ref. [4]) were not sensitive enough due to the presence of interfering endogenous compounds in the gastric juice matrix that were not present in plasma. Different chromatographic column materials were evaluated such as porous graphitic carbon and a polymeric reversed-phase column, but none of these resolved the interfering peaks from that of amoxi-

cillin or were able to provide satisfactory peak shapes. An ion-pair extraction sample pretreatment procedure using perfluorinated carboxylic acids was investigated as a method to improve the selectivity of the extraction process, but this also proved unsuccessful. LC electrospray mass-spectrometry methods using various buffer systems (including volatile ion-pair forming buffers) were able to resolve interfering components, but a high background noise and a relatively weak signal from the amoxicillin limited sensitivity to around 1 μg mL^{-1} in gastric juice.

The eventual development of a successful method for measuring low concentrations of amoxicillin in gastric samples was based on the earlier work of Barbhaiya et al. [14] who identified a specific fluorophore resulting from the degradation of ampicillin, a close structural relative of amoxicillin. Initial experiments with this method showed promise in terms of both selectivity and sensitivity improvements compared with the HPLC–UV methods. We modified the original method with the addition of mercury (bis)chloride to improve the formation of the fluorescent compound and by adding a liquid–liquid sample preparation stage. One major advantage of the method was the conversion of amoxicillin from a hydrophilic zwitterion to a relatively hydrophobic fluorescent derivative that could be readily and selectively removed from the biological matrix using a simple liquid–liquid extraction method, leaving the polar interfering compounds behind. The simplicity of the mobile phase (methanol–water) combined with the high specificity of fluorescence detection resulted in excellent specificity and sensitivity for this HPLC method. The derivatization procedure appeared to give equivalent conversion to fluorescent products for both amoxicillin and the internal standard for all the biological matrices and with simple aqueous standards. Optimization of the derivatization procedure showed that heating at 50 °C for 40 min gave the optimum fluorescence yield in accordance with previously published data [12]. The lack of authentic standards for the fluorescent derivatives prevented quantitative determination of the yield of fluorescent product. However, the same concentration of amoxicillin in different biological matrices produced similar peak areas for the fluorescent derivatives by HPLC, suggesting that the recovery was at least consistent between matrices.

Representative chromatograms of blank plasma, gastric juice aspirate and stomach tissue and for samples containing amoxicillin are shown in Fig. 2. The retention times for amoxicillin and that of the internal standard ampicillin were 2.7 and 5.1 min, respectively. No interfering peaks were noticeable in

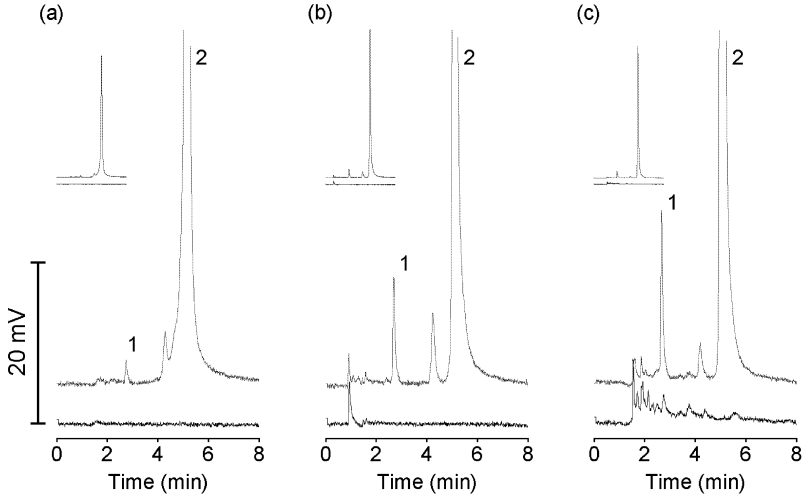

Fig. 2. Details of chromatograms of extracts of blanks (lower lines) and amoxicillin spiked samples of: (a) gastric aspirate (0.10 μg mL^{-1}), (b) plasma (4.99 μg mL^{-1}), and (c) gastric tissue (1.00 μg g^{-1}), with inserts showing full scale plots. Peak 1 = amoxicillin derivative and peak 2 = ampicillin (internal standard) derivative. Mobile phase methanol–water (55:45, v/v), 0.4 mL min^{-1}, column Kromasil ODS (150×3.2 mm, 3 μm particle size) at 40 ° C, fluorescence detection λ_{ex} = 365 nm, λ_{em} = 445 nm.

the baselines of the chromatograms of blank plasma, gastric juice aspirate and gastric tissue, demonstrating the high selectivity of the method. Samples of the biological matrices containing drugs or chemicals likely to be present for experimental purposes (metronidazole, clarithromycin, pentagastrin, omeprazole, pronase) did not interfere with either the drug or internal standard. The very high selectivity of the method probably derives from the very specific internal rearrangement to a fluorescent product which appears to occur only with the amino penicillins such as amoxicillin and ampicillin.

Table 1 summarises the accuracy and precision of the analysis after extraction of QC standards from the appropriate biological matrices. Nine-point calibration lines were used to validate the assay in plasma, gastric juice aspirate and gastric tissue over the ranges outlined above. The lower limits of detection (LOD) (signal-to-noise ratio of 3:1) were found to be 0.02 μg mL^{-1}, 0.3 μg mL^{-1} and 0.04 μg g^{-1}, respectively, for gastric juice aspirate, plasma and gastric tissue. The lower limits of quantitation (LOQ) for the assays were defined from the linearity tests as the lowest concentration of amoxicillin to give an RSD of 20% or less for intra-day precision and accuracy and were found to be 0.1 μg mL^{-1} for gastric juice, 0.5 μg mL^{-1} for plasma and 0.075 μg mL^{-1} for gastric tissue. The apparently higher LOD and LOQ values for the plasma assay were due to the small standard volumes used in this assay (50 μL) which limited the lowest absolute amount detectable. The three assays showed good linearity over the range of concentrations used in the calibration samples. The slope and intercept values of the equations of the calibration lines in the respective biological matrices were as follows (presented as mean±standard deviation, $n=6$):

Gastric juice $y = 0.091(\pm 0.018)x - 0.0051(\pm 0.0050)$

$(R^2 = 0.9993 \pm 0.0006)$

Plasma $y = 0.0086(\pm 0.0017)x + 0.0011(\pm 0.0049)$

$(R^2 = 0.9993 \pm 0.0004)$

Gastric tissue $y = 0.0071(\pm 0.0019)x - 0.0079(\pm 0.0355)$

$(R^2 = 0.9975 \pm 0.0044)$

where y is the ratio of drug/internal standard peak area and x is the concentration of amoxicillin.

No absolute figures for recovery could be calculated since no standard fluorescent degradation product was available for use as an external standard and hence the efficiency of the fluorescent conversion reaction could not be determined. Inter- and intra-day accuracy and precision at a range of concentrations were all within acceptable limits, suggesting that the conversion reaction was reproducible and linear over a wide range of amoxicillin concentrations.

3.2. Amoxicillin pharmacokinetic study in rats

Typical chromatograms from dosed rats are shown in Fig. 3. The plasma concentration time curve of amoxicillin following i.v. administration to a rat pretreated with pentagstrin is depicted in Fig. 4. Fig. 4 also shows the amoxicillin concentration time profile in gastric juice aspirate, with the amoxicillin concentration being considerably lower in gastric aspirate than in plasma throughout the experiment. The tissue concentration of amoxicillin at the end of the experiment was found to be 32.7 μg g^{-1}, slightly more than half the amoxicillin concentration in plasma (41.9 μg mL^{-1}). The results were subsequently used to calculate the amount of amoxicillin transferred into the gastric juice (gastric transfer fraction) and other pharmacokinetic parameters [16].

This method has also been applied successfully to the measurement of amoxicillin in human gastric biopsy samples (typical weight 5–10 mg, unpublished data). In total, more than 3500 biological samples have been successfully analysed for amoxicillin using this method in rat and human pharmacokinetic and drug delivery studies.

4. Conclusions

We have developed a rapid, practical and specific HPLC assay for the determination of amoxicillin in small volumes of rat plasma, gastric juice aspirate and gastric tissue. The short time of analysis, sensitivity and reproducibility of the method make it particularly useful for the pharmacokinetic studies where low concentrations (<1.0 μg mL^{-1}) of

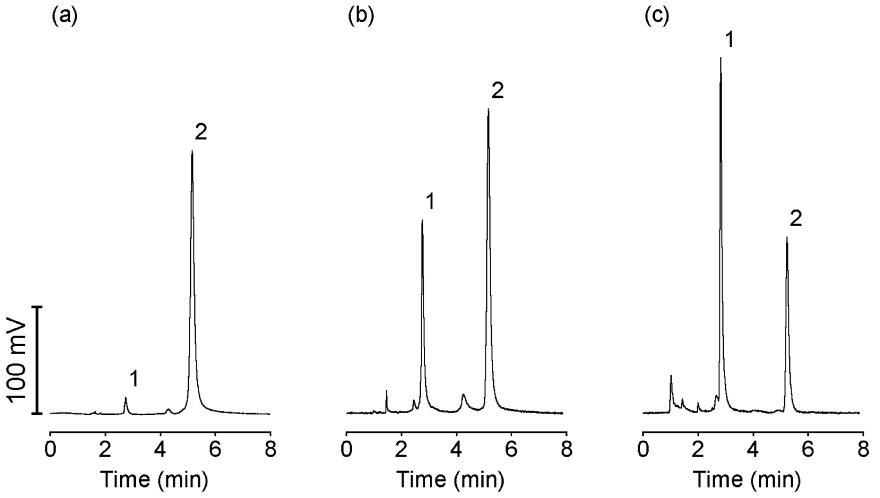

Fig. 3. Examples of chromatograms of (a) gastric aspirate (0.50 μg mL^{-1}), (b) plasma (43.0 μg mL^{-1}), and (c) gastric tissue (32.7 μg g^{-1}) samples from rats dosed intravenously with amoxicillin. Peak 1 = amoxicillin derivative and peak 2 = ampicillin (internal standard) derivative. HPLC conditions as in Fig. 2.

amoxicillin are expected, and where only small amounts of sample are available.

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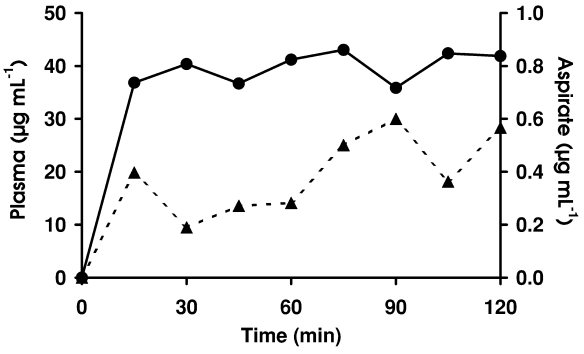

Fig. 4. Typical amoxicillin concentration vs. time profiles for gastric aspirate (▲) and plasma (●) from a rat treated with pentagastrin. Amoxicillin was administered initially by a 11 mg kg^{-1} i.v. bolus followed by a continuous i.v. infusion (20 mg kg^{-1} h^{-1}).

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