Liquid Chromatographic Determination of Amoxicillin Concentrations in Bovine Plasma by Using a Tandem Solid-Phase Extraction Method

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We report a means of determining amoxicillin in bovine plasma by liquid chromatography with UV detection at 235 nm. Purification and concentration of extracts were accomplished by a tandem solid-phase extraction procedure with two reversed-phase columns. Separation of amoxicillin from interferences was improved by the incorporation of a crown ether in the solvent systems used both for the solid-phase extraction and the final high-pressure liquid chromatography. Cefadroxil was added as an internal standard. The average recovery of amoxicillin from plasma was 78.2 ± 3.0%, and the within-run and between-run coefficients of variation ranged from 1.8 to 7.0%. The detection limit was estimated at 0.1 μg/ml. This method was used to determine amoxicillin in bovine plasma after intramuscular administration of the drug.

The high-pressure liquid chromatographic (HPLC) determination of amoxicillin in plasma represents a greater analytical challenge than does HPLC determination of ampicillin. A sensitive ampicillin assay with detection in the low UV region (210 nm) requires special approaches for sample pretreatment and chromatography, to eliminate the interference from polar plasma constituents (11). Amoxicillin exhibits a maximum at λmax 230 nm, but it is considerably more polar than ampicillin and, consequently, is more susceptible to such interferences. To improve the selectivity of amoxicillin assays, the compound can be chemically (1, 2, 5, 9) or electrochemically (8) converted to derivatives that absorb UV light at higher wavelengths (1, 5) or that fluoresce (2, 8, 9).

In our previous ampicillin assay (11), enhanced selectivity and sensitivity were not derived from improved detection but from the use of a tandem solid-phase purification and concentration of the sample. The performance of a single solid-phase extraction method is apparently insufficient to separate amoxicillin efficiently from interfering plasma constituents (6, 7). The principle of two consecutive solid-phase extractions was maintained in the amoxicillin assay reported here. However, the original approach devised for ampicillin did not prove to be transferable to amoxicillin. The modified method uses two different reversed-phase extractions rather than one ion-exchange and one reversed-phase step. A key factor for obtaining the required degree of chromatographic selectivity both on the extraction columns and in the final HPLC separation is the incorporation of a crown ether in the respective solvent systems.

MATERIALS AND METHODS

Chemicals and reagents. Amoxicillin trihydrate and cefadroxil (for structural formulas, see Fig. 1) were purchased from Sigma (St. Louis, Mo.). HPLC-grade acetonitrile was from ROMIL (Loughborough, United Kingdom). Crown ether (18-crown-6) was obtained from Janssen Chimica (Beersse, Belgium). Bond Elut CH (cyclohexyl) and C18 (500 mg, 2.8 ml) came from Baker (Phillipsburg, N.J.) or Analytichem (Harbor City, Calif.). All other reagents were analytical grade and were obtained from UCB (Braine-l’Alleud, Belgium) or Merck (Darmstadt, Germany).

Samples. Plasma samples were taken from three lactating cows. They were given a single dose of 10 mg of amoxicillin per kg of body weight by a deep intramuscular injection in the neck region (23, 25, and 30.5 ml, respectively, of a 20% preparation [Alfasan, Woerden, The Netherlands] divided over two injection sites). Blood was sampled at 0, 1, 2, 3, 4, 8, 12, and 24 h postinjection by means of a catheter in a jugular vein. The blood was collected in Venoject Li heparin tubes (Terumo Europe, Leuven, Belgium), and the plasma was separated by centrifugation at 1,600 × g and was kept frozen at −80°C until analysis.

Apparatus and chromatographic conditions. The liquid chromatograph consisted of a Varian 5020 pump (Varian Associates, Palo Alto, Calif.), an N60 valve injector (Valco Houston, Tex.) fitted with a 100-μl loop, an LC-95 variable-wavelength detector (Perkin-Elmer, Norwalk, Conn.) set at 235 nm (0.02 absorbance units full scale), and an SP 4100 integrator (Spectra-Physics, San Jose, Calif.) set at attenuation 16. The column (15 by 0.46 cm) packed with 5-μm Hypersil ODS (Hichrom, Reading, United Kingdom) was eluted with 0.005 M potassium dihydrogen phosphate–0.1 M 18-crown-6 in acetonitrile (84:16; vol/vol). The flow rate was 1 ml/min, and the temperature was ambient.

Sample preparation. (i) Double reversed-phase extraction. To 0.5 ml of plasma was added 50 μl of the internal standard solution (cefadroxil; 15 μg/ml, 5 ml of 1.8% sodium chloride, and 0.1 ml of 1 M 18-crown-6 in methanol. This mixture was applied on top of a CH (cyclohexyl) extraction column that had been preconditioned successively with methanol (5 ml) and water (5 ml). The column was washed with 1 ml of water-methanol (9:1; vol/vol) containing 0.1 M 18-crown-6. Elution was carried out with 3 ml of methanol-water (45:55; vol/vol). The eluate was transferred to a conical evaporation tube, and the methanol was evaporated by applying vacuum (water aspiration) under continuous vortexing of the tubes (Rotary Evapo-Mix; Büchler Instruments, Fort Lee, N.J.). To the aqueous residue was added 0.1 ml of 1 M 18-crown-6.

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in methanol and sufficient water to give a total volume of approximately 3 ml. This mixture was applied on top of a C18 extraction column that had been preconditioned with methanol (5 ml) and water (5 ml). The column was washed with 1 ml of water-methanol (9:1; vol/vol) containing 0.1 M 18-crown-6 and was eluted with 3 ml of methanol. The eluate was evaporated to dryness by using the Evapo-Mix apparatus, the residue was redissolved in 0.25 ml of the chromatographic solvent, and a 100-μl aliquot was injected.

(ii) Ion-exchange plus reversed-phase extraction. A 0.5-ml sample of bovine plasma supplemented with 50 μl of the internal standard solution (cefadroxil; 15 μg/ml) was mixed with 0.075 ml of 0.15 M phosphoric acid and 3 ml of methanol. After centrifugation, the supernatant was isolated and concentrated under vacuum (Rotary Evapo-Mix). To the aqueous residue was added 4 ml of 0.01 M hydrochloric acid, and the mixture was applied on top of a propylsulfonic acid extraction column that had been preconditioned successively with methanol (5 ml) and 0.01 M HCl (5 ml). The column was washed with 2 ml of 0.01 M HCl and was eluted with 3 ml of 0.067 M potassium dihydrogen phosphate. After the addition of 0.1 ml of 0.1 M 18-crown-6 in methanol and 2 ml of water, the solution was transferred to a C18 extraction column that had been preconditioned successively with methanol (5 ml) and water (5 ml). The remainder of the procedure was as described above for double reversed-phase extraction.

Quantitation. Stock solutions of amoxicillin in 0.067 M KH₂PO₄ contained 4.4 and 44 μg/ml (expressed as anhydrous amoxicillin). The concentration of the internal standard (cefadroxil dissolved in 0.067 M KH₂PO₄) was 15 μg/ml. All solutions were stored at 4°C and were controlled daily by chromatography. Samples (0.5 ml) of blank plasma were supplemented with known amounts of amoxicillin (0.18 to 4.4 μg/ml) and the internal standard (1.5 μg/ml) and were analyzed as the unknowns. Calibration curves were constructed by plotting peak height ratios (amoxicillin/cefadroxil) versus the corresponding amoxicillin concentrations. The amoxicillin concentrations in unknown samples were calculated by extrapolation from the standard curves.

Method validation. (i) Linearity. The slopes, intercepts, and correlation coefficients of the calibration curves were calculated by linear regression analysis.

(ii) Reproducibility. Three plasma pools supplemented with amoxicillin (nominal values, 0.42, 1.66, and 4.16 μg/ml) were divided into 0.7-ml aliquots and frozen at −80°C. Samples (0.5 ml) were repetitively analyzed on different days (one sample a day) to calculate between-run precision. A control sample containing 1.60 μg/ml was analyzed in one run (n = 8) to test within-run precision. In addition, the day-to-day variation on the slope of the calibration curves was calculated.

(iii) Recovery. The recovery was determined by repetitively analyzing plasma supplemented with known amounts of amoxicillin (0.42, 1.66, and 4.16 μg/ml), but with the addition of the internal standard after the evaporation of the methanolic eluate of the C18 extraction column. A mixture of the same amount of amoxicillin and the internal standard served as a reference. Similarly, the recovery of the internal standard cefadroxil was evaluated, with amoxicillin added at the end.

(iv) Detection limit. The detection limit was estimated from the size of the smallest amoxicillin peak obtained in plasma extracts. It was defined as the concentration in plasma that resulted in a detectable peak of approximately four times the noise level.

Pharmacokinetic analysis. Model-independent parameters were evaluated. The peak concentration of drug in serum and the time at which it was reached were the observed values. The elimination half-life was calculated from the terminal log-linear phase of the plasma concentration-time curve. The area under the curve was calculated by the trapezoidal rule.

RESULTS

Chromatography and plasma profiles. Figure 2A and B represent chromatograms of postdose bovine plasma and blank plasma, respectively. Amoxicillin and the internal standard cefadroxil eluted after 5.6 and 4.9 min, respectively. An acceptable blank trace was obtained, but a minor peak coeluted with amoxicillin (Fig. 2B). This interference had to be taken into account in the standardization.

Sample preparation. There was virtually no difference in the overall cleanliness of the extracts obtained with the two
tandem solid-phase extractions (double reversed-phase or ion-exchange plus reversed-phase procedure). However, the total recovery of amoxicillin in the double reversed-phase procedure was about 20% higher than that in the ion-exchange plus reversed-phase procedure (78% versus <60%). In the latter procedure, the recovery from the reversed-phase extraction column was nearly quantitative, but approximately 20% of the amoxicillin was not retained on the PRS column. The loss of another 20% of the amoxicillin before the application of the deproteinized extract could not exactly be accounted for, but it occurred in connection with the protein precipitation and/or evaporation of the methanol.

**Method validation.** The linear range of the standard curves extended to at least 17 μg/ml. However, in the daily routine, standard curves were constructed only in the range of 0.17 to 4.4 μg/ml. Linear regression analysis of standard curves always yielded correlation coefficients that exceeded 0.999. Data with regard to within-run and between-run precisions are given in Table 1, and day-to-day variations on the slope of the standard curves are given in Table 2. Table 3 lists recovery data for amoxicillin and the internal standard. The detection limit was set equal to the concentration corresponding to the small interfering plasma peak, i.e., roughly 0.1 μg/ml.

**Application.** The method was applied to a small-scale pharmacokinetic study of amoxicillin in three cows. Figure 3 shows a representative plasma amoxicillin-versus-time plot. Basic pharmacokinetic parameters for the three cows are listed in Table 4.

### DISCUSSION

The same two principles that formed the backbone of our previous ampicillin assay (11), i.e., the incorporation of a crown ether into the chromatographic eluent and a tandem solid-phase extraction, also were the basis of the determination of amoxicillin. However, in both respects amoxicillin showed an erratic behavior compared with that of ampicillin, so that the original chromatographic system and sample pretreatment could not be used. The original eluent containing 0.067 M KH₂PO₄, methanol, acetonitrile, and a crown ether failed to separate amoxicillin from large interfering plasma peaks, even when the percentage of acetonitrile was lowered to accommodate for the higher polarity of amoxicillin. Furthermore, amoxicillin also could not be separated from cefadroxil, which is structurally the most straightforward choice as an internal standard. Two modifications were necessary. First, a decrease of the molarity of potassium phosphate from 0.067 to 0.005 M led to a considerable improvement in resolution between amoxicillin and the interferences, mainly because of a specific increase in the retention of amoxicillin. This is consistent with the enhanced interaction between crown ethers and aminopenicillins at lower concentrations of potassium salts (10). Second, by omitting the methanol from the eluent, i.e., by dissolving the crown ether in the acetonitrile, amoxicillin and cefadroxil became separated at the baseline.

Unlike for ampicillin, the original tandem solid-phase extraction gave a recovery of amoxicillin from plasma that did not exceed 30%. Most of the loss (>50%) occurred on the ion-exchange extraction column, which gave poor retention, although the pKₐ of amoxicillin is nearly identical to that of ampicillin (2). This anomalous behavior could be attributed to the ionic strength of the mixture applied on the PRS column. With an amoxicillin standard dissolved in 0.01 M HCl, the recovery from PRS was 95%, whereas 0.05 M HCl lowered this value to 46%. However, the recovery of amoxicillin from plasma mixed with 0.01 M HCl was nil.

### TABLE 1. Reproducibility of amoxicillin determination by analysis of control samples

<table>
<thead>
<tr>
<th>Precision</th>
<th>Mean ± SD concn (μg/ml)</th>
<th>Coefficient of variation (%)</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run</td>
<td>1.596 ± 0.029</td>
<td>1.8</td>
<td>8</td>
</tr>
<tr>
<td>Between-run*</td>
<td>0.445 ± 0.031</td>
<td>7.0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1.780 ± 0.071</td>
<td>4.0</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>4.391 ± 0.175</td>
<td>4.0</td>
<td>10</td>
</tr>
</tbody>
</table>

* For the between-run precision values, theoretical (nominal) values were 0.416, 1.664, and 4.160 μg/ml, respectively.

### TABLE 2. Day-to-day variation on the slope of the calibration curves

<table>
<thead>
<tr>
<th>Slope (mean ± SD)</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.381 ± 0.016b</td>
<td>4.1</td>
</tr>
<tr>
<td>0.381 ± 0.007c</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* A total of 11 samples were tested.
  b Obtained on spiked samples.
  c Obtained by direct injection of standards.

### TABLE 3. Recovery of amoxicillin and cefadroxil (internal standard) from bovine plasma

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc (μg/ml)</th>
<th>Recovery (% [mean ± SD])</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>0.42</td>
<td>77.5 ± 3.6</td>
<td>7</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>1.66</td>
<td>80.2 ± 3.7</td>
<td>8</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>4.16</td>
<td>76.7 ± 2.7</td>
<td>8</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>Avg recovery</td>
<td>78.2 ± 3.0</td>
<td>23</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>1.5</td>
<td>74.6 ± 3.8</td>
<td>10</td>
</tr>
</tbody>
</table>

**FIG. 3.** Levels of amoxicillin in the plasma of a nonlactating cow (cow 2) as a function of time after administration of a dose of 10 mg/kg of body weight.
because the acid was too dilute to overcome the buffering action of the plasma. The final pH was only 3.5, which is well above the pKₐ of amoxicillin. The removal of the plasma proteins partly overcame this problem, but it could not prevent the persistent loss of about 40% of amoxicillin (20% on the PRS column and 20% elsewhere). Because the double reversed-phase cleanup method gave extracts of comparable purity but higher yields, and because it was also simpler (no need for protein precipitation and subsequent evaporation of methanol), the double reversed-phase procedure was selected for final use. Initially, for application on the cyclohexyl column, the plasma sample was diluted only with water and was mixed with the methanolic solution of the crown ether. The mean recovery was 80.0% ± 5.0% (x = 0.88 μg/ml; n = 6). All samples in connection with the pharmacokinetic study in cows were analyzed under those conditions, without any anomalous results. However, when the method was again used more than a year later, occasional abnormal losses of the internal standard were observed. The problem was readily solved by including sodium chloride in the sample before its application on the cyclohexyl cartridge. Sodium chloride reportedly increases the recovery of aminopenicillins in the presence of crown ethers on Sep Pak C₁₈ extraction columns (12). To avoid any further inconsistencies in recovery, possibly caused by batch-to-batch variations in the extraction columns, sodium chloride was maintained in the definitive procedure. The extent of purification was sufficient to permit concentration of the extract (final volume before injection 250 μl), so that the equivalent of 200 μl of plasma was injected onto the column. In less selective assays that use UV detection, this quantity is usually limited to 2.5 to 20 μl and the detection limits are on the order of 0.5 μg/ml (3, 4, 7, 13, 14). The limit of detectability for the method described here was about 0.1 μg/ml, which is a factor of 10 higher than the corresponding value for our ampicillin assay (11). The difference was mainly due to the presence of the small endogenous plasma peak that coeluted with amoxicillin. However, although this peak proved to be the sensitivity-limiting factor, it occurred in a reproducible way. That is, the peak height ratio (unknown versus internal standard) was fairly constant (0.048 ± 0.0057) and the peak was present in all samples, including those of human plasma. To compensate for the positive error, each run was standardized with drugfree plasma supplemented with amoxicillin and always included the analysis of a blank sample. The choice of cefadroxil, the cephalosporin equivalent of amoxicillin, as an internal standard has the same rationale as the use of cephalixin in our ampicillin assay (11). Both compounds behave in a similar way in the assay, as evidenced by their comparable recoveries (Table 3) and the equal slopes of the standard curves obtained by analyzing spiked samples and those obtained by direct injection (Table 2). Previous amoxicillin assays do not include internal standardization (1, 2, 4, 5, 7, 9, 13, 14) or use a compound that is structurally unrelated to the drug (3, 6).

The new method described here was applied to studies of the pharmacokinetics of amoxicillin in cows. A total of 20 samples, including 5 standards and 1 control sample, could be analyzed in a normal working day. Because of the complexity of the sample pretreatment, the present method has the same limitations as our corresponding ampicillin assay (11) in terms of sample throughput. Advantages of the method include its minimal technical requirements (no need for chemical or electrochemical derivatization or column switching) and HPLC column lifetime. The latter is likely to be reduced considerably by repeated injections of impure extracts obtained by a simple deproteinization of plasma. Compared with other such assays, the one described here offers the advantage of enhanced selectivity and sensitivity.

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