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Journal of Chromatography A, 812 (1998) 227–236

JOURNAL OF CHROMATOGRAPHY A

# Development and validation of amoxicillin determination by micellar electrokinetic capillary chromatography

Y.M. Li, A. Van Schepdael*, Y. Zhu, E. Roets, J. Hoogmartens

*Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Faculteit Farmaceutische Wetenschappen, K.U. Leuven, Van Evenstraat 4, B-3000 Leuven, Belgium*

**Abstract**

A micellar electrokinetic capillary chromatography (MECC) method has been developed and validated for the analysis of amoxicillin and its potential impurities. The influence of various parameters on the separation such as pH and concentration of the buffer, the concentration of sodium dodecyl sulfate (SDS), and organic solvent were investigated. final electrophoresis conditions were as follows: running buffer, 70 m*M* sodium dihydrogenphosphate, 125 m*M* SDS and 5% acetonitrile, adjusted to pH 6.0; length of fused-silica capillary, 44 cm (effective length 36 cm)×50 μm I.D.; voltage, 15 kV; temperature, 25°C; detection wavelength, 230 nm. The method allows amoxicillin to be completely separated from its potential impurities. The robustness of the method has been examined by means of a full-fraction factorial design. The parameters for validation, namely relative standard deviation, linearity, precision, limit of detection and limit of quantitation are also reported. The performance of MECC was compared with that of liquid chromatography. 

*Keywords:* Validation; Pharmaceutical analysis; Micellar electrokinetic capillary chromatography; Amoxicillin; Penicillins; Antibiotics

## 1. Introduction

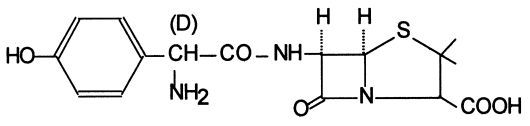
Amoxicillin is a semi-synthetic penicillin with activity against both gram-positive and gram-negative bacteria. It is available as an injectable, capsule or oral suspension. This molecule is relatively unstable both in aqueous solution and storage, leading to the formation of a variety of degradation products. It may also contain precursors from semi-synthesis, such as D-4-hydroxyphenylglycine (**2**) and 6-aminopenicillanic acid (6-APA, **3**), or side products from semi-synthesis, such as L-amoxicillin (**6**), 4-hydroxyphenylglycylamoxicillin (**9**) and *N*-pivaloyl-4-hydroxyphenylglycine (**13**). The structures of amoxicillin and a number of its potential impurities are shown in Fig. 1.

Liquid chromatography (LC) has been used extensively for the separation of amoxicillin from its potential impurities [1–4] and from other penicillins [5–8]. The assay of amoxicillin in biological samples can also be performed by LC [9,10].

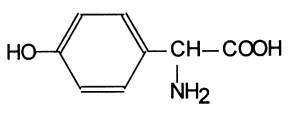
Capillary electrophoresis (CE) is a powerful separation and quantitation technique that often provides higher resolving power, shorter analysis time, and lower operational cost than LC. In recent years, the use of CE for analysis of pharmaceuticals and drugs has gained considerable importance and was discussed extensively in some reviews [11–13]. The perspectives of micellar electrokinetic capillary chromatography (MECC) in drug analysis were reviewed by Nishi and Terabe [14]. The CE analysis of

*Corresponding author.

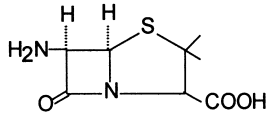
0021-9673/98/$19.00 
PII: S0021-9673(98)00287-8



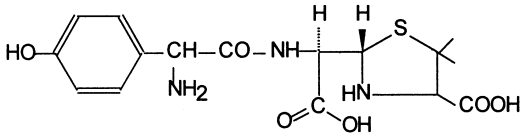
1. amoxicillin



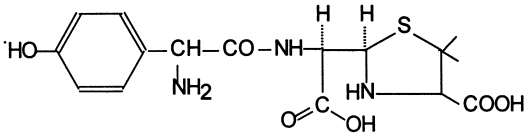
2. 4-hydroxyphenylglycine



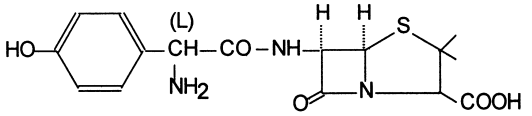
3. 6-APA



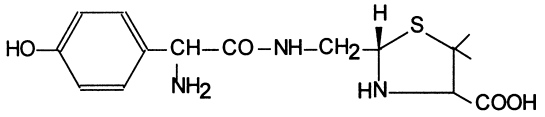
4. amoxicilloic acid (5S, 6R)



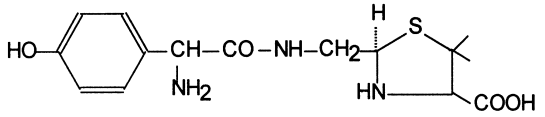
5. amoxicilloic acid (5R, 6R)



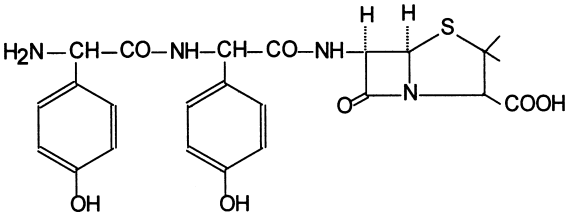
6. L-amoxicillin



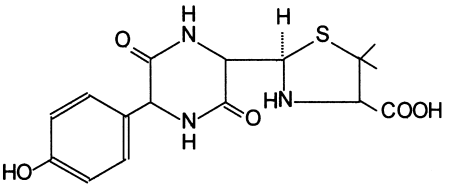
7. amoxilloic acid (5S)



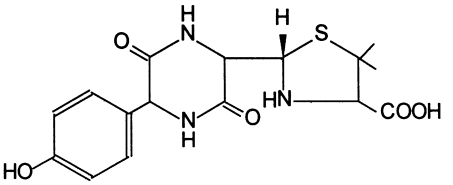
8. amoxilloic acid (5R)



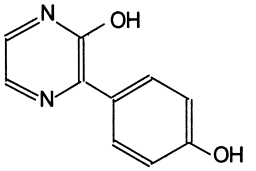
9. 4-hydroxyphenylglycylamoxicillin



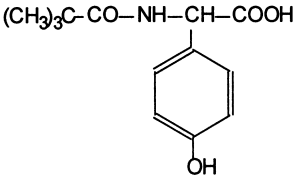
10. amoxicillin (5R) piperazine-2',5'-dione



11. amoxicillin (5S) piperazine-2',5'-dione

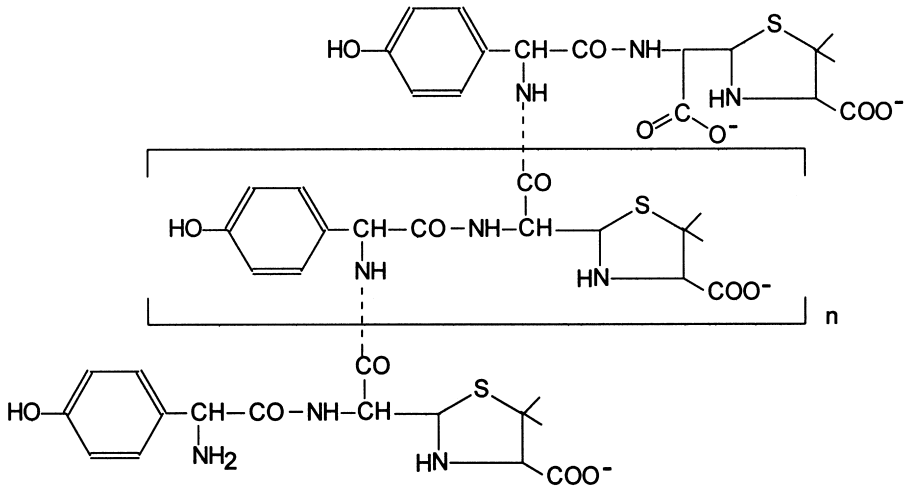


12. 2-hydroxy-3-(4-hydroxyphenyl)pyrazine

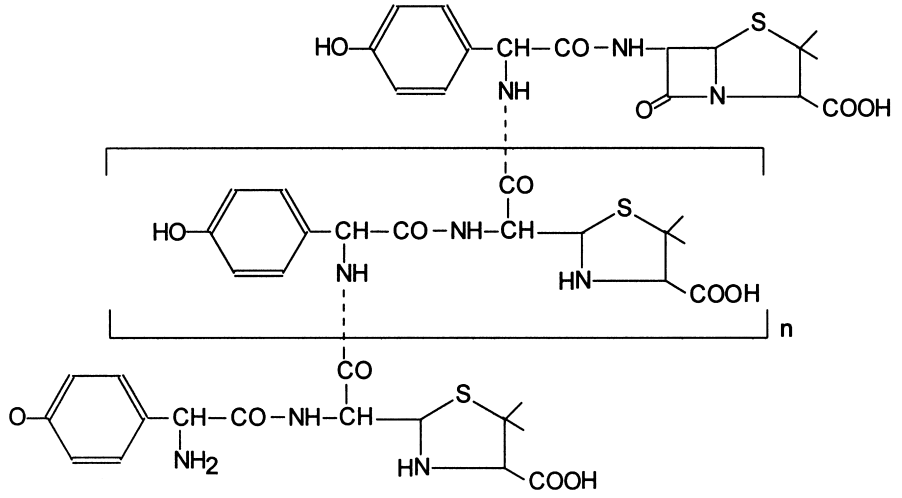


13. N-pivaloyl-4-hydroxyphenylglycine

Fig. 1. Structures of amoxicillin and its potential impurities.



**14. dimeroates** (n = 0)
**trimeroates** (n = 1)



**15. dimer** (n = 0)
**trimer** (n = 1)

Fig. 1. (*continued*)

antibiotics was reviewed by Bobbitt and Ng [15]. Our laboratory has reported some CE methods for separation of tetracycline and penicillin antibiotics from their related substances [16,17]. The use of CE for separating amoxicillin from five of its related compounds has also been reported [18].

The method presented here implements MECC because of its better selectivity. It enables the

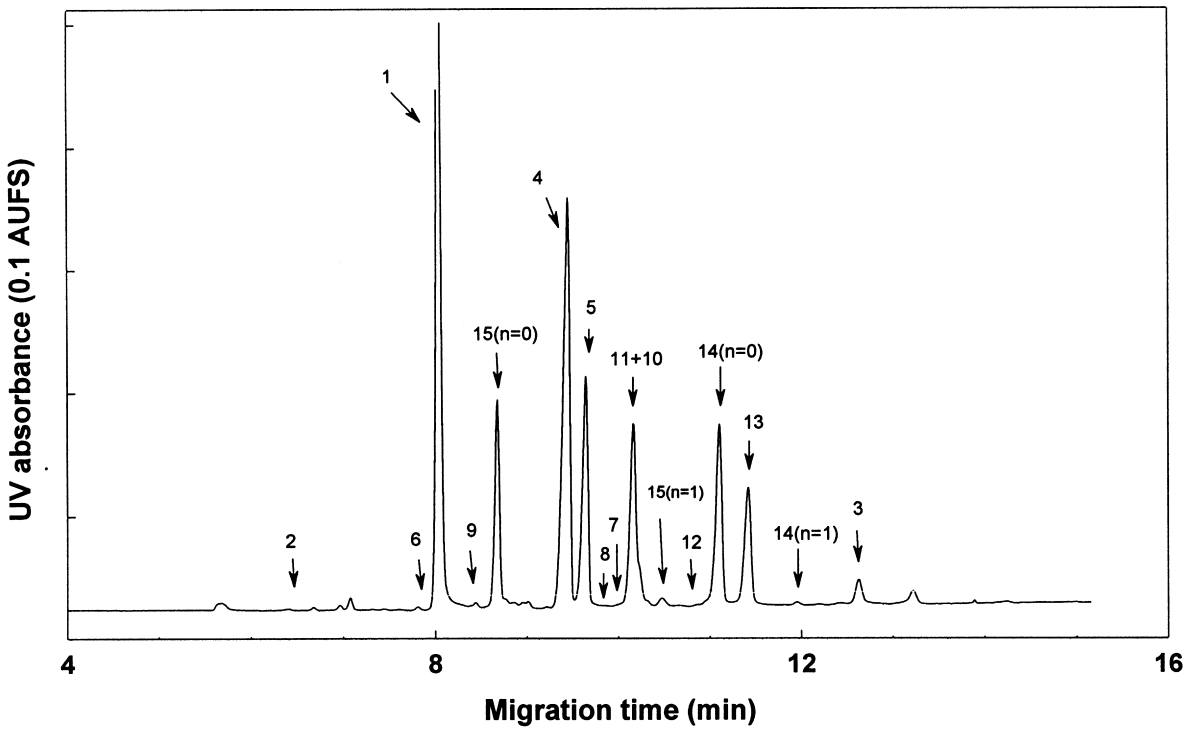



Fig. 2. Electropherogram of an old commercial sample of amoxicillin sodium salt. BGE, 70 m*M* sodium dihydrogenphosphate, 125 m*M* SDS and 5% acetonitrile, adjusted to pH 6.0; capillary: uncoated fused-silica, 44 cm (effective length 36 cm)×50 μm I.D.; temperature, 25°C; voltage, 15 kV; detection wavelength, 230 nm. See Fig. 1 for peak identification.

complete separation of amoxicillin from its potential impurities within a short analysis time. The robustness of this method was examined by applying a full-fraction factorial design at two levels [19,20]. Quantitative data is also reported.

## 2. Experimental

### *2.1. Instrumental and operating conditions*

CE was performed on Spectraphoresis 500 equipment (Thermo Separation Products, Fremont, CA, USA), coupled to a 3396 series II integrator (Hewlett-Packard, Avondale, PA, USA). UV detection was at 230 nm. Injection was done hydrodynamically for 4 s. pH measurements were performed on a Consort pH meter (Turnhout, Belgium) using calibration buffers constituted according to the European Pharmacopoeia [21]. When necessary, the pH of running buffers was adjusted using 1 *M* NaOH before making up to volume. The capillary was washed at the beginning of the day with 0.1 *M* NaOH for 5 min followed by a water wash for 5 min at 60°C. Before every analysis, the capillary was washed for 5 min with running buffer. All samples were dissolved in Milli-Q water with dimethyl sulfoxide (DMSO, 0.2%, v/v) as electroendosmotic flow (EOF) marker.

### *2.2. Materials*

All reagents were of analytical grade (Merck, Darmstadt, Germany or Acros Organics, Geel, Belgium). A fused-silica capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA): 44 cm (effective length 36 cm)×50 μm I.D. Every new capillary was conditioned with a 1 *M* NaOH rinse for 20 min, followed by water for 5 min at 60°C. Throughout the study, Milli-Q water was delivered by a Milli-Q$^{50}$ system (Millipore, Milford, MA,

USA). All the solutions were filtered through 0.2-μm nylon filters (Alltech, Laarne, Belgium).

Amoxicillin sodium and impurity **3** were obtained from Gist Brocades (Delft, Netherlands). Compound **2** was obtained from Acros Organics. Potential impurities **6** and **13** were obtained from Antibiotics and Biochemie (Spain). Amoxicilloic acid (5*S*,6*R*) (**4**) and amoxicilloic acid (5*R*,6*R*) (**5**) were prepared as described by Munro et al. [22]. The preparation of amoxilloic acid (5*S*) (**7**) and amoxilloic acid (5*R*) (**8**) was described recently by Zhu et al. [23]. 2-Hydroxy-3-(4-hydroxyphenyl)pyrazine (**12**) was prepared in a similar way as described by Lebelle et al. [24]. Amoxicillin(5*R*)piperazine-2′,5′-dione (**10**) and amoxicillin(5*S*)piperazine-2′,5′-dione (**11**) were prepared as described by Roets et al. [25] and Haginaka and Wakai [26]. 4-Hydroxyphenylglycylamoxicillin (**9**) was prepared in a similar way as described by Zhu et al. [27]. The oligomeroates (**14**) and oligomers (**15**) were prepared as described by Roets et al. [25] and Bundgaard and Larsen [28].

## 3. Results and discussion

### 3.1. Method development

All developmental experiments were performed in an uncoated fused-silica capillary. Using an artificial mixture consisting of amoxicillin and its main impurities, the influence of buffer pH (pH 5.5–7.5), phosphate concentration (40–80 m*M*), sodium dodecyl sulfate (SDS) concentration (50–150 m*M*) and acetonitrile concentration (0–10%) were investigated. As a result of this developmental work, the following electrophoretic conditions were chosen: running buffer, 70 m*M* sodium dihydrogenphosphate, 125 m*M* SDS and 5% acetonitrile adjusted to pH 6.0; voltage, 15 kV; temperature, 25°C; detection wavelength, 230 nm. This method allows amoxicillin to be completely separated from all its impurities. Fig. 2 shows a typical electropherogram of an old commercial sample of amoxicillin sodium salt, stored at room temperature. The selectivity of this MECC method is better than that of LC [4] and the MECC method also takes less time; 20 min including the washing procedure vs. more than 40 min for LC. Furthermore, the LC method needs a gradient elution, which is another disadvantage.

### 3.2. Robustness

The four important electrophoretic parameters, governing the separation process, were examined by applying a full-fraction factorial design at two levels. This involves $2^4=16$ different experimental measurements. One central point combination was included in the design, therefore, 17 measurements had to be performed as well as duplicate experiments 18–34.

The most relevant electrophoretic parameters examined were: (1) buffer pH; (2) phosphate concentration; (3) concentration of SDS and (4) concentration of acetonitrile. The values for the design are given in Table 1. As response variables in the factorial design, relative migration times were measured versus DMSO. Analysis of the measured response variables made it possible to obtain standardized pareto charts and response surface plots.

A standardized pareto chart consists of bars with a length proportional to the absolute value of the estimated effects, divided by the standard error. The codes A, B, C and D correspond to the buffer pH, the phosphate concentration, the concentration of SDS and the concentration of acetonitrile. The combination of two codes indicates the interaction

Table 1
Factorial design

| Electrophoretic parameter | Low value (−1) | Central value (0) | High value (+1) |
|---|---|---|---|
| Buffer pH | 5.5 | 6.0 | 6.5 |
| Phosphate concentration (m*M*) | 60 | 70 | 80 |
| SDS concentration (m*M*) | 100 | 125 | 150 |
| Acetonitrile concentration (%, v/v) | 2.5 | 5.0 | 7.5 |

Nominal values corresponding to −1, 0 and +1.

effect between the two parameters. The bars are displayed in order of the size of the effects, with the largest effects on top. The chart includes a vertical line at the critical *t*-value for an $\alpha$ of 0.05. The effects for which the bars are smaller than the critical *t*-value are considered as not significant and not affecting the response variables. These effects may be positive or negative.

Standardized pareto charts, representing the estimated effects of parameters and parameter interactions on relative migration times of L-amoxicillin (**6**), amoxicillin (**1**) and 4-hydroxyphenylglycylamoxicillin (**9**) are shown in Fig. 3. These substances were chosen because they migrate most closely to the main component. Fig. 3a and b show that the concentration of SDS and acetonitrile ($CH_3CN$) are parameters that have a significant influence on the migration of L-amoxicillin (**6**) and amoxicillin (**1**). Although the buffer pH (A) has no significant influence, there is a significant positive interaction with the $CH_3CN$ concentration (AD). This means that an increase in concentration of $CH_3CN$ affects the migration time of L-amoxicillin and amoxicillin more at a higher buffer pH than at a lower buffer pH. However, there is a significant negative interaction between the concentrations of SDS and $CH_3CN$ (CD), which means that an increase in concentration of SDS affects migration time of L-amoxicillin and amoxicillin more at lower concentration of $CH_3CN$ than at higher concentration. Fig. 3c shows that it is difficult to increase the resolution between L-amoxicillin and amoxicillin through adjustment of SDS and

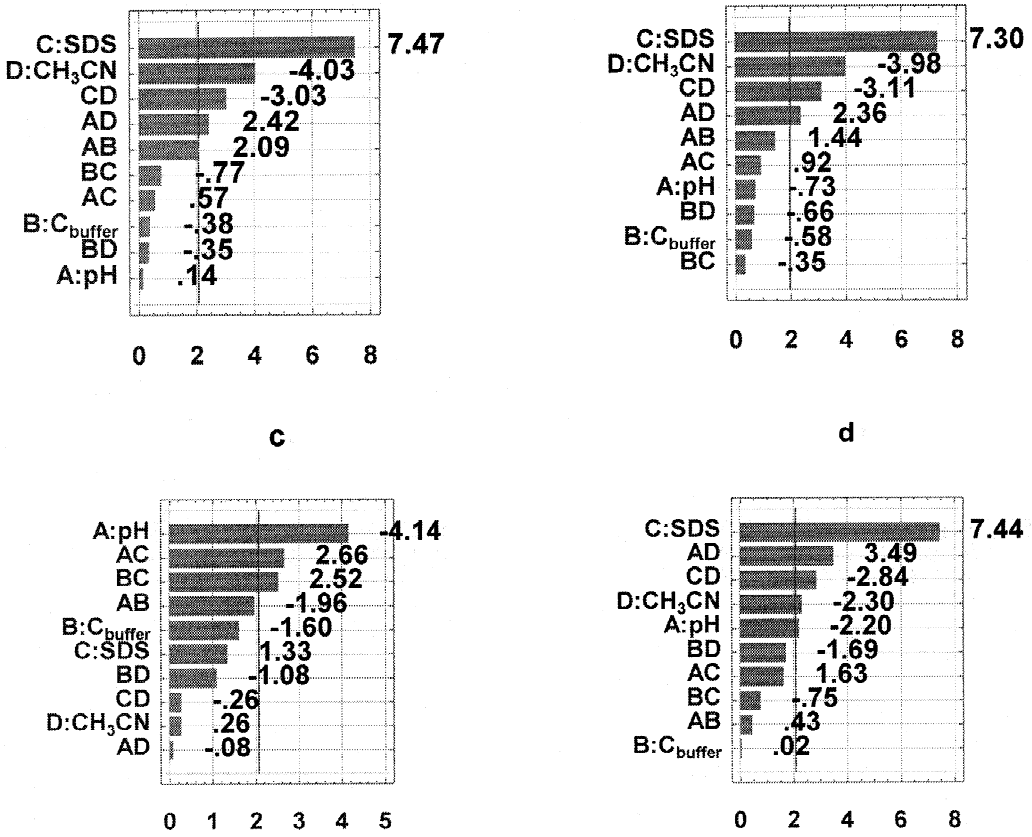



Fig. 3. Standardized pareto chart, representing the estimated effects of parameters and parameter interactions on the (a) relative migration time of L-amoxicillin (**6**), (b) relative migration time of amoxicillin (**1**), (c) selectivity between L-amoxicillin and amoxicillin, (d) relative migration time of 4-hydroxyphenylglycylamoxicillin (**9**). A=Buffer pH; B=phosphate concentration; C=concentration of SDS; D=concentration of acetonitrile.

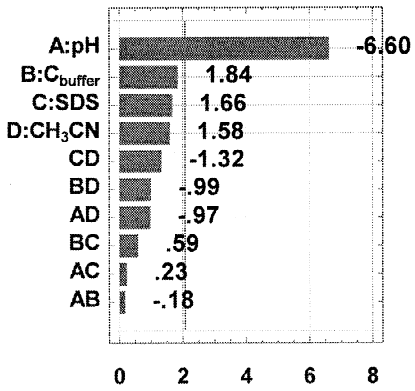



Fig. 4. Standardized pareto chart, representing the estimated effects of parameters and parameter interactions on the relative migration time of amoxicillin piperazine-2′,5′-dione (**10**+**11**).

$CH_3CN$. The selectivity can be modified, however, by changing the pH. Indeed, Fig. 3c shows that the pH is the only parameter with a significant influence on the selectivity between L-amoxicillin and amoxicillin. From Fig. 3d, it can be seen that the concentration of SDS and the interaction of $CH_3CN$ with pH and SDS concentration, significantly influenced the migration of 4-hydroxyphenylglycylamoxicillin (**9**). This compound migrates immediately after amoxicillin. An increase in concentration of SDS will lead to a significant increase of migration time. An increase in acetonitrile and pH will lower migration time.

The migration of one of the compounds investigated, amoxicillin piperazine-2′,5′-dione showed a striking dependence on buffer pH. The standardized

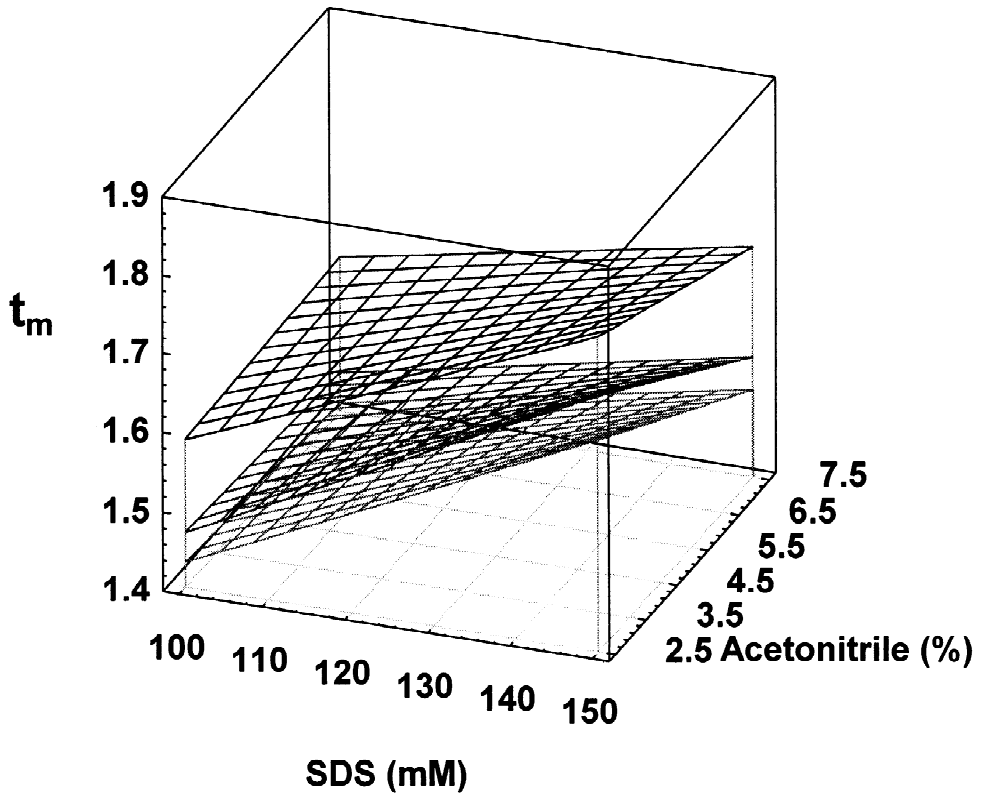



Fig. 5. Estimated response surface plot for relative migration times ($t_m$) of L-amoxicillin (lower plane), amoxicillin (middle plane) and 4-hydroxyphenylglycylamoxicillin (upper plane). The pH and buffer concentration were kept constant at pH 6.0 and 70 m*M*, respectively.

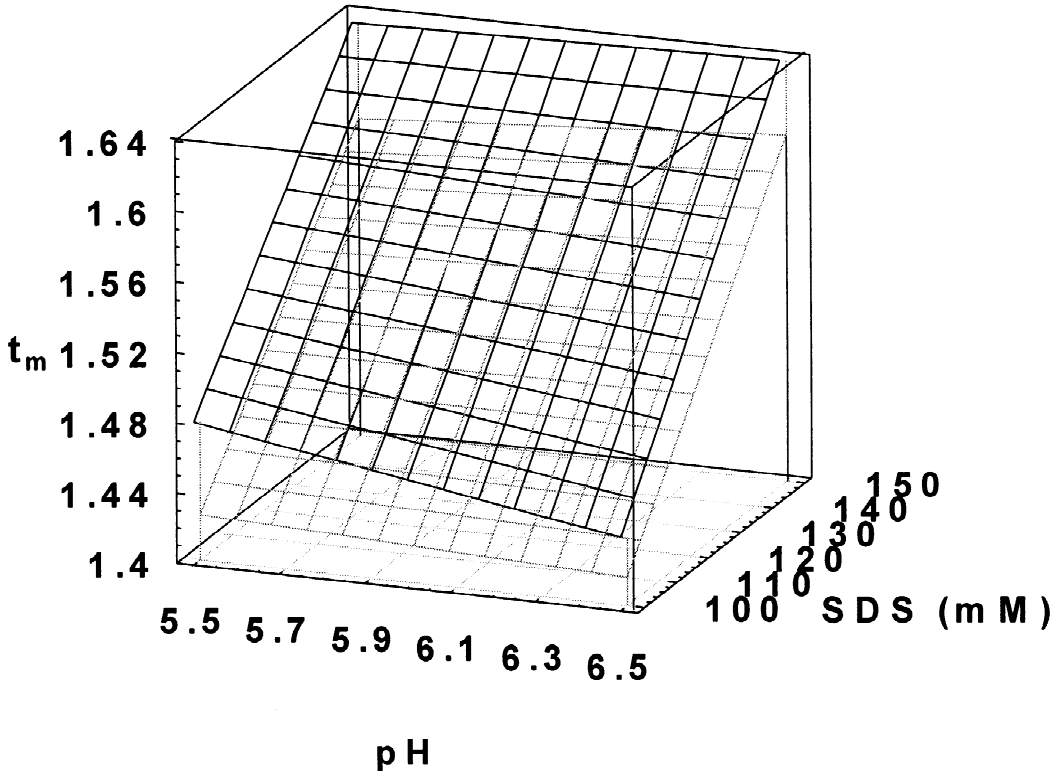



Fig. 6. Estimated response surface plot for relative migration times ($t_m$) of L-amoxicillin and amoxicillin. The buffer concentration and acetonitrile concentration were kept constant at 125 m*M* and 5% (v/v), respectively.

pareto chart for this compound is depicted in Fig. 4 and demonstrates that an increase in pH leads to a markedly decreased migration time. In order to explain this, the p$K_a$ values of amoxicillin piperazine-2′,5′-dione (**10**+**11**) and amoxilloic acid (**7**+**8**) were determined. The migration time of the latter was affected much less by pH. The following values have been obtained for amoxicillin piperazine-2′,5′-dione, 1.6 (carboxylic acid function), 3.3 (thiazolidine function) and 9.3 (fenol function), and for amoxilloic acid, 1.3 (carboxylic acid function), 4.0 (thiazolidine function), 7.8 (amine function) and 9.6 (fenol function). However, this structure is not available as the fully protonated molecule and must be considered as zwitterionic structure. Considering the p$K_a$ values of 3.3 and 7.8, amoxicillin piperazine-2′,5′-dione is a stronger acid than amoxilloic acid. For a stronger acid, the distribution to the micelle will be reduced, causing a significant decrease in migration time.

In order to determine the influence of the most important parameters, as shown by the pareto charts, response surface plots were constructed. Surface plots allow one to visualise the evolution of the relative migration time in function of two variable parameters. Fig. 5 shows how the relative migration times of L-amoxicillin (**6**), amoxicillin (**1**) and 4-hydroxyphenylglycylamoxicillin (**9**) vary as a function of the SDS concentration and the acetonitrile concentration. The pH and buffer concentration were kept constant at 6.0 and 70 m*M*, respectively. Since the planes do not overlap, it can be concluded that this method is robust. In view of the fact that the selectivity between L-amoxicillin and amoxicillin was largely influenced by pH (see Fig. 3c), a response surface plot was also constructed for pH combined with concentration of SDS. Fig. 6 depicts how the relative migration times of L-amoxicillin and amoxicillin change with respect to pH and concentration of SDS. Here too, planes do not overlap. It should be pointed out, however, that small variations in the electrophoretic parameters will strongly affect

Table 2
Quantitative data for MECC of amoxicillin

| Parameter | Amoxicillin |
|---|---|
| Within-day repeatability ($n=6$)[a] | |
| Migration time | R.S.D.=1.4% |
| Corrected area | R.S.D.=0.84% |
| Day-to-day repeatability ($n=3$)[a] | |
| Migration time | R.S.D.=3.5% |
| Corrected area | R.S.D.=1.7% |
| Linearity[b] | $y=256282x-3464$<br>$r=0.9992$ |
| LOD ($S/N=3$)[c] | 3.3 pg (0.02%) |
| LOQ, R.S.D.=9.3% ($n=9$)[c] | 13.3 pg (0.08%) |

[a] Sample concentration, 1.0 mg/ml.
[b] $y$=Corrected area, $x$=amoxicillin concentration (mg/ml), range=0.25–1.50 mg/ml, number of concentrations=6, total number of analyses=12.
[c] Hydrodynamic injection, 9.9 s.

the separation between 4-hydroxyphenylglycyl-amoxicillin (**9**) and dimer (**15**, $n=0$).

### *3.3. Quantitative analysis*

The quantitative features of this method were examined and the results are shown in Table 2. Relative standard deviations (R.S.D.s) are higher than with LC where R.S.D. values of 0.15% (within-day) and 0.3% ($n=8$, day-to-day) were found [4]. As to the limit of detection (LOD), this value was the same for LC and CE (0.02%) [4]. The limit of quantitation (LOQ) reached beneath 0.1% for LC (0.05% [4]) as well as for CE.

Since the separation between L-amoxicillin, amoxicillin and 4-hydroxyphenylglycylamoxicillin is robust, and since the quantitative features are good, this method can be proposed for the assay of amoxicillin. Determination of potential impurities could be found to be delicate because their migration can depend very much on slight variations in the preparation of the background electrolyte (BGE) (see above). However, it has been shown that the separation power of the system is very good.

## 4. Conclusions

A MECC method was developed for analysis of amoxicillin. Compared to LC, it offers the advantages of speed and better selectivity, but LC still performs better in quantitative analysis. A full fraction factorial design pointed out that acetonitrile and SDS concentration are parameters with a large effect on relative migration times. The response surface plots showed that the separation of the main compound amoxicillin from its neighbouring impurities was robust.

## Acknowledgements

Fruitful discussions with Dr. J.O. De Beer, Wetenschappelijk Instituut Volksgezondheid Louis Pasteur, Brussels, Belgium, are gratefully acknowledged. A. Jacobs, Janssen Research Foundation, Beerse, Belgium is acknowledged for p$K_a$ determinations. The authors thank the Flemish Fund for Scientific Research (FWO) for financial support.

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