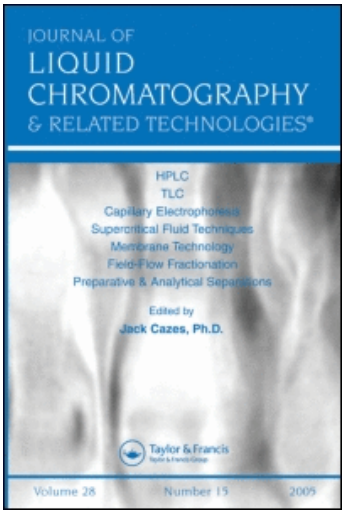


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High Speed HPLC Determination of *Bis*(Pivaloyloxymethyl)-PMEA and Its Degradation Products, Mono(POM)-PMEA and PMEA

P. Augustijns[a]; P. Annaert[a]; S. Adriaens[a]; E. De Clercq[b]; R. Kinget[a]
[a] Galenical and Clinical Pharmacy, Katholieke Universiteit Leuven, Leuven, Belgium [b] Rega Institute for Medical Research Katholieke Universiteit Leuven, Leuven, Belgium

To cite this Article Augustijns, P. , Annaert, P. , Adriaens, S. , De Clercq, E. and Kinget, R.(1996) 'High Speed HPLC Determination of *Bis*(Pivaloyloxymethyl)-PMEA and Its Degradation Products, Mono(POM)-PMEA and PMEA', Journal of Liquid Chromatography & Related Technologies, 19: 14, 2271 — 2283
To link to this Article: DOI: 10.1080/10826079608017156
URL: http://dx.doi.org/10.1080/10826079608017156

J. LIQ. CHROM. & REL. TECHNOL., 19(14), 2271–2283 (1996)

HIGH SPEED HPLC DETERMINATION OF BIS(PIVALOYLOXYMETHYL)-PMEA AND ITS DEGRADATION PRODUCTS, MONO(POM)-PMEA AND PMEA

P. Augustijns,[1] P. Annaert,[1] S. Adriaens,[1]
E. De Clercq,[2] R. Kinget[1]

[1] Galenical and Clinical Pharmacy
[2] Rega Institute for Medical Research
Katholieke Universiteit Leuven
B-3000 Leuven, Belgium

ABSTRACT

A high-speed HPLC gradient method for the determination of the antiviral ester prodrug bis(POM)-PMEA and its degradation products mono(POM)-PMEA and PMEA, is described. Perfusion chromatography enabled us to increase the flow rate up to 4 mL/min, resulting in high sample turn-over. Using a gradient system, the three compounds were separated from each other and the sample matrix within six minutes, while column equilibration was obtained in less than 3 minutes. Tetrabutylammonium was used as a counterion for PMEA. Notwithstanding the high flow rate, excellent reproducibility was obtained: the intraday as well as the interday precision, expressed as the relative standard deviation, for concentrations ranging from 5 to 50 μM PMEA and bis(POM)-PMEA, was lower than 5%. The applicability of the method is demonstrated by studying the temperature- and pH-dependent degradation of bis(POM)-PMEA.

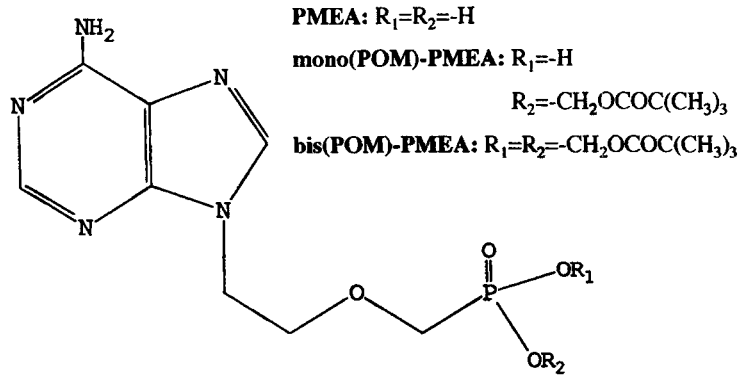


Figure 1. Structure of PMEA and bis(POM)-PMEA.

INTRODUCTION

9-(2-phosphonylmethoxyethyl)adenine (PMEA) is an acyclic nucleoside phosphonate analogue (Figure 1), which shows promising antiviral activity against herpes-, retro- and hepadnaviruses.[1-4] Unfortunately, oral bioavailability is limited by low permeability through biological membranes. Therefore, several ester prodrugs have been developed which show enhanced membrane permeability.[5-7] The bis(pivaloyloxymethyl) ester of PMEA (bis(POM)-PMEA, Figure 1) is such an oral prodrug of PMEA.[8] It is presently in clinical trials in patients infected with human immunodeficiency virus (HIV) or hepatitis B virus (HBV). Bis(POM)-PMEA is converted to mono(POM)-PMEA by enzymatic and chemical degradation, and further, to PMEA by enzyme-catalysed ester hydrolysis.[6] Concentrations of bis(POM)-PMEA and its degradation products can be measured using an HPLC system with UV detection (260 nm). The difference in polarity of the compounds to be determined, requires the use of a gradient system. Disadvantages of a gradient method using a conventional column are a relatively long analysis time, followed by extended column re-equilibration. As pharmacokinetic studies, as well as stability studies, yield a large number of samples to be analysed, a long analysis time results in long apparatus occupation times.

In this study, a high-speed HPLC method is presented for the simultaneous determination of bis(POM)-PMEA, mono(POM)-PMEA and PMEA by using perfusion chromatography. The applicability of the analysis method described is illustrated by studying the temperature- and pH-dependent degradation of bis(POM)-PMEA.

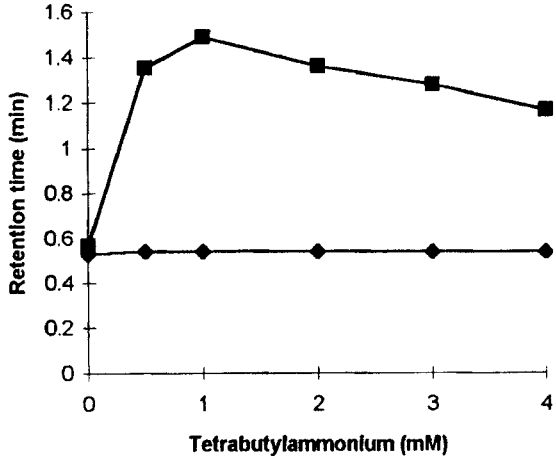


Figure 2. Effect of the concentration of tetrabutylammonium in the mobile phase on the retention of PMEA (potassium phosphate 2 mM, pH 9.0, flow rate 3 mL/min) (● : solvent peak; ■: PMEA).

MATERIALS AND METHODS

Reagents

Bis(POM)-PMEA was synthesized at Gilead Sciences. PMEA was kindly provided by Dr. A. Holý (Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic). Acetonitrile (far UV grade) was purchased from BDH (Poole, England). Tetrabutylammonium dihydrogen phosphate (99 %) and potassium dihydrogen phosphate (Suprapur) were from Fluka (Neu-Ulm, Switzerland) and Merck (Darmstadt, Germany), respectively.

Chromatographic Conditions

The HPLC system was equipped with a Waters (Millford, Mass.) Model 600 Controller, Model 717 plus auto-injector and a Model 480 Lambda Max UV detector (260 nm). Chromatograms were recorded and processed using the Maxima 820 computer programme (Waters). The column used was a 4.6-mm diameter x 100 mm length (1.7 mL column bed volume) POROS© R1/H (PerSeptive Biosystems, Cambridge, MA). The packing consisted of 10 μm

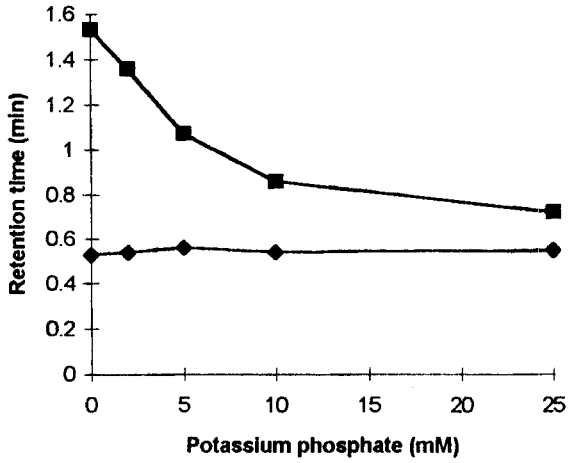


Figure 3. Effect of the concentration of potassium phosphate in the mobile phase on the retention of PMEA (tetrabutylammonium 2 mM, pH 9.0, flow rate 3 mL/min) (● : solvent peak; ■ : PMEA).

flow-through particles of cross-linked poly(styrene-divinylbenzene). Mobile phase A was an aqueous buffer containing 1 mM phosphate and 0.5 mM tetrabutylammonium (pH adjusted to 8.0 with NH_3 5%). Mobile phase B contained 30 % acetonitrile and 70 % water. Separation was carried out with an initial isocratic stage of 1 min in mobile phase A and a linear gradient from 3 to 90 % mobile phase B over 3.6 min, followed by an isocratic stage of 0.8 min and a return to initial conditions over 0.8 min. Column re-equilibration in solvent A took 2.8 min. The flow rate of the mobile phase varied between 3 and 4 mL/min. Both mobile phases were filtered and degassed continuously by He sparging. Samples were stored at a temperature of 4 °C in the autosampler, while the separation was carried out at ambient temperature. The injection volume amounted to 25 μL. System back pressure in mobile phase A was approximately 600 psi.

Stock Solutions and Calculations

Solutions of PMEA and bis(POM)-PMEA were made up in an isotonic buffer solution (pH as mentioned in Figure legends). Concentrations of PMEA and bis(POM)-PMEA were determined using a calibration curve made up by standards, in which concentration was plotted against peak area. No internal

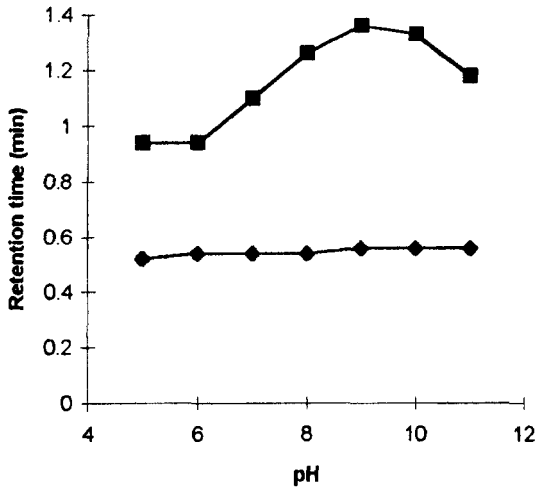


Figure 4. Effect of the pH of the mobile phase on the retention of PMEA (potassium phosphate 2 mM, tetrabutylammonium 2 mM, flow rate 3 mL/min)(◆: solvent peak; ■ : PMEA).

standard was used. Concentrations of mono(POM)-PMEA were determined using the calibration curve of bis(POM)-PMEA, because at the time of method development no mono(POM)-PMEA was available. This was justified because no change in UV response could be observed when bis(POM)-PMEA was gradually degraded into mono(POM)-PMEA.

RESULTS AND DISCUSSION

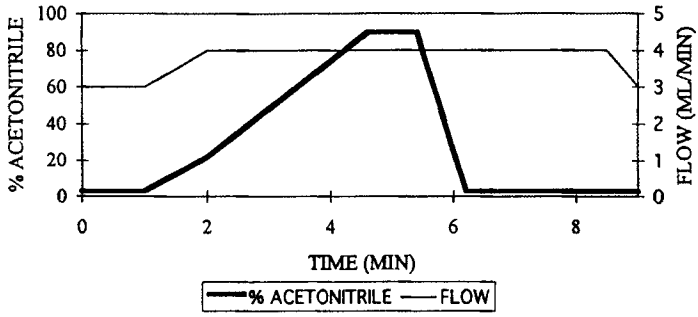
PMEA offers great promise as antiviral agent because of its broad-spectrum activity against retro-, herpes- and hepadnaviruses. However, like other acyclic nucleoside phosphonates, PMEA shows low cellular uptake and poor oral bioavailability. Therefore, prodrugs have been developed with improved cellular uptake and oral bioavailability. This prodrug approach has yielded bis(POM)-PMEA. This compound had originally been selected as the lead compound out of a series of prodrugs because of its permeability characteristics across Caco-2 monolayers,[9] which is an in vitro model for the intestinal mucosa.[10] Oral bis(POM)-PMEA has proved effective in suppressing murine retroviral infections in SCID mice;[11] following oral administration of bis(POM)-PMEA, only PMEA, but not bis(POM)-PMEA, was recovered from plasma, which suggests that, following uptake by the intestinal mucosa, bis(POM)-PMEA was readily cleaved to free PMEA (oral bioavalability being

53 %). Indeed, in aqueous solution, bis(POM)-PMEA is chemically degraded to mono(POM)-PMEA, while, in biological systems, bis(POM)-PMEA is first degraded to mono(POM)-PMEA and further on to PMEA by enzyme-catalysed ester hydrolysis.

Determination of the concentrations of these compounds is usually performed by HPLC.[11] Polarity differences between the parent compound and its metabolites necessitate the use of a gradient system. Disadvantage of a conventional column when using a gradient, are the relatively long analysis time and extended re-equilibration after returning to the initial conditions. To increase sample turn-over, a high speed HPLC gradient method was developed using perfusion chromatography. This enabled us to reduce analysis time without loosing resolution or reproducibility by increasing the flow rate of the mobile phase. High flow rates are made possible by the bimodal pore structure of the packing material, which is a base matrix of poly(styrene-divinylbenzene). Through-pores of 6000-8000 Å allow the mobile phase to flow through the packing material, while diffusion is possible in smaller pores (800-1500 Å) that are connected to them.[12] The presence of the pores allows high speed analysis, without a dramatic increase in backpressure.

In a first set of experiments, the effect of various separation parameters, relating to both mobile phase and sample were investigated. The retention of PMEA ($pKa_1 = 2.0$; $pKa_2 = 6.8$) on the column, was strongly dependent on the concentration of tetrabutylammonium as a counterion for PMEA, the concentration of phosphate ions and the pH of the mobile phase. The influence of different experimental conditions on the retention of PMEA was investigated in the isocratic mode at a flow rate of 3 mL/min. Increasing the concentration of the counterion resulted in a satisfactory separation at 0.5 mM, reaching a maximum retention at a concentration of 1 mM (Figure 2; other parameters: 2 mM phosphate, pH 9.0). Potassium phosphate reduced the retention of PMEA in the presence of counterion, which indicated that a low capacity buffer had to be used (Figure 3; other conditions: tetrabutylammonium 2 mM, pH 9.0). As the column material allowed an increase of the pH of the mobile phase to 14, the pH effect could be studied over a broad pH range.

Optimum separation from the sample matrix was obtained at pH 9.0 (Figure 4; other conditions: tetrabutylammonium 2 mM, phosphate 2 mM). However, this high pH could not be used for the simultaneous determination of PMEA and its parent compound bis(POM)-PMEA when using a gradient system, because on column degradation of bis(POM)-PMEA was observed at this high pH. Based on these observations, mobile phase A was composed of 0.5 mM counterion, 1 mM phosphate and a pH of 8.0. After an isocratic stage of 1 min at the initial conditions (3 mL/min), the gradient was started as illustrated in Figure 5 (upper panel). Perfusion chromatography enabled us to



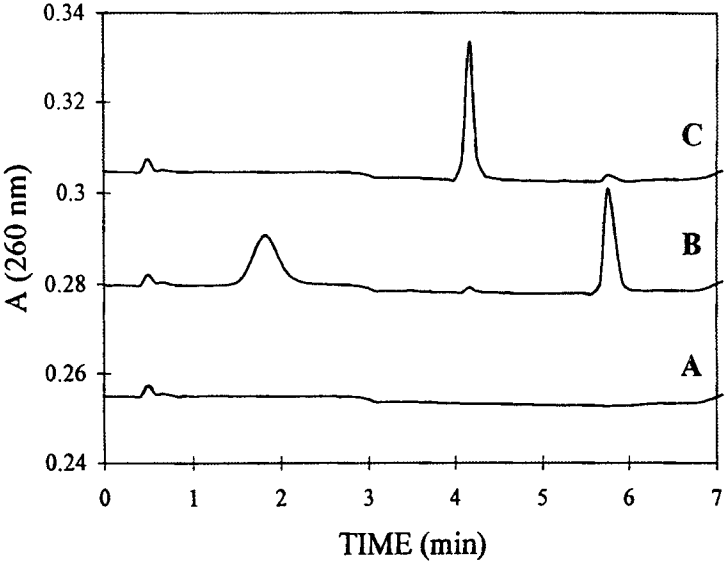


Figure 5. Top panel: Time course of the gradient and flow rate. Lower panel: chromatograms of a blank sample (A), a mixed PMEA / bis(POM)-PMEA sample (B), and a mono(POM)-PMEA sample (C) (PMEA, rt = 1.8 min; mono(POM)-PMEA, rt = 4.1 min; bis(POM)-PMEA, rt = 5.7 min).

Table 1

Reproducibility of PMEA Analysis

Concentration Added (μM)	Concentration Found ± SD	RSD*
Intraday variation (n=5)		
50	50.2 ± 0.3	0.55
40	40.1 ± 0.3	0.84
30	29.8 ± 0.4	1.33
20	20.1 ± 0.2	1.16
10	9.9 ± 0.2	2.09
5	5.4 ± 0.2	3.44
Interday variation (n=7)		
50	50.5 ± 0.7	1.31
40	40.0 ± 0.6	1.38
30	29.8 ± 0.9	2.87
20	19.8 ± 0.5	2.25
10	10.3 ± 0.3	3.25
5	5.4 ± 0.2	3.82

* relative standard deviation

increase the flow rate during the gradient to 4 mL/min. PMEA, mono(POM)-PMEA and bis(POM)-PMEA were well separated from each other and the sample matrix within six minutes, while column equilibration was obtained in less than three minutes. Typical chromatograms recorded under optimized conditions are given in Figure 5 (lower panel). All samples used in this study were made up in isotonic buffers. PMEA as well as bis(POM)-PMEA showed linearity over a range of 5 to 50 μM (r^2>0.999). Detection limits were 2.5 μM PMEA and 0.625 μM bis(POM)-PMEA, corresponding to absolute amounts of approximately 60 and 15 pmol. Intraday and interday precision were determined as shown in Tables 1 and 2.

The applicability of the method is demonstrated by studying the temperature- and pH-dependent degradation of bis(POM)-PMEA. Aqueous solutions of bis(POM)-PMEA are known to be unstable, with rapid hydrolysis of one of the pivaloyl ester bounds, followed by spontaneous elimination of

Table 2

Reproducibility of bis(POM)-PMEA Analysis

Concentration added (μM)	Concentration Found ± SD	RSD*
Intraday variation (n=5)		
50	49.7 ± 0.4	0.77
40	39.6 ± 0.3	0.72
30	29.5 ± 0.2	0.69
20	19.6 ± 0.2	0.77
10	9.7 ± 0.1	1.08
5	5.0 ± 0.1	1.33
Interday variation (n=7)		
50	50.4 ± 1.1	2.20
40	39.9 ±1.2	2.92
30	30.0 ± 0.9	3.00
20	20.0 ± 0.4	2.04
10	10.3 ± 0.2	1.43
5	5.4 ± 0.3	4.99

* = relative standard deviation.

formaldehyde, yielding mono(POM)-PMEA. The ester hydrolysis was monitored by a decline in parent compound and the appearance of mono(POM)-PMEA. Concentrations of mono(POM)-PMEA were determined using the calibration graph of bis (POM)-PMEA. This was justified because no change in UV response (260 nm) could be observed when bis(POM)-PMEA was gradually degraded into mono(POM)-PMEA (data not shown). Chemical degradation of bis(POM)-PMEA as a function of time is displayed in Figure 6. The stability of aqueous bis(POM)-PMEA solutions at different pH values is illustrated in Figure 7. Bis(POM)-PMEA was shown to be stable for at least one day at pH 3. At pH 7.4, a 40 % decline of bis(POM)-PMEA was observed after an eight-hour period. An analogous experiment at varying temperatures of the aqueous solution (pH 7.4) revealed that even at 4°C, a decline of 5 % was observed after 24 hr (Figure 8). Therefore, all samples in further studies (usually pH 7.4) were

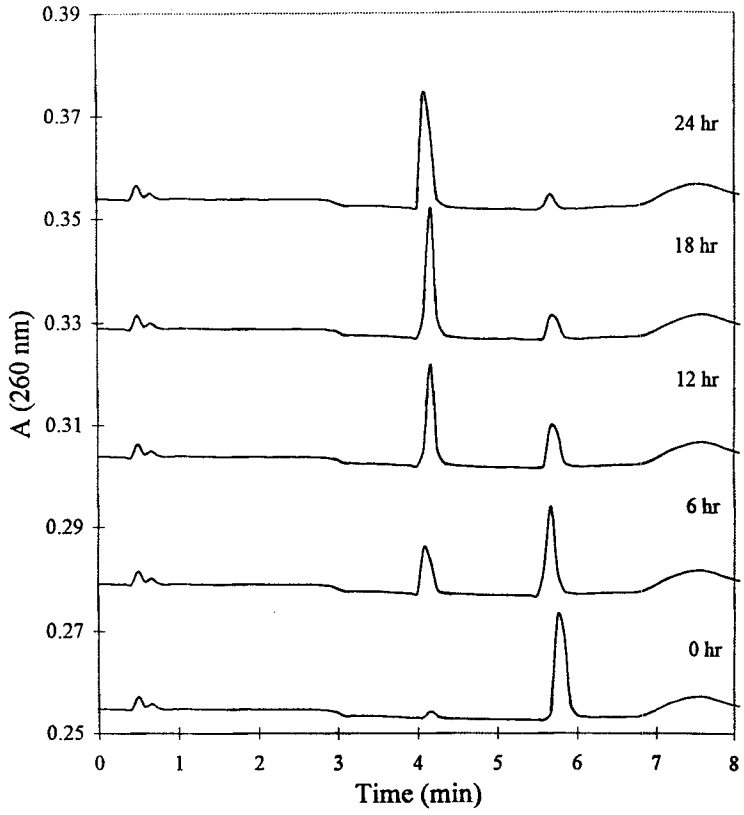


Figure 6. Chromatograms displaying the chemical degradation of bis(POM)-PMEA at a concentration of 50 μM (37°C, pH 7.4) as a function of time (mono(POM)-PMEA, rt = 4.1 min; bis(POM)-PMEA, rt = 5.7 min).

stabilized by adding 200 μL HCL 0.05 N to 800 μL sample, thus adjusting the pH to 3.3. Peak splitting of PMEA was observed if 50-μL samples were injected; therefore, the injection volume was restricted to 25 μL. The formation of PMEA was not observed in this study; the appearance of PMEA in *in vivo* or in *in vitro* studies results from enzyme-catalysed ester hydrolysis. As the back pressure was only 600 psi, it may even be possible to further increase the flow rate of the mobile phase. In conclusion, the high speed HPLC gradient method presented in this study offers sufficient sensitivity for the quality control of bis(POM)-PMEA preparations.

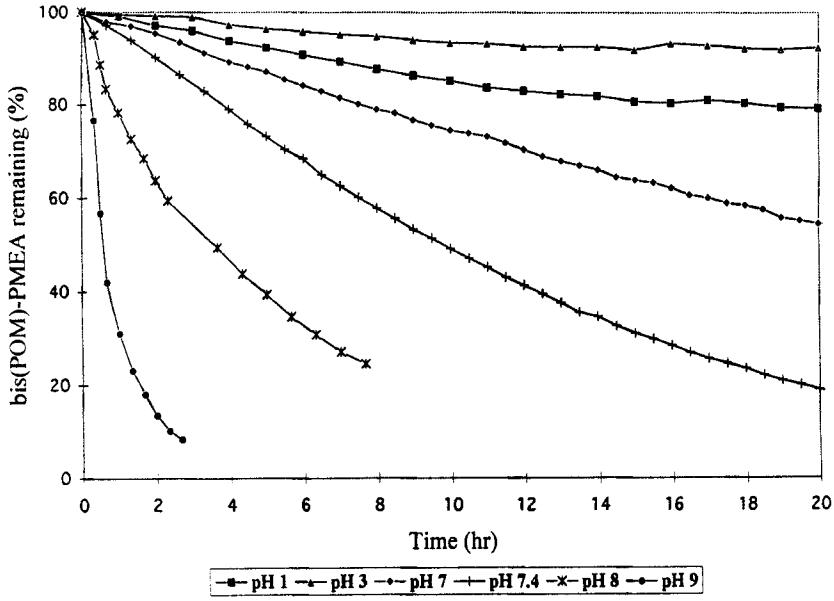


Figure 7. Influence of pH on the degradation of bis(POM)-PMEA

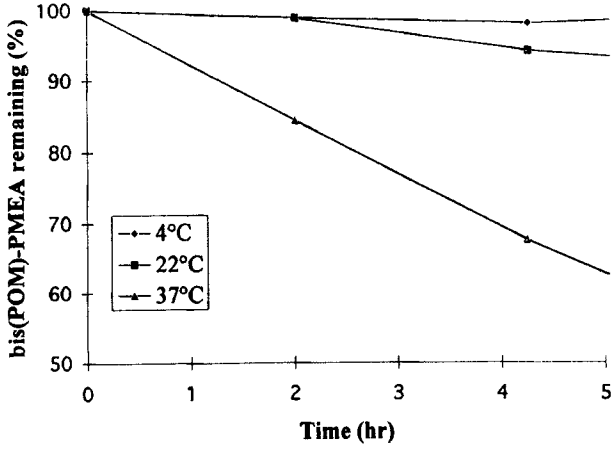


Figure 8. Influence of temperature on the degradation of bis(POM)-PMEA.

The short analysis time allows to increase the sample turn-over and to decrease the apparatus occupation time. However, the method may not provide the sensitivity needed for the analysis of samples obtained from pharmacokinetic studies *in vivo*.

ACKNOWLEDGEMENT

This study was supported by a grant from the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek (NFWO). P. Annaert acknowledges the receipt of a fellowship from the Flemisch Institute for the promotion of scientific -technological research in the industry (IWT).

REFERENCES

1. E. De Clercq, A. Holý, I. Rosenberg, T. Sakuma, J. Balzarini, P. C. Maudgal, Nature, **323**, 464-467 (1986).

2. E. De Clercq, Biochem. Pharmacol., **42**, 963-972 (1991).

3. E. De Clercq, Clin. Microbiol. Rev., **8**, 200-239 (1995).

4. L. Naesens, J. Balzarini, E. De Clercq, Rev. Med. Virol., **4**, 147-159 (1994).

5. H. T. Serafinowska, R. J. Ashton, S. Bailey, M. R. Harnden, S. M. Jackson, D. Sutton, J. Med. Chem., **38**, 1372-1379 (1995).

6. R. V. Srinivas, B. L. Robbins, M. C. Connelly, Y.-F. Gong, N Bischofberger, A. Fridland, Antimicrob. Agents Chemother., **37**, 2247-2250 (1993).

7. J. E. Starrett, D. R. Tortolani, J. Russell, M. J. M. Hitchcock, V. Whiterock, J. C. Martin, M. M. Mansuri, J.Med.Chem., **37**: 1857-1864 (1994).

8. K. C. Cundy, J. A. Fishback, J.-P.Shaw, M. L. Lee, K. F. Soike, G. C. Visor, W.A. Lee, Pharm. Res., **11**, 839-843 (1994).

9. J. P. Shaw, K. C. Cundy, Pharm. Res., **10** (Suppl.), S-294 (1993).

10. I. J. Hidalgo, T. J. Raub, R. T. Borchardt, Gastroenterology, **96**: 736-749 (1989).

11. L. Naesens, J. Balzarini, N. Bischofberger, E. De Clercq, Antimicrob. Agents Chemother., in press (1995).

12. F. E. Regnier, Nature, **350**, 634-635 (1991).

Received January 15, 1996
February 5, 1996
Manuscript 4068