Interaction between hydroxypropyl methylcellulose and biphasic calcium phosphate after steam sterilisation: capillary gas chromatography studies.

Xavier Bourges, Michel Schmitt, Yves Amouriq, Guy Daculsi, Gilbert Legeay, Pierre Weiss

Interaction between hydroxypropyl methylcellulose and biphasic calcium phosphate after steam sterilisation: capillary gas chromatography studies

Xavier Bourges [1,2], Michel Schmitt [2], Yves Amouriq [2], Guy Daculsi [2], Gilbert Legeay [1], Pierre Weiss [2].

1- Centre de Transfert de Technologie du Mans, Département Matériaux, 20 rue Thalès de Milet, 72000 Le Mans, France.

2- INSERM E 99-03, Laboratoire des matériaux d'intérêt biologique, 1 place Alexis Ricordeau, 44042 Nantes Cedex, France.

Short Title: Chemical stability of injectable bone substitute

Correspondence to:

Pierre Weiss

INSERM 99-03, Laboratoire des matériaux d'intérêt biologique,
UFR d'odontologie
1 place Alexis Ricordeau, 44042 Nantes Cedex, France.

Abstract

The purpose of this study was to check the chemical stability of an injectable bone substitute (IBS) composed of a 50/50 w/w mixture of a 2.92% hydroxypropyl methylcellulose (HPMC) solution in deionised water containing biphasic calcium phosphate (BCP) granules (60% hydroxyapatite/40% β-tricalcium phosphate w/w). After separation of the organic and mineral phases, capillary gas chromatography (GC) was used to study the possible modification of HPMC due to the contact with BCP granules following steam sterilisation and 32 days of storage at room temperature. HPMC was extracted from IBS in aqueous medium, and a dialytic method was then use to extract calcium phosphate salts from HPMC. The percentage of HPMC extracted from BCP was 98.5%±0.5% as measured by a UV method. GC showed no chemical modifications after steam sterilisation and storage.

Keywords: Hydroxypropyl methylcellulose, Biphasic calcium phosphate, Capillary gas chromatography, Injectable bone substitute.

Introduction

A new injectable bone substitute (IBS) composed of calcium phosphate ceramic granules [biphasic calcium phosphate (BCP): 60% hydroxyapatite and 40% β-tricalcium phosphate] has been developed for endoscopic techniques [1-3] and tested in rabbit bone. Good results were achieved for bone repair [4-6] in which IBS showed osteocoalescence properties [7-10]. Hydroxypropyl methylcellulose (HPMC) was used in solution as the BCP granule carrier. This cellulose ether, which is synthesised by reaction with halogen methyl and propylene oxide, is used in ophthalmic surgery for its biocompatibilty and rheological properties [11-13]. HPMC is soluble in water and shows very high viscosity at low concentrations (4,000 mPa.s at 2% in solution and 20,000 mPa.s at 3% in solution) and good stability during steam sterilisation [14]. Preliminary studies of HPMC-BCP interactions were performed using Fourier

transformed infrared spectroscopy (FTIR), X-ray diffraction and X-Ray photoelectron spectroscopy (XPS) [15-17]. After one month of storage, the FTIR spectra of the composites showed slight oxidation peaks (C=O) and carboxylic acid function peaks only with high extractable pH calcium phosphate ceramic. Accordingly, a low extractable pH calcium phosphate BCP ceramic and a recently developed HPMC product used in ophthalmic surgery were chosen for the experiments. Thus, HPMC properties were not the same as those in previous studies [11,15]: the substitution degree in methoxyl groups was greater and the molecular weight lower (M_w=290,000g/mol). The purpose of this study was to use capillary gas chromatography (GC) to check the stability of HPMC after mixture with BCP and steam sterilisation. HPMC required several treatments before analysis. Methanolysis, first described by Clamp and Chambers [20], allowed monosaccharides to be obtained from polysaccharides. Hydroxyl groups were then derived from monosaccharides and phosphates by trimethylsilylation, which separates similar molecules and transforms mineral phosphate into organic phosphate detectable by flame ionisation. It was essential to eliminate salts from the BCP dissolution As phosphate reacts with cellulose in an alcoholic acid medium [21], it was necessary to use a dialytic technique to separate HPMC from phosphate salts.

Materials and methods

HPMC purchased from Colorcon (E4M-Kent-England) was used to identify the peaks obtained by gas-liquid chromatography. Methylcellulose (A4M-Colorcon-Kent-England), hydroxypropyl-β-cyclodextrin 97+% (Acros-France) and glucose (Fluka-France) were treated by methanolysis and trimethylsilylation in the same manner as HPMC. For preliminary study of the interaction between HPMC and disodium hydrogenophosphate (Merck-France), a solution was prepared at 0.05 M and added to HPMC (2.92% in weight). The gel was left for 3 days at room temperature. One part was sterilised, and the other (unsterilised) served as a

control. One gram of each was dried at 50°C for 48 h. The samples were then treated by methanolysis and trimethylsylilation for gas-liquid chromatography analysis.

An HPMC solution (2.92% in weight) was prepared for the IBS. Three days later, 25 g of this solution were added to 25 g of 100-200 µm MBCP® granules (Biomatlante-France). Samples (7 g each) of IBS were then sealed in vials (10 ml) with butyl rubber stoppers and sterilised in an autoclave at 121°C for 21 min. As HPMC has a gel point [22] and needs to recover its structure, studies were begun 4 days after steam sterilisation.

Two extractions of HPMC from IBS were performed. For the first, 6 g of IBS were introduced in 27 g of deionised water. The suspension was then stirred for 3 h and centrifuged at 20,000 rpm for 30 min with a Beckman L 7-65 (Palo Alto, CA) to isolate the aqueous phase. For the second extraction, the BCP phase from the first was added to 27 g of deionised water. The solution was stirred overnight and ultracentrifuged at 20,000 rpm for 30 min. The second aqueous phase was mixed with the first, and the resulting solution was filtered on Millipore 0.4 µm (Type HA) and dried at 50°C for 24 h.

A UV assay method [23] was used to check the extraction percentage. Twenty-four hours before use, 4 HPMC standard solutions (concentrations between 20 and 150 µg/ml) and a blank (deionised water) were prepared. Two millilitres of each standard and the blank were pipetted and added to 0.1 g of pure BCP. For samples, 0.1 g of extracted BCP from IBS was added to 2 ml of deionised water. Five millilitres of diphenylamine solution, prepared by dissolving 3.75 g of diphenylamine (Sigma-France) in 150 ml of glacial acetic acid (Fisher-France) and dilution of the solution with 90 ml of concentrated hydrochloric acid (Fisher-France), were added to each tube. The tubes were mixed and immediately placed in an oil bath at 105°C for 30 min. After cooling, absorbances were measured at 635 nm with a Shimadzu UV-1605 spectrophotometer (Germany).

Dialysis in aqueous medium, a less complicated method than precipitation, was used to extract phosphate salts from HPMC previously extracted from IBS. Thirty milligrams of dry HPMC from IBS were placed in a dialysis bag [24] with 4 ml of deionized water. The bag was then immersed in 500 ml of deionised water under stirring. The water was changed after 3 h, and stirring was stopped after 14 h. HPMC solutions were then dried at 50°C for 24 h.

For GC sample preparation, 20 mg of dry HPMC were added to 1.7 mg of trisodium citrate [20] used as internal standard (Acros-Belgium). The samples were taken up in 0.7 ml of methanolic HCl solution prepared by adding 0.6 ml acetyl chloride to 15 ml of methanol and sealed in vials with silicon/aluminium stoppers. Methanolysis was performed at 80°C for 24 h before the methanolic HCl solution was removed under a nitrogen stream. The trimethylsilylation reagent prepared in the laboratory was composed of pyridine, hexamethyldisilazane and trimethylchlorosilane (9:3:1, v/v/v). Samples (0.5 ml) of this reagent were sealed directly in vials and heated at 80°C for 2 h. The derivatised samples were rotary evaporated at 50-60°C, and the residue was immediately dissolved in 0.5 ml of dichloromethane. GC was performed with 1.5 µl of this solution. To identify the different peaks obtained for HPMC, 20 mg of methylcellulose, glucose and hydroxypropyl-β-cyclodextrin were treated and passed through the gas chromatograph. One millilitre of disodiumhydrogenophosphate solution at 0.05M was dried in a desiccator and treated by methanolysis and then trimethylsilylation.

The gas chromatograph (Perkin-Elmer Autosystem) coupled to a PE Nelson 1020 computer was equipped with a 30 m X 0.25 mm ID fused silica column coated with 0.25µm of poly (Simplicity-5, 5% phenyl–95% methylsiloxane, Supelco-France). The carrier gas was nitrogen at a flow rate of 1.2 ml/min. The injector and the flame ionisation detector were set to 220°C and 300°C respectively. Injection was performed with a splitting ratio of 50:1, and oven temperature was raised from 150°C to 240°C at 3°C/min and 240°C for 5 min.

Results

1-Extraction

Samples of 100 mg of dry IBS was theoretically made up of 2.818 mg of dry HPMC and 97.182 mg of BCP. The standards were passed through a UV spectrophotometer, and sample concentration was determined by linear regression. The extraction percentage was 98.5%±0.5% (n=5). Four days after sterilisation, the extraction percentage did not differ between sterilised and non-sterilised IBS.

2-Gas chromatography

Peak retention times are shown in table 1.

	Molecules type	Peaks retention times (min)													
HPMC	MG	9,94	10,34	10,8	11,94	12,72	13,18	13,34	14,36	14,8	15,02	16,6	17,87	18,21	
	Unsubstitued	19,45	20,04												
	HPG	21,12	21,78	22,77	24,93	25,16	25,38	26,9	27,1	27,45	27,88	28,2	28,32	31,65	32,07
	HPMG	18,46	18,7	20,2	20,42	20,82	21,38	21,84	22,14	23,24	23,54	25,63			
	TMSP	4,9													
	IS	9,68													
	N	7,91	8,68	9,37	11,1	11,36	15,62								

Table 1: Peaks retention times of the trimethylsilylphosphate(TMSP), trisodium citrate (IS), and the different monosaccharides from HPMC: methyl glycosides (MG), hydroxypropyl glycosides (HPG), hydroxypropyl methylglycosides (HPMG) and molecules appeared in contact between HPMC and phosphates (N).

A peak at 5.6 min corresponded to trimethylsilylphosphate (TMSP). Treatment allowed mineral phosphate to be transformed into organic phosphate, which was detected by flame ionisation. With glucose, two peaks appeared, corresponding to α and β glucopyranose. Methanolysis causes the racemisation of this monosaccharide [25,26]. As the other monosaccharides were considered to have the same racemisation, the separation of the units

substituted in the methyl and hydroxypropyl groups or the hydroxypropyl groups alone was not very efficient, but allowed the different peaks to be defined.

With the HPMC-Na_2HPO_4 solution, six news peaks appeared with or without sterilisation and trisodium citrate (internal standard). Retention for the new peaks was 7.91, 8.68, 9.37, 11.10, 11.36, and 15.62 min, showing a slight reduction in methylglycoside concentration. GC analysis of HPMC after IBS extraction (32 days) gave the same new peaks as the HPMC-Na_2HPO_4 solution, except for the absence of a peak at 9.37 min, which indicated that the trimethylsilylphosphate concentration was different. After phosphate salt extraction by dialysis, the new peaks disappeared, and the chromatogram returned to its original configuration. Only a small amount of trimethylsilylphosphate was detected, which was not sufficient to induce HPMC modification during methanolysis and trimetylsilylation.

Discussion

This paper reports a method for extracting HPMC from IBS. The major difficulty was to concentrate HPMC to the maximum. This polymer was used at a low concentration in water (2.92% w/w), and cellulose ether represented only 1.46% of dry material after BCP addition. Water extraction avoids chemical modifications, and the 98.5% of HPMC extracted from IBS was largely sufficient for analysis by gas-liquid chromatography. HPMC quantification by the UV method after acidic treatment in the presence of diphenylamine transformed solid BCP into ionic form, allowing access to all of the HPMC by BCP dissolution. This type of quantification requires a lower amount of polymer then that in IBS. Four days after sterilisation, no differences were observed between the product with or without sterilisation. After one month, no chemical modifications were observed in the cellulose derivative. Cellulose showed oxidation only in a highly oxidising solution. In our conditions, BCP was very stable (pH nearly neutral), but slight dissolution was observed in gas chromatography. GC analysis of the different sugars and polysaccharides was necessary

to identify HPMC distribution. Highly substituted sugars were difficult to separate, but formed only a minority and were less susceptible to chemical modification. Thus, cellulose derivatives are difficult to oxidise in solution at near-neutral pH. Cellulose oxidation often occurs in oxidising media such as H_3PO_4 85% [21], which gives uronic acids on C-6. Our results indicated that all modifications disappeared after phosphate extraction.

Conclusion

Chemical degradation of cellulose ether is not likely to occur in IBS conditions. Thus, GC could be an ideal method for checking the industrial batch purity of HPMC and confirming the stability and chemical integrity of the macromolecule after mixture with BCP and steam sterilisation and before implantation into the human body.

References

1. G. Daculsi, *Biomaterials* **19**,1473 (1998).
2. G. Daculsi, P. Weiss, J.M. Bouler, O. Gauthier and E. Aguado, *Bone* **25(2 suppl)**,59S (1999).
3. G. Daculsi, P. Weiss, J. Delecrin, G. Grimandi, N. Passuti and F. Guerin, Patent WO95/21 634 (1995).
4. O. Gauthier, J.M. Bouler, , P. Weiss, J. Bosco, E. Aguado, and G. Daculsi, *Bone* **25(2 suppl),**71S (1999).
5. O. Gauthier, J.M. Bouler, P. Weiss, J. Bosco, G. Daculsi and E. Aguado, *J. Biomed. Mater. Res.* **47(1)**,28 (1999).
6. O. Gauthier, D. Boix, G. Grimandi, E. Aguado, J.M. Bouler, P. Pilet and G. Daculsi, *J. Periodont.* **70**,375 (1999).
7. G. Daculsi, R.Z. LeGeros, E. Nery, K. Lynchand and B. Kerebel, *J. Biomed. Mater. Res.* **23,**883 (1989).
8. G. Daculsi, R.Z. LeGeros, M. Heughebaert and I. Barbieux, *Calcif. Tissue Int.* **46,**20 (1990).
9. E. Nery, R.Z. LeGeros, K.L. Lynch and K. Lee, *J. Periodontol.* **63**,729 (1992).

10. O. Malard, J.M. Bouler, J.Guicheux, D.Heymann, P. Pilet, C. Coquard, and G. Daculsi, *J. Biomed. Mater. Res.* **46(1)**,103 (1999).

11. G. Grimandi, P. Weiss, F. Millot and G. Daculsi, *J. Biomed. Mater. Res.* **39**,660 (1998).

12. T. Liesegang, *Surv. Ophthalmol.* **34**, 268 (1990).

13. T. Miyamoto, S. Takahashi, H. Ito and H. Inagaki, *J. Biomed. Mater. Res.* **23**,125 (1989).

14. S. Duggirala and P. Deluca, *PDA J. Pharm. Sc. Tec.* **50**,290 (1996).

15. P. Weiss, M. Lapkowski, R.Z. Legeros, J.M. Bouler, A. Jean and G. Daculsi, *J. Mat. Sc.: Mat. Med.* **8,**621 (1997).

16. P. Weiss, O. Gauthier, J.M. Bouler, G. Grimandi and G. Daculsi, *Bone* **25(2 suppl)**,67S (1999).

17. A. Dupraz, TP. Nguyen, M. Richard, G. Daculsi and N. Passuti, *Biomaterials* **20**,663 (1999).

18. J.F. Lawrence and J.R. Iyenga, *J. Chromatogr.* **350**,237 (1985).

19. G. Vanlaecke, H. Cuppens, L. Leyssensand and J. Raus, *J. Pharm. Biomed. Anal.* **7**,1641 (1989).

20. J.R. Clamp and R.E. Chambers, *Biochem. J.* **125**,1006 (1971).

21. D. Klemm, B. Philipp, T. Heinze, U. Heinze and W. Wagenknecht, *Comprehensive cellulose chemistry vol.2*, Wiley-VCH, Weinheim (1998).

22. C. Chevillard, *Colloïd. Pol. Sc.* **275,**537 (1997).

23. Hydroxypropyl methylcellulose. *The United States Pharmacopeia*, 21st rev. United States Pharmacopeial Convention, Inc, Rockville, MD, 1984.

24. C. Karlsson, A. Carlsson, M. Stenberg and H. Nygren, *Colloid Polym. Sci.* **270**,377 (1992).

25. C. Biermann, *Adv. Carbohydr. Chem. Biochem.* **46**,251 (1988).

26. J. Bleton, P. Mejanelle, J. Sansoulet, S. Goursaud and A. Tchalpa, *J. Chromatogr. A.* **720**,27 (1996).

Captions

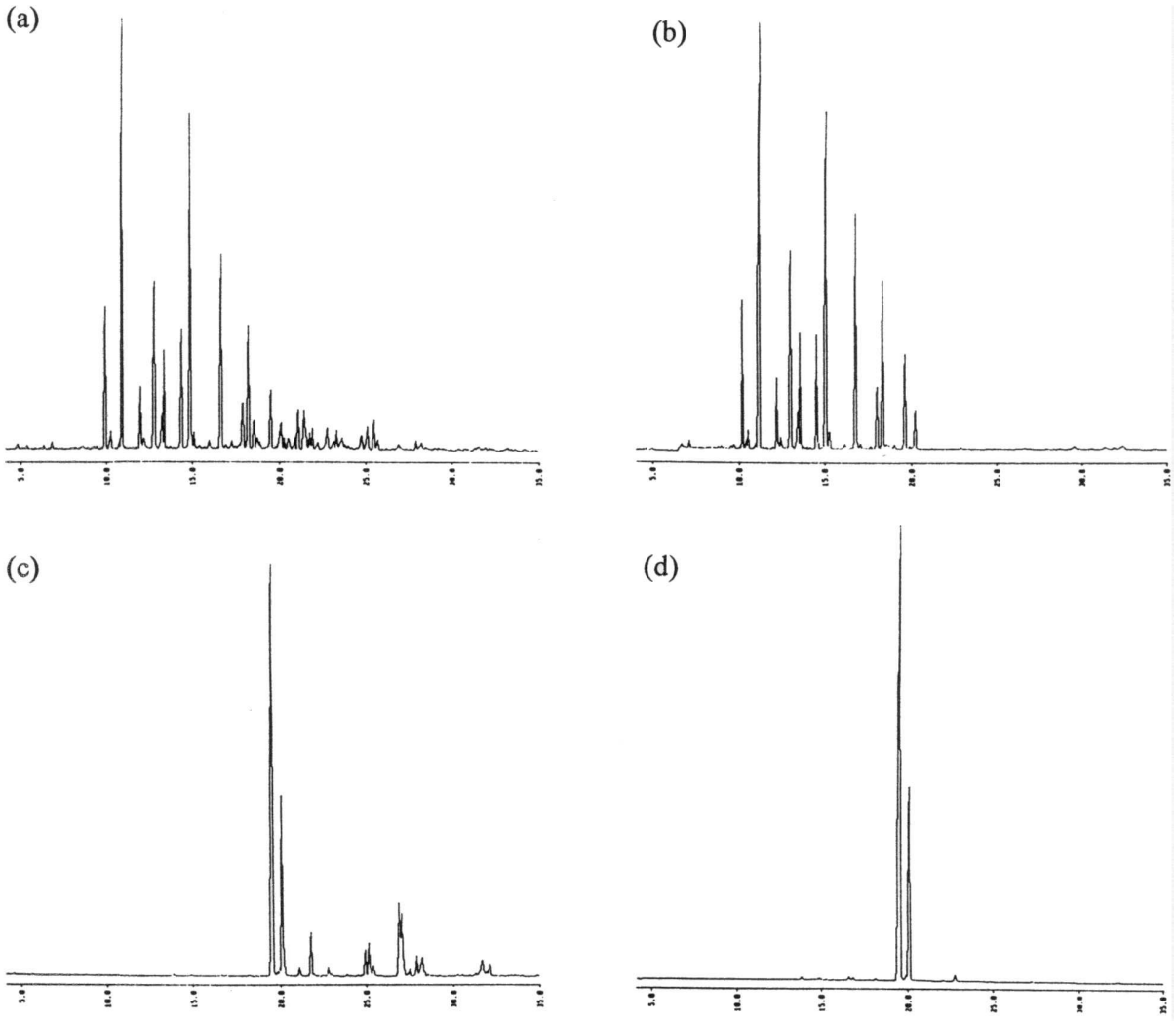

Figure 1: Chromatograms obtained after methanolysis and trimethylsilylation of hydroxypropyl methylcellulose(a), methylcellulose (b), hydroxypropyl-ß-cyclodextrin (c) and glucose (d).

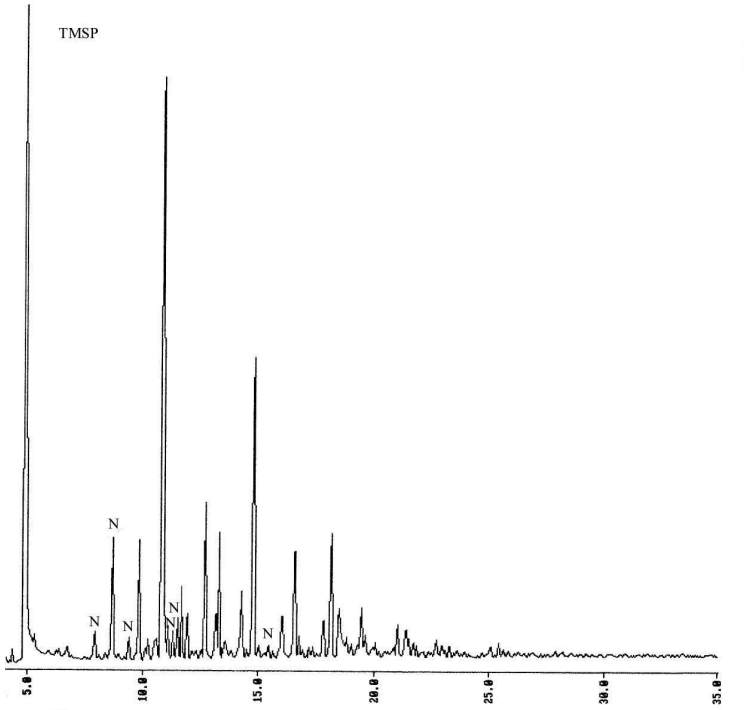

Figure 2: Chromatogram after methanolysis and trimethylsilylation of HPMC-Na_2HPO_4 gel without sterilisation: (TMSP) trimethylsilylphosphate, (N) new molecules.

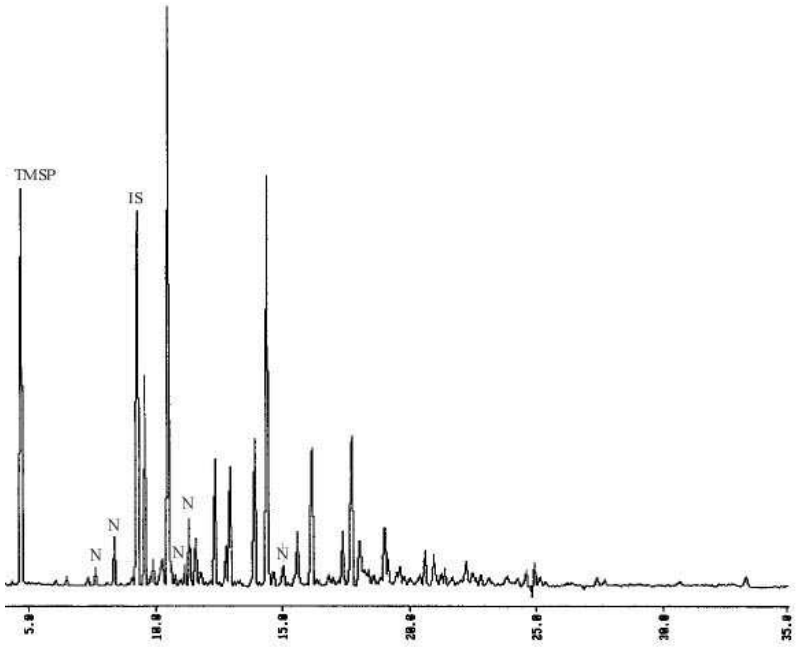

Figure 3: HPMC chromatogram after extraction from IBS sterilised (32 days old): (TMSP) trimethylsilylphosphate, (N) new molecules from HPMC, (IS) internal standard.

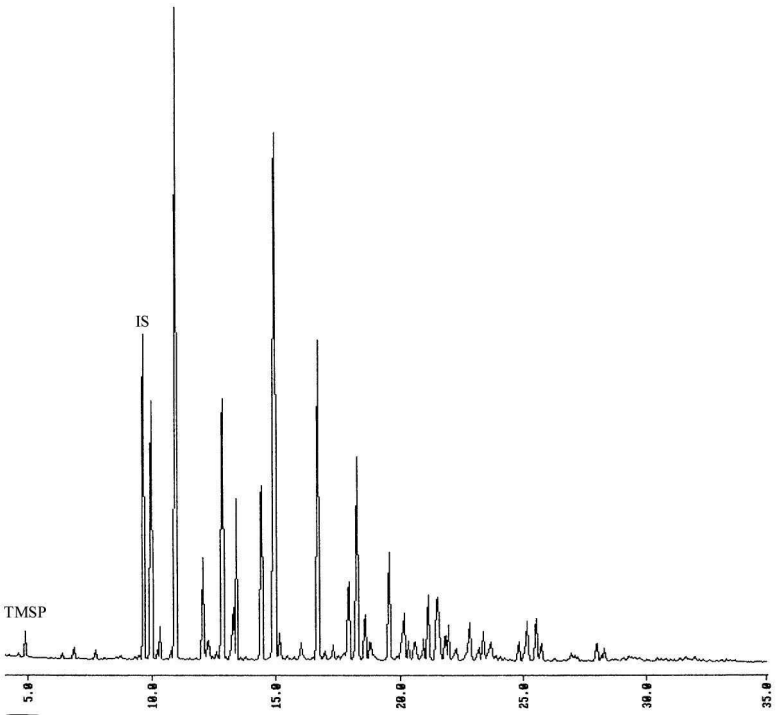

Figure 4: HPMC chromatogram after extraction from IBS sterilised (32 days old) and dialysis: (TMSP) trimethylsilylphosphate, (IS) internal standard.