Journal of Pharmaceutical & Biomedical Analysis,
Vol. 1, No., 1, pp. 99–104, 1983
Printed in Great Britain

0731–7085/83/010099–06$03.00/0

Short Communication

Separation and determination of the sodium, benzathine and procaine salts of benzylpenicillin by reversed phase high-performance liquid chromatography

M. PUTTEMANS*, M. LIPPENS, L. DRYON and D. L. MASSART

Farmaceutisch Instituut, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium

Keywords: *High-performance liquid chromatography; benzylpenicillin salts; ion-pair reversed-phase separation; tetrabutylammonium phosphate; quality control.*

Introduction

In the quality control of pharmaceuticals containing penicillin, there is a need for an analytical method which is faster and more specific than the microbiological [1] or the iodometric methods [2, 3] in current use. When dealing with mixtures of penicillins prior separation is indispensable. High-performance liquid chromatography (HPLC) has been described by several authors for the determination of penicillins in pharmaceuticals [3–7] or in biological fluids [5, 8]. Both ion-exchange chromatography [3] and reversed-phase HPLC [4–10] have been proposed. The aim of the present work was to separate and quantitate mixtures of the sodium, procaine and benzathine salts of benzylpenicillin in one chromatographic procedure. Simultaneous resolution of these substances is difficult to achieve adequately in reversed-phase chromatography because of the presence of the acid benzylpenicillin, together with the two bases procaine and benzathine. Resolution can, however, be obtained by the careful choice of mobile phase pH and composition. Another method to influence the retention of ionizable compounds involves the addition of a counterion to the mobile phase. This increases the retention of ions of opposite charge and decreases the retention of ions with the same charge [11]. Both these approaches are considered in the present work with regard to their application in the routine analysis of benzylpenicillin salts in pharmaceutical formulations.

* To whom correspondence should be addressed.

Experimental

Apparatus

Two coupled Varian LC 8500 chromatographs, equipped with a solvent programmer and a manual loop-valve injector (10 μl loop) were employed. The columns used were MicroPak MCH-10 (Varian Ltd), filled with 10 μm octadecylsilica (300 × 4 mm i.d.).

A Varian UV 254–280 detector was used at 254 nm. The signal was recorded with a Varian 9176 recorder and integrated using a Varian CDS 111 integrator. The pH values were measured with an Orion Ionalyser 601 and a combined glass electrode.

Chromatographic method

Phosphate buffers (0.05 M) were prepared from analytical grade sodium phosphate and phosphoric acid (Merck, F.R.G.) and doubly-distilled water. HPLC grade methanol was obtained from Fluka AG (Buchs, Switzerland). Mobile phases were degassed in an ultrasonic bath prior to use. Tetrabutylammonium phosphate (TBA) (0.5 M in phosphate buffer, pH 7.5) was obtained from Altex.

Gradient elution system: initial eluent composition (phase A), 50% methanol in phosphate buffer (0.1 M) pH 4.8; eluent composition (phase B), 50% methanol in phosphoric acid (0.1 M). The gradient profile adopted was : $t = 0$, %B = 0; 2 min, 0%; 6 min, 40%; 9 min, 40%; 9.1 min, 0%.

All retention times, capacity factors, peak areas or heights and plate numbers were calculated as the average of three determinations. The flow rate was 1 ml/min unless otherwise stated.

Standards

Sodium benzylpenicillin, procaine benzylpenicillin and benzathine benzylpenicillin were used as received from Bristol Belgium. The benzylpenicillin content was determined by the method of the European Pharmacopoeia [2], the results (expressed as sodium benzylpenicillin equivalent) being: sodium benzylpenicillin, 100.0 ± 0.1%; procaine benzylpenicillin, 62.0 ± 0.1%; benzathine benzylpenicillin, 75.0 ± 0.1%. The benzathine content of benzathine benzylpenicillin was found to be 24.5 ± 0.1% according to the European Pharmacopoeia [2]. The procaine content of procaine benzylpenicillin was determined by HPLC as follows: standards of procaine in the range 0–80 mg/dl methanol were chromatographed in the gradient elution system, together with three solutions of procaine benzylpenicillin of about 65 mg/dl in methanol. The procaine content was 37.5 ± 0.2%. The potency of each substance in international units was: sodium benzylpenicillin 1670 IU/mg, procaine benzylpenicillin 1011 IU/mg, benzathine benzylpenicillin 1240 IU/mg. Standards of sodium benzylpenicillin were dissolved in methanol and calibration curves made between 0 and 200 mg/dl, the regression statistics being:

$y = 415.1\,x - 397$; the 95% confidence interval, expressed as a percentage of the median, was 6.1%.

The regression data for procaine in procaine benzylpenicillin (0–100 mg/dl) were:

$y = 3018\,x + 537.9$; confidence interval, 2.1%.

The regression data for benzathine in benzathine benzylpenicillin (0–200mg/dl) were:

$y = 381.5\,x - 686.5$; confidence interval, 7.1%.

Calibration data for benzathine and procaine were obtained from combined chromatographic runs, while sodium benzylpenicillin standards were run separately.

Samples

Unit doses of Bactocilline (Smith–Kline Belgium) and Tri-Penadur (Smith–Kline Belgium) were dissolved in methanol and diluted to 100.0 ml with methanol. Aliquots (10 µl) of this solution were injected, the chromatogram recorded and peak areas calculated. The content of procaine and of benzathine benzylpenicillin was measured directly from the calibration graphs, whereas the sodium benzylpenicillin was determined by difference, taking the total amount of benzylpenicillin and subtracting the calculated benzylpenicillin content of benzathine and procaine benzylpenicillin. Bactocilline 1200 is formulated to contain in 1 g for i.m. injection: 800,000 IU procaine benzylpenicillin, 400,000 IU sodium benzylpenicillin, and 1,000,000 IU streptomycin sulphate. Tri-Penadur (1,200,000 IU for i.m. injection) has the following composition: benzathine benzylpenicillin 600,000 IU, procaine benzylpenicillin 300,000 IU, and potassium benzylpenicillin 300,000 IU.

Results and Discussion

Since benzylpenicillin is rather hydrophilic, it has short retention times in a reversed-phase system, as shown in Table 1. The pH exerts little influence on retention, but the

Table 1
Influence of the pH and the percentage of methanol of the mobile phase on the capacity factor (k') of benzylpenicillin, benzathine and procaine

	k' values					
	40% Methanol		50% Methanol		60% Methanol	
pH	5.1	6.5	5.1	6.5	5.1	6.5
Benzylpenicillin	1.2	1.1	0.6	0.4	0.2	0.0
Benzathine	> 25	> 25	> 25	> 25	4.3	21.5
Procaine	2.4	9.4	1.6	6.3	1.4	4.0

percentage of methanol in the mobile phase has a more profound effect. For the sodium salt only one peak is obtained, but for the other salts two peaks are observed, corresponding to the benzathine or procaine moieties. As shown in Table 1, the bases are more strongly retained than benzylpenicillin by the reversed-phase material. One solution of the separation problem arising from the large differences in retention times of these three components was to employ a gradient of decreasing pH. Although the system described above gives complete resolution of the three components, it is inconvenient in that it requires long re-equilibration times, causes progressive degradation of the column material and leads to a rising baseline. The effect of adding a counterion, tetrabutylammonium (phosphate), on the capacity factor of benzylpenicillin, procaine and

benzathine at various values of pH and methanol concentration is shown in Table 2. At pH 7 benzathine has a capacity factor larger than 30 in all solvent systems examined. For benzylpenicillin, an anionic compound, the capacity factor increases with increasing TBA concentration. The retention of procaine, a cationic compound, decreases when

Table 2
Influence of the tetrabutylammonium (TBA) concentration on the capacity factor of: A, benzylpenicillin; B, procaine; and C, benzathine at different values of pH and methanol concentration

	pH 7						pH 3					
Concentration of TBA (M)	30% Methanol			40% Methanol			30% Methanol			40% Methanol		
	A	B	C	A	B	C	A	B	C	A	B	C
0	2.7	25.6	> 30	0.7	17.4	> 30	9.1	2.4	1.4	3.3	1.4	0.8
1×10^{-3}	3.6	13.3	> 30	0.8	8.2	> 30	11.1	1.1	0.6	3.5	0.7	0.5
2.5×10^{-3}	4.5	11.8	> 30	1.3	8.2	> 30	14.3	0.8	0.5	4.3	0.6	0.5
5×10^{-3}	5.1	10.8	> 30	1.4	8.0	> 30	16.3	0.7	0.5	4.8	0.5	0.4
7.5×10^{-3}	5.1	10.8	> 30	1.3	7.4	> 30	17.3	0.6	0.5	5.3	0.5	0.5
1×10^{-2}	5.1	10.9	> 30	1.2	7.7	> 30	17.8	0.6	0.5	5.1	0.5	0.8

TBA is added. Indeed, a very marked decrease of the capacity factor of procaine, possibly due to the repulsion of this cationic compound by TBA adsorbed on the column, is observed. This effect is most noticeable when small amounts (10^{-3} M) of tetrabutylammonium are added to the mobile phase. After an initial change, the capacity factor stabilizes when the TBA concentration exceeds 5×10^{-3} M. From Table 2 it may be concluded that a mixture of the three compounds cannot be resolved at pH 7 within a reasonable period of time and therefore the same experiments were performed at pH 3. Table 2 shows that the elution order differs at pH 7 and pH 3. Moreover, the eluent, consisting of 40% methanol and phosphate buffer (pH 3), permits the resolution of sodium benzylpenicillin, while benzathine and procaine are poorly retained. In order to resolve these two bases, an eluent containing 30% methanol is more appropriate (Fig. 1). The tetrabutylammonium ion is an indispensable component of the mobile phase. It influences both the retention time of the solutes and their peak shape. Indeed, the efficiency of the system, expressed as the number of plates per metre (N), increases upon addition of the counterion, an influence which is apparent especially on the procaine and benzathine peaks. The value of N increases from 3900 to 11,600 for procaine and from 2300 to 7300 for benzathine, for a TBA concentration between 0 and 10^{-2} M, with a mobile phase consisting of 30% methanol and phosphate buffer (pH = 3) This variation cannot be explained by the slight decrease in retention time alone.

Optimum separation conditions were chosen as follows: methanol–0.1 M phosphate buffer (pH = 3) (30:70 v/v), containing 5×10^{-4} M tetrabutylammonium phosphate.

An explanation of this phenomenon may be found in the possible presence of free silanol groups on the octadecylsilica stationary phase, these polar groups being known to cause peak broadening. Since silica can adsorb counterions [12], tetrabutylammonium groups could be attracted to any available free silanol groups, so that peak tailing would be reduced. At pH 7 the influence of a counterion upon the peak shape of procaine is much less important than at pH 3, which also points towards the free silanol group mechanism. For benzylpenicillin an increase of N (from 4000 to 6800) with TBA

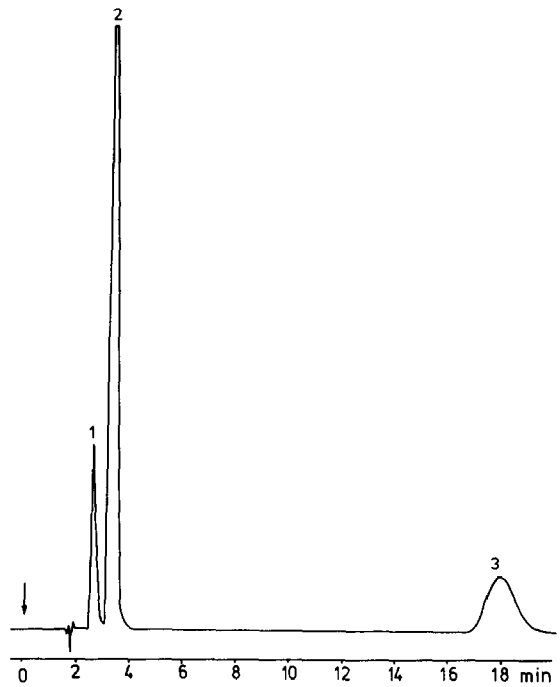

Figure 1
Separation of a mixture of benzylpenicillin salts in Tri-Penadur by ion pair reversed-phase HPLC: 1, benzathine; 2, procaine; 3, benzylpenicillin. Eluent composition: methanol–phosphate buffer (pH 3) (30:70 v/v) with 5×10^{-4} M tetrabutylammonium phosphate. Flow rate, 1.5 ml/min.

concentration increase from 0 to 10^{-2} M is observed at pH 7, while at pH 3 N decreases from 8800 to 6100. This may be explained by a strong increase in the capacity factor, which leads to peak broadening. When the same experiments were carried out on an Altex Ultrasphere-I.P. reversed-phase column, where the packing material is said to be fully capped, the influence of the counterion on the peak shape was found to be negligible. This would also point towards the presence of free silanol functions on the MCH 10 reversed-phase material.

Determination in pharmaceutical preparations

Mixtures of procaine benzylpenicillin and sodium benzylpenicillin were determined in Bactocilline and mixtures of the three salts were determined in Tri-Penadur, as illustrated in Fig. 1. Both preparations were injectable solutions. Dilutions of samples and standards were made in methanol. The results of a six-fold analysis are given in Table 3. For Tri-Penadur there was found to be no interference from streptomycin sulphate. The mean values obtained conform with the prescribed content, since they are within the 5% limits permitted for these preparations in Belgium.

Acknowledgements: The authors acknowledge Bristol Belgium for gifts of standards, Smith–Kline Belgium (Recherche Industrielle Thérapeutique) for gifts of Tri-Penadur and Bactocilline and K. Broothaers-Decq for technical assistance.

Table 3
Analysis of benzylpenicillin salts in commercial preparations

Sample	Compound	Theoretical (IU)	Found (IU)	Mean ± SD (IU)	RSD
Bactocilline	Sodium benzylpenicillin	400,000	403,120 425,570 407,880 415,910 417,910 430,410	416,800 ± 10,300	2.47%
	Procaine benzylpenicillin	800,000	794,470 850,630 836,760 825,230 836,900 846,630	831,770 ± 20,310	2.44%
Tri-Penadur	Potassium benzylpenicillin*	300,000	280,800 296,460 288,060 289,640 293,670 281,910	288,090 ± 6310	2.19%
	Procaine benzylpenicillin	300,000	298,490 300,180 318,280 300,430 314,640 310,820	307,140 ± 8510	2.77%
	Benzathine benzylpenicillin	600,000	578,470 593,430 607,180 594,930 588,740 593,510	592,710 ± 9320	1.57%

* Potassium benzylpenicillin was assayed as its sodium equivalent.

References

[1] XXX Belgian Pharmacopoeia V (1974).
[2] XXX European Pharmacopoeia (1975).
[3] P. O. Roksvaag, H. I. Brummenaers and T. Waaler, *Pharm. Acta Helv.* **54**, 180–185 (1979).
[4] K. R. Bagon, *J. High Resol. Chromatogr.* **2**, 211–215 (1979).
[5] V. Hartmann and M. Rödiger, *Chromatographia* **9**, 266–272 (1976).
[6] W. A. Vadino, E. T. Sugita, R. L. Schnaare, H. V. Ando and P. J. Niedergall, *J. Pharm. Sci.* **68**, 1316–1318 (1979).
[7] M. Le Belle, K. Graham and W. L. Wilson, *J. Pharm. Sci.* **68**, 555–556 (1979).
[8] T. B. Vree, V. A. Hekster, A. M. Baars and E. Van der Kleijn, *J. Chromatogr.* **145**, 496–501 (1978).
[9] F. Nachtmann, *Chromatographia* **12**, 380–385 (1979).
[10] F. Salto, *J. Chromatogr.* **161**, 379–385 (1979).
[11] J. H. Knox and J. Jurand, *J. Chromatogr.* **149**, 297–312 (1978).
[12] J. Crommen, *J. Chromatogr.* **186**, 705–724 (1979).

[*First received for review 15 March 1982; revised manuscript received 13 July 1982*]