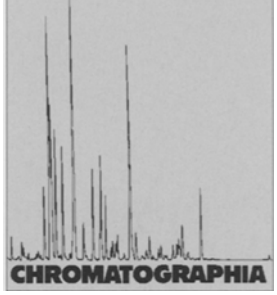
Assay of *para*-Aminobenzoic Acid Formed by Hydrolysis of Procaine in CP1B Solution



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Key Words

Thin-layer chromatography
Cardioplegia solution
Procaine
p-Aminobenzoic acid

Summary

CP1 is a cardioplegia solution containing procaine, a substance that is unstable at pH > 7. For this reason the product it is supplied as two solutions that are mixed immediately before use.

para-Aminobenzoic acid (PABA) was separated by horizontal high-performance thin-layer chromatography (HPTLC) on silica gel 60 nanoplates with fluorescence indicator. This method is specific, repeatable, and reproducible.

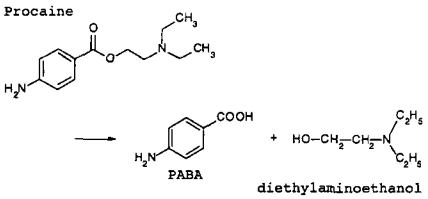
The autoclave sterilization conditions currently used are the least aggressive towards procaine. Under normal storage conditions, the concentration of procaine in CP1B complies with standards (47.5 – 52.5 mmol L^{-1}) for a period of four years. The shelf-life of 2 years is thus satisfactory.

Introduction

Cardioplegia solutions, or cardioplegics, are drugs used in heart surgery to stop the heart from beating, thereby facilitating the work of surgeons. Candidate drugs must reduce the energy requirements of the myocardium, create a favorable environment for the resumption of energy production and combat the harmful effects of ischemia [1].

Although there are many formulations, there is no unanimous consensus among surgical teams [2]. Five solutions are currently used for cardioplegia in the Paris Hospital System (Assistance Publique des Hôpitaux de Paris; AP-HP): CP1® (PCH) or Fabiani's solution, CP9® (PCH) or Menasche's solution, SLF 103® (Aguettant), Plegisol® (Abbott), and SLF 11® (Fresenius), equivalent to Bretchneider's solution. The compositions of these preparations are listed in Table I. CP1 accounted for 90% of cardioplegia solutions purchased from the Pharmacie Centrale des Hôpitaux de Paris in 1996, of which 30% were used by the AP-HP. The classic composition of the formulations includes potassium, the plegia active ingredient, calcium, which blocks muscle contraction, and other ingredients intended to maintain pH, osmolality, and protect cell integrity [1]. The role of procaine, found in CP1 only, is essentially to stabilize the cell membrane [3, 4].

Procaine is the ester of *para*-aminobenzoic acid (PABA) and diethylaminoethanol. It can be hydrolyzed, a process which is accelerated by alkaline pH [5], yielding PABA and diethylaminoethanol according to the breakdown reaction:



Because CP1 solution is buffered at pH 8 with Tris(hydroxymethyl)aminomethane (THAM), it is packaged as two solutions to be mixed just before use – the vehicle CP1A (1000 mL) containing THAM, and concentrated CP1B (10 mL) which contains magnesium chloride and the procaine.

The rate of hydrolysis is also considerably accelerated by heat [6]. Cardioplegia solutions are, however, injected directly into the heart of the patient and must thus be sterile. CP1B ampoules are currently sterilized by autoclaving for 30 min at 110 °C. Hydrolysis of procaine yields *para*-aminobenzoic acid [6], which colors the solution yellow; its systemic toxicity at low doses has not been clearly established [7]. It is thus important to quantify procaine breakdown to determine the extent of exposure of patients to this breakdown product and to verify the compliance of

Table I. Composition (mmol L^{-1}) of the cardioplegia solutions available at the PCH.

	CP1	CP9	SLF 103	PLEGISOL	SLF 11
Na^+	144	0	147	110	12
K^+	20	16	20	16	10
Ca^{2+}	4	0	2	1.2	0
Mg^{2+}	16	3	16	16	2
Cl^-	201	22	203	160	34
THAM	2.8	0	0	0	0
HCl	qs pH = 8	0	0	0	0
Procaine	1	0	0	0	0

procaine with standards, both after autoclaving and after storage.

The aim of this work was to determine the stability of procaine in CP1B as a function of sterilization conditions and sample storage times. After validation of the analytical method, PABA (procaine breakdown product) was assayed in samples of the same batch of CP1B subjected to four different sterilization cycles, and in 32 batches of CP1B manufactured over 5 years and stored at room temperature protected from light.

Experimental

Products and Equipment

Reagents

All starting materials used were analytical grade, supplied by Prolabo (Paris, France) and Sigma (St Louis, MO, USA). Fluorescent silica gel nanoplates for thin-layer chromatography (HPTLC plates; 100 mm × 200 mm, silica gel $60F_{254}$) were supplied by Merck (Darmstadt, Germany).

Standard Solution

Six aqueous standard solutions of PABA were prepared for the calibration ranges (50.5, 58.0, 58.3, 59.7 and 63.5 mg L^{-1}) and the control (79 mg L^{-1}). They contained 1.36 g L^{-1} procaine, 60 g L^{-1} potassium chloride and 145 g L^{-1} magnesium chloride, hexahydrate. This composition was thus very close to that of CP1B. Chromatographic development of the standards was comparable with that of the samples. These solutions were stored at +4 °C protected from light.

Sample

The study was conducted with drug samples stored at room temperature and protected from light. All batches (32 in total) manufactured in a five-year period were examined; one was rejected because its procaine concentration was non-compliant.

A special batch of 2500 ampoules of CP1B was manufactured by the PCH production unit for determination of the effect of sterilization conditions on procaine hydrolysis. The batch was divided into five sub-batches, corresponding to different sterilization cycles: non-sterilized, 30 min at 110 °C, 45 min at 110 °C, 20 min at 120 °C, and 30 min at 120 °C. Manufacturing and sterilization conditions were chosen to enable work with methods validated according to Good Laboratory Practice [8].

Equipment

An automatic sample applicator (AS30 TLC applicator) and the densitometer for quantitative analysis (CD60 Densitometer) were obtained from Desaga (Heidelberg, Germany). The horizontal linear development chamber was obtained from Camag (Muttenz, Switzerland).

Methods

Procaine Assay

Procaine was assayed by UV spectrophotometry at 290 nm, a method that has been validated for the quality control of manufactured batches. In compliance with the decree of December 9, 1992 [9], the procaine concentration was ± 5% of the target value, or 50 ± 2.5 mmol procaine per liter CP1B. The results of procaine assays conducted when each batch was validated are found in the corresponding analysis certificates. Because this assay is not specific for procaine, the results are actually the total quantities of procaine and PABA in ampoules of CP1B.

PABA Assay

PABA was separated by high-performance thin-layer chromatography (HPTLC) on fluorescent silica gel nanoplates. The assay was conducted as described in the test for related substances in the monograph of the European Pharmacopoeia, 3rd edition [10]. Fluorescence inhibition was determined by densitometry at 275 nm. This wavelength was selected after acquisition of a solid-phase absorption spectrum between 230 and 350 nm. At 275 nm, absorbance by fresh plates was low whereas absorption by PABA was close to the maximum.

Sample Application

The automatic applicator used enabled quantification of PABA, because of the precision of the volume applied and the homogeneity of the resulting applications. Sample (2 µL) bands were 5 mm long; the first band was 15 mm from the edge of the plate and adjacent applications were separated by 15 mm. The spraying rate was 10 µL min^{-1}. If several applications were made at the same site the interval between applications was 10 s. The 100 mm × 200 mm silica gel plates used enabled 12 applications, or four range points and 4 samples in duplicate, to be made on opposite edges of the plate. Among the samples applied, a control enabled the assay to be validated.

The calibration range was prepared with the automatic applicator, using one of the five standard PABA solutions described above. The quantities applied for the four range points were between 100 and 510 ng PABA.

Development

Chromatographic development was performed horizontally (Camag system) in a ventilated cabinet. Runs took less than 15 min, with samples applied in parallel on each side of the plate. The mobile phase was freshly prepared acetic acid–dibutyl ether–hexane, 4:80:16 (*v/v*). Under these conditions, procaine remained at the origin of the plate and PABA migrated ca 12 mm. The plate was dried in a ventilated cabinet and the migration distance of PABA was measured under a UV lamp (254 nm) for precise location of PABA spots to enable programming of the densitometer.

Densitometric Assay

Densitometry measures the fluorescence extinction of spots with reference to the fresh plate. Peak areas were calculated by means of the CD60 software. A linear regression line not passing through the ori-

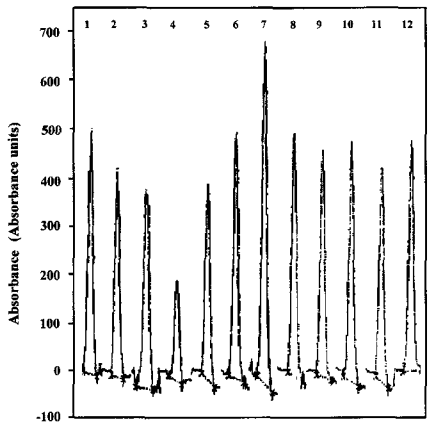


Figure 1
Chromatogram of *para*-aminobenzoic acid (PABA).

gin (a peculiarity of thin-layer chromatography) was then calculated. This function determines the quantity of PABA applied as a function of the measured peak area.

Each sample was applied in duplicate and the quantity of PABA was calculated from the mean area of the two peaks. The coefficient of variation between two measurements of the same sample did not exceed 5%.

The control was applied in duplicate in all assay series. The series assayed was regarded as validated if the control did not differ from its target value by more than 10%, and if the coefficient of variation between the two measured values did not exceed 5%.

The results enabled the molar concentrations of PABA and procaine in ampoules of CP1B to be calculated by use of the molar masses of procaine and PABA, 272.8 and 137.1 g mol^{-1}, respectively.

Analysis of the Results

After development, typical chromatograms contained two fluorescence extinction bands that could be seen under UV light at 254 nm. These bands are described in the section *Test for Related Substances* in the procaine monograph of the European Pharmacopoeia, 3rd edition [10]. Under the conditions used, procaine did not migrate, remaining at the site of application (origin of the plate). PABA, the only breakdown product described in the procaine monograph, migrated 12 mm on average under the conditions used. The densitometric output from a typical chromatogram is depicted in Figure 1.

Table II. Technical validation of the HPTLC assay of PABA.

Linearity Range	$y = b.x + a$	Reproducibility Assay	R_F	Repeatability Assay
50.5 mg L^{-1}	$b = 1.56$ $a = 94.23$ $r = 0.996$	Standard solution at 316 ng per application	All the range points of the linearity study	Standard solution
59.7 mg L^{-1}	$b = 1.62$ $a = 110.98$ $r = 0.995$			
58 mg L^{-1}	$b = 1.95$ $a = 42.72$ $r = 0.994$	Results from thirty assays: $m = 302$ ng $CV = 3\%$	Results from sixty assays: $m = 0.316$ $CV = 3\%$	Results from six assays: $CV = 4.8\%$
58.3 mg L^{-1}	$b = 1.44$ $a = 2.45$ $r = 0.995$			
63.5 mg L^{-1}	$b = 1.28$ $a = 51.92$ $r = 0.996$	Recovery 95.60%		

m, mean; CV, coefficient of variation; r, correlation coefficient.

Results

Validation of the Method

Validation of an analytical procedure ensures the quality of a result by scientific study of the reliability of the method by use of a statistical tool. At present there is no validation method suited to quantitative thin-layer chromatography. Despite this, the general principle for validation of an analytical method applied to the assay of a medicinal substance [11] was used as a reference in this work. The validation of assay methods using high-performance liquid chromatography described by the SFSTP [12] is a practical basis for the method used. The validation results are summarized in Table II.

Specificity

The specificity of an HPTLC separation comes from the choice of the plate and mobile phase; these enable the separation of the components of the sample assayed to be optimized. PABA, the main breakdown product of procaine, contains the aminobenzoic ring that is responsible for the spectral properties of the parent compound, and thus absorbs at the same wavelength. Procaine is thus the only substance in CP1B that can interfere with the assay of PABA. The method used in this work enabled PABA to be separated from procaine, as described in the procaine monograph of the European Pharmacopoeia, 3rd edition [10]. It is thus specific for PABA.

Sensitivity

The limits of detection and quantification of the method were determined by use of a PABA concentration range of eight points from 29 to 580 ng. The limit of detection is the smallest quantity of substance that can be detected. It was defined as the quantity de PABA that furnished a signal-to-noise ratio 3. The limit of detection was found to be 29 ng PABA.

The limit of quantification is the smallest value that can be considered as valid for an assay. It was arbitrarily set at 3.3 times the limit of detection. The limit of quantification was 100 ng PABA.

Linearity

The limit of linearity was determined graphically by use of the calibration plot prepared for the PABA range prepared for study of sensitivity and was found to be 510 ng PABA. Five calibration ranges of four points each were prepared by means of five PABA standard solutions (50.5, 58.0, 58.3, 59.7 and 63.5 mg L^{-1}). The four range points, equivalent to 2, 4, 6, and 8 µL standard solution, were applied in triplicate to each plate. The regression coefficient for each plot, taken individually, was consistently > 0.994.

Reliability

Repeatability. The same standard solution was assayed six times on the same plate. The coefficient of variation was 4.8% and so the method can be regarded as repeatable.

Table III. Effects of sterilization conditions on the hydrolysis of procaine in CP1B.

Sterilization conditions	[Procaine] (mmol L^{-1})	[PABA] (mmol L^{-1})	[PABA] (mmol L^{-1})	PABA (%)
Non-sterilized	50	< *LOD*	< *LOD*	0
110 °C, 30 min	50	0.067	0.49	1
110 °C, 45 min	50	0.1	0.73	1.5
120 °C, 20 min	50	0.114	0.83	1.7
120 °C, 30 min	50	0.123	0.90	1.8

LOD, limit of detection.

Table IV. HPTLC assay of PABA in different batches of CP1B.

Analysis certificates Storage time (years)	$[Procaine]_{1m}$ (mmol L^{-1} $year^{-1}$)	PABA assays $PABA_m$ (% $year^{-1}$)	$[Procaine]_{2m}$ (mmol L^{-1} $year^{-1}$)
1	49.8	2.1	48.8
2	49.4	3	47.9
3	50.3	3.2	48.7
4	50.3	3.5	48.6
5	49.5	4.3	47.3

$[Procaine]_{1m}$, mean initial procaine concentration on release of batch; $[Procaine]_{2m}$, mean procaine concentration calculated from amount of breakdown into PABA; $PABA_m$, mean amount of PABA.

Reproducibility. Reproducibility of chromatographic development can be assessed by calculating the retention factor (R_F) which for each spot is the distance migrated by the solute divided by the distance migrated by the solvent. The mean R_F calculated from the 60 range points used in the linearity study was 0.316 (CV = 3%). The R_F can thus be regarded as reproducible.

The concentration of the standard PABA solution (79 mg L^{-1}) was determined 30 times (4 µL per application), including 16 times to validate different series of assays. The mean value found was 302 ng PABA with a CV of 3%. The method can thus be regarded as reproducible.

Accuracy

The accuracy of the assay was determined by use of results obtained from the standard solution in the reproducibility study. The mean value obtained for this solution (302 ng PABA) differed from the target value by 4.4%, and the maximum variation compared with the target value was 10%. The method is thus at least 90% accurate.

Effects of Sterilization on Procaine Hydrolysis

The concentration of PABA in CP1B before sterilization was lower than the limit of detection of the method. PABA is thus formed as a result of breakdown of procaine during steam sterilization. PABA was assayed in each solution sterilized by one of the four sterilization cycles tested. The measured concentration remained consistently lower than 1 mmol PABA per liter CP1B (Table III). The quantity of procaine remaining in the CP1B solution was, therefore, consistently higher than the lower acceptable limit of 47.5 mmol L^{-1}, and the batch was always compliant. The results show that the extent of breakdown of procaine into PABA increased with autoclaving time and temperature. Sterilization conditions currently applied to CP1B, 30 min at 110 °C, are the least aggressive towards procaine.

Assay of PABA in CP1B

The maximum concentration of PABA measured in one of the batches of CP1B after five years of storage was 0.314 g L^{-1}, i. e. 2.3 mmol L^{-1}. Results from the study of the stability of procaine as a function of storage time are listed in Table IV.

Discussion

The PABA assayed is a procaine breakdown product and not an active ingredient, and is present in very small quantities. The precision of the method used is sufficient for assay of an identified breakdown product. The limit tests in the monograph of the European Pharmacopoeia 3rd edition call for a calculated maximum PABA content of 500 µg per gram procaine base [10]. The use of densitometric plate reading as method of detection enabled the limit of quantification to be reduced to 100 ng PABA.

When performing linearity studies, the SFSTP recommends preparing six different ranges, each containing five range points assayed six times [12]. In our work, five different ranges were prepared, each containing four points applied three times. The resulting calibration curves had different slopes (between 1.28 and 1.95; CV = 16%) and Y-intercept values (between 2.45 and 111; CV = 71%). The calibration curves are thus not reproducible. This difference between the equations shows that the results are highly dependent on operating conditions, in particular the quality of chromatography plates that are not perfectly homogeneous. These results show the necessity of preparing a validated calibration range for quality control for each assay series on the same plate.

The reproducibility of R_F values shows the homogeneous particle size distribution of the silica gel nanoplates and the excellent reproducibility of development.

The CV of repeatability (4.8%) was abnormally higher than that of reproducibility (3%). This can be explained statistically – the standard was assayed 30 times for reproducibility, and only six times for repeatability. Nevertheless, the CV values for repeatability and reproducibility are satisfactory (< 5%). The same is true for the minimum accuracy of the method (90%). The maximum expected error (10%) is acceptable for assay of an identified impurity present in very small quantities.

Procaine breakdown detected in the study of the sterilization of CP1B was found to be temperature-dependent, but remained relatively low, not exceeding 2.5% (Table III). The procaine concentration thus remained within acceptable levels in all the samples (47.5 to 52.5 mmol L^{-1}). Steam sterilization under the conditions described does not massively destroy procaine as might have been expected. An alternative sterilization procedure is thus not necessary. The plot of a hydrolysis curve (Figure 2) shows the effect of sterilization conditions on procaine hydrolysis.

The results in Table IV show that the concentration of PABA increased with time. Procaine is thus unstable in solution. After a given storage time, its residual level in CP1B depends on its initial level.

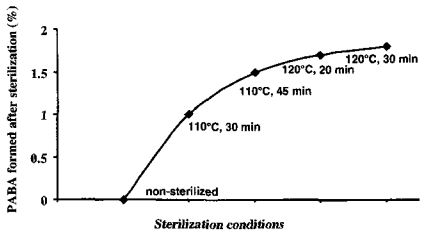


Figure 2
Effect of sterilization conditions on the appearance of PABA formed by hydrolysis of procaine in CP1B.

After five years of storage at room temperature protected from light, the procaine concentration in one batch only was found to be lower than standards (46.4 mmol L^{-1}) and this batch was rejected. Two other batches were at the lower limit of acceptability. The shelf life of two years after the date of manufacture is thus totally satisfactory. After two years of storage, mean procaine breakdown was 3%, a level that remained almost constant during the third and fourth years.

Conclusion

As might have been expected, there was no evidence of considerable breakdown of procaine during sterilization of manufactured batches of CP1B. The concentration of procaine in the finished product remained compliant with the standards set by the European Pharmacopoeia 3rd edition. The PABA formed is a breakdown product, the concentration of which must be determined, justifying this work. On the other hand, and at the current state of scientific knowledge, it is not a real public health risk – it is the active ingredient in other pharmaceutical preparations administered systemically.

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