

Clinical aspects of allergic disease

Skin testing with penicilloate and penilloate prepared by an improved method: Amoxicillin oral challenge in patients with negative skin test responses to penicillin reagents

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Background: Penicillin skin testing has been limited by the lack of commercially available penicilloate and penilloate reagents.

Objective: This project was proposed to produce a stable, well-characterized supply of penicilloate and penilloate for intrastate use by our health maintenance organization and to document clinical safety and efficacy.

Methods: An improved method of extraction for penicilloate and penilloate, which changed the solvents used during recrystallization, was developed. With these newly prepared reagents, penicillin skin testing was performed on 348 subjects. Skin testing was immediately followed by an oral challenge of 250 mg of amoxicillin in 215 of 288 (75%) subjects displaying a negative response to a battery of penicillin skin tests.

Results: Nuclear magnetic resonance and mass spectrometry of the newly produced penicilloate and penilloate showed no evidence of organic contamination. Penicillin skin testing resulted in 17.2% (60 of 348) positive test results, with 20% of the subjects with positive results only responding to the newly produced minor determinants. The rate of mild adverse reactions to penicillin skin testing was 1.1% (4 of 348). The rate of mild acute adverse reactions was 5.1% (11 of 215), and the delayed reaction rate was 0.9% (2 of 215) with the amoxicillin challenge.

Conclusions: This improved penicillin minor determinant extraction method allows for the reproducible production of very pure preparations of penicilloate and penilloate. Large-scale penicillin skin testing, followed by amoxicillin challenge if results are negative is feasible in a large group model health maintenance organization operating within a single state with the use of internally produced penicilloate and penilloate and commercially available penicillin, amoxicillin, and penicilloyl polylysine. (*J Allergy Clin Immunol* 1997;100:586-91.)

Abbreviations used

AC:	Amoxicillin challenge
PenST:	Penicillin skin testing/test
PrePen:	Penicilloyl polylysine
TBS:	Tris-buffered saline

Key words: Allergy, amoxicillin, challenge, health maintenance organization, minor determinants, penicillin, penicilloate, penilloate, skin test

Penicillin allergy is the most common self-reported drug allergy. In a sequential sample of 827 patients seeking treatment at one of our urgent care clinics, 86 (10.4%) reported a history of an adverse reaction to a penicillin class antibiotic. Penicillin skin testing (PenST) with only the commercially available reagents (penicillin G, amoxicillin, and penicilloyl polylysine [PrePen]) cannot accurately diagnose all potentially true-positive cases of penicillin allergy. Skin test reactivity restricted to the two minor determinants, penicilloate and penilloate, can account for 10% to 30% of all patients with penicillin allergy identified by the most comprehensive available PenST and may include those patients with the highest probability of anaphylaxis on penicillin exposure.¹

Most large studies have demonstrated that fewer than one in five subjects with a history of an adverse reaction to a penicillin class antibiotic have positive PenST results.²⁻⁴ Unnecessary avoidance of penicillin class antibiotics is thought to be contributing to increased resistance of bacteria to nonpenicillin class antibiotics, such as resistance of *Staphylococcus aureus* to vancomycin. If a subject is not allergic, then penicillin class antibiotics are among the most well-tolerated antibiotics with respect to other side effects. Therefore knowing whether large numbers of "penicillin-allergic" individuals are truly allergic to penicillins may improve the quality of medical care provided.

In general, PenST is safe, and the incidence of false-negative results is thought to be low, but there are still concerns. PenST well in advance of antibiotic use has been shown to be safe in children by Mendelson et al.⁵ The problem with false-negative test results can be

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reduced by using a challenge dose of amoxicillin in subjects with negative PenST results, but even patients with positive results occasionally tolerate oral challenges with penicillins. Whether PenSTs and amoxicillin challenge (AC) resensitize subjects with currently negative results at a low frequency remains to be determined.

One of the limiting factors to comprehensive universal PenST has been the lack of commercially available minor determinants, penicilloate and penilloate. To circumvent this problem and to provide a stable supply of well-characterized reagents for our organization, we produced a total of 100 gm each of penicilloate and penilloate by using an improved extraction method. In this article we demonstrate the safe clinical use of PenSTs in a large group model health maintenance organization, using internally produced penicilloate and penilloate prepared by an improved method of extraction.

METHODS

Preparation of penicilloate

To a solution of penicillin G at 25°C (100 gm 1.0 equivalent benzylpenicillin sodium salt, Sigma) in distilled water (200 ml, 1.4 mol/L), a solution of 3 mol/L NaOH (103 ml, 1.1 equivalent) was added dropwise such that the acidity level was maintained at pH 12 (approximately 2 to 3 hours, 1 drop every 10 seconds). An automatic stirring apparatus in a 3 L round-bottom flask was used to maintain stirring consistency. A pH meter, calibrated with pH 10 buffer at a low temperature was used to maintain the pH at 12 and the temperature between 25°C and 30°C. After the solution was added with constant stirring, the reaction was cooled to 0°C (approximately ½ hour by immersion of the flask in an ice bath), and a 6N HCl solution (approximately 56 ml, 1.2 equivalent) was added dropwise (1 drop every 10 seconds) to gradually lower the pH to 4.5. (It is important to maintain constant stirring with an automatic stirrer and keep the temperature below 10°C during addition of HCl.) When the pH reached 4.5, the solution formed a cloudy white precipitate. Stirring was continued with addition of 6N HCl, but at a reduced rate of 1 drop every 20 seconds, until the pH reached 3.8. When the pH reached 3.8, the precipitate was collected with a spatula and placed on a scintered glass filter with low vacuum. The precipitate was washed with ice water (4 times, 100 ml total) and acetone (4 times 80 ml total). The crude solid was air-dried and then recrystallized in a solution of acetone (740 ml, 0.38 mol/L), water (225 ml, 1.26 mol/L), and MeOH (370 ml, 0.76 mol/L) and was added to the solid in this order. If the solvents were not added in this order, the compound would become hydrated and could not be crystallized. Next, after 10 minutes of vigorous stirring at 30°C with a stir bar in a 4 L Erlenmeyer flask, a second aliquot of MeOH (74 ml, 3.8 mol/L) was added, and the entire crude preparation was vigorously stirred until it dissolved (approximately 1.5 hour/100 gm). Once dissolved, the crude preparation was filtered through paper into another 4 L flask and then ethyl acetate, cooled to 15° to 20°C (1.2 L, 0.25 mol/L) and was added in one step. Crystals forming after 5 minutes were noted. The solution was then stored at 5°C for 2 days in a cold room. The crystals were then collected on a scintered glass funnel, and the filtrate was saved (to be condensed and used for the next step) and washed with 1:1

solution of acetone/water (3 times, 50 ml). The resulting fine, pure, white needles were then dried over high vacuum for 5 days in an Obderhall drying tube apparatus with P₂O₅ as the drying agent at 25°C. The observed yield varied between 40% and 55%. The solid was then weighed in a glove/dry box under argon atmosphere at 25°C, separated into 1 gm aliquots, and placed in 10 ml silicon-stoppered bottles. The pure solid was stored at -78°C.

Penicilloate assay data

mp. 154-155°C; ¹HNMR (250 MHz, CDCl₃)—8.813-8.789 (m, 4H, ArH), 8.750-8.710 (m, 1H, ArH), 6.804-6.794 (d, J = 5.0 Hz, 1H, CON-CH-CO₂), 6.425 (bs, 1H, CONH), 6.020-6.10 (d, J = 5.0 Hz, 1H N-CH-5), 5.249 (s, 1H, N-CH-CO₂), 5.130-5.061 (dd, J = 11.5 Hz, 2H, Ph-CH₂-CON), 4.800 (s, 1H, NH), 3.031 (s, 3H, CH₃), 2.823 (s, 3H, CH₃); ¹³CNMR (125 MHz, CDCl₃) — 157.7, 155.2, 120.4, 114.1 (2c), 113.3 (2c), 111.6, 59.1, 49.8, 40.9, 32.3, 32.2, 27.4, 11.0, 10.4; Mass Calculated for C₁₆H₂₀O₅N₂Na (N+Na) 375, found 375.

Preparation of penilloate

The filtrate, collected from the recrystallization step with penicilloate, was condensed by means of rotovaporation to remove the remaining acetone, MeOH, and ethyl acetate. The resulting condensate (in water solution) was then diluted with additional water (one third of initial condensate volume) and then boiled for 5 minutes in a 2 L Erlenmeyer flask with a stir bar. With vigorous stirring the pH was slowly lowered to 1.5 with 10N HCl (added in single drops until the solution cleared). The pH was monitored with a pH meter and calibrated for low pH and high temperature; the pH meter was washed frequently to prevent sticky residue buildup. This is the most difficult step. Once the solution reached pH 1.5, it was cooled to room temperature, 25°C, and then ethanol (one third of the reaction volume) was added with constant stirring. The solution was then boiled for 5 minutes with stirring and then removed from the hot plate to cool. (Note: any precipitate formed in the pH step should have dissolved to produce a clear solution at this stage). The solution was allowed to cool to 25°C, and the crystal formation was observed after 10 minutes. The mixture was crystallized overnight at 25°C, and the crystals were collected on a scintered glass filter, washed with cold water (2 times, 50 ml) and then ether (2 times, 50 ml). The resulting pure white needles (longer than penicilloate) were dried over high vacuum for 5 days in an Obderhall drying tube with P₂O₅ as the drying agent at 25°C. The observed yield, based on the remaining precursor penicilloate (recovered from the previous step), varied between 50% and 60%. The solid was then weighed in a glove-dry box under argon atmosphere at 25°C and divided into 1 gm aliquots in 10 ml silicon-stoppered bottles. The solid was stored at -78°C.

Penilloate assay data

mp. 107-108°C; ¹HNMR (250 MHz, CDCl₃)—8.785-8.774 (m, 5H, ArH), 6.438 (bs, 1H, N-CH-S), 5.220 (s, 1H, N-CH-CO₂), 4.99 (s, 2H, Ph-CH₂-CON), 4.827-4.799 (m, 3H, CON-CH₂-C-S and NH), 3.108 (s, 3H, CH₃), 2.767 (s, 3H, CH₃); ¹³CNMR (125 MHz, CDCl₃) — 158.3, 155.2, 120.4, 114.0 (2c), 113.9, 113.4, 111.7, 56.7, 48.5, 42.1, 29.8, 27.5, 12.0, 11.2; Mass Calculated for C₁₅H₂₁O₃N₂S (N+H) 309, found 309.

Tris-buffered saline

A solution of 0.14 mol/L NaCl and 0.02 mol/L Tris base (Sigma) was brought to pH 7.4 with 1 mol/L HCl and filter-

TABLE I. Type and specificity of positive penicillin skin test reactions

Reagent	Puncture	Intradermal
PrePen		24
Penicilloate	2	2
Penilloate	1	6
Penicilloate + penilloate		1
Penicillin + PrePen		6
Penicillin		2
Amoxicillin		1
All other combinations*	3	12
Total	6	54

*All of these subjects had positive responses to at least one of the commercially available skin test materials: amoxicillin (3 of 15), PrePen (11 of 15), and penicillin (1 of 15).

sterilized through a 0.22 μ m filter. Tris-buffered saline (TBS) was used as diluent and control for all skin testing.

Protection of human subjects

This protocol was approved by the Kaiser Permanente Southern California Institutional Review Board. Written informed consent was obtained from all subjects.

Legal issues

Many states have laws and regulations that impose the equivalent of Food and Drug Administration regulations on investigational new drugs and devices that are manufactured and evaluated entirely within the state. The California Health and Safety code has several sections that address the testing and approval process for new drugs and devices within the state and sections that provide protection for human subjects involved in medical experimentation. These sections generally incorporate by reference the collateral or parallel requirements of federal law. However, for the purposes of this study, both the authors and the institutional review board that approved this study relied on federal regulations, rather than state law.

Skin testing

Skin testing was performed by using prick puncture and intradermal techniques with the following materials.

1. Penicilloyl (PrePen) from Schwarz Pharma (used per package directions)
2. Sodium penicillin (benzylpenicillin) for intravenous injection (0.01 mol/L in TBS)
3. Sodium amoxicillin from Sigma (0.01 mol/L in TBS)
4. Penilloate (0.01 mol/L in TBS)
5. Penicilloate (0.01 mol/L in TBS)
6. Control in TBS
7. Histamine from Eli Lilly (1 mg/ml)

All materials were prepared and maintained in a sterile fashion. The testing solutions 1, 3, 4, and 5 were divided into aliquots and placed in 1 ml single-dose vials. Approximately 0.2 ml of solution was placed in each vial and stored frozen at -78° C. Vials were thawed one time only just before use. The stock bottles of control saline and histamine solutions were stored at 4° C. Prick puncture testing was performed by using a bifurcated needle. Intradermal testing was performed with 0.02 ml of material administered through a 27-gauge tuberculosis syringe. Skin test results were recorded in millimeters as the mean diameter of wheal over mean diameter of flare or

TABLE II. Time to onset of index adverse reaction after penicillin class antibiotic*

Hours	PenST positive			PenST negative		
	n	%	95% CI	n	%	95% CI
<1	6	13.0	(6.1, 13.9)	46	16.0	(13.8, 18.2)
1-24	15	25.0	(19.4, 30.6)	86	29.9	(27.2, 32.6)
25-72	13	21.7	(16.4, 27.0)	39	13.5	(11.5, 15.5)
>73	12	20.0	(14.8, 25.2)	42	14.6	(12.5, 16.7)
Unknown	14	23.3	(17.8, 28.8)	75	26.0	(23.4, 28.6)
Total	60	100		288	100	

CI, Confidence interval.

*All comparisons are not significant.

surrounding erythema. Skin test reactions were read at 15 minutes after application. Positive responses consisted of a wheal of 5 mm or more in diameter with surrounding erythema greater than the wheal, a negative response to control solution and a positive response to histamine. Amoxicillin skin tests were only performed on the 264 subjects who entered the study at the San Diego site.

Oral challenge

Amoxicillin was chosen as the penicillin class antibiotic for challenge because 81.5% of all penicillin class antibiotics used in 1995 in our health maintenance organization were amoxicillin-based. Amoxicillin also accounted for 69% of the reported index adverse reactions in which the subject could recall the name of the antibiotic. AC was offered to all subjects with negative PenST results, and 215 of 288 (74.6%) accepted. AC consisted of a 250 mg oral dose of amoxicillin. At the San Diego site 70.0% of subjects (157 of 224) were challenged, and at the other sites, 90.6% of subjects (58 of 64) were challenged. All patients were observed for 1 hour. The leading reason for challenge not occurring was patient refusal, either because of the additional time required for observation or fear of adverse reaction despite negative PenST results. Patients reported delayed reactions by phone.

Patient selection

Patients were either self-selected for evaluation of penicillin allergy or sent by their primary care physician or a consulting physician to an allergy department for evaluation. Two hundred sixty-four patients were enrolled by the primary allergy department (San Diego) involved in this study, and the remaining 84 patients were enrolled by seven other allergy departments in the Kaiser Permanente Southern California Region. Subjects were offered PenSTs if they had a history of an adverse reaction to a penicillin class antibiotic that did not include Stevens-Johnson Syndrome, toxic epidermal necrolysis, serious hepatic disorder, hematopoietic disorder, or anemia. These excluded disorders are not IgE-mediated, and the PenST would be of no predictive value regarding the possibility of a recurrence of the same condition. Patients receiving long-term β -blocker therapy were not skin tested if the β -blockers could not be stopped. Patients with dermographism at the time of skin testing were not entered into the study.

RESULTS

Penicilloate and penilloate production

The details of the improved extraction methodology in the Methods section are based on the work of Levine

TABLE III. Primary type of index adverse reaction after penicillin class antibiotic*

Reaction	PenST positive			PenST negative		
	<i>n</i>	%	95% CI	<i>n</i>	%	95% CI
Anaphylaxis	2	3.3	(1.0, 5.6)	17	5.9	(4.6, 7.3)
Anxiety	1	1.7	(0.0, 3.4)	10	3.5	(2.3, 4.5)
Fixed drug eruption	2	3.3	(1.0, 5.6)	16	5.6	(4.2, 7.0)
Hives	47	78.3	(73.0, 83.6)	200	69.4	(66.7, 72.1)
Gastrointestinal	1	1.7	(0.0, 3.4)	8	2.8	(1.8, 3.8)
Unknown and other	7	11.7	(7.6, 15.9)	37	12.9	(10.9, 14.9)
Total	60	100		288	100	

CI, Confidence interval.

*All comparisons are not significance.

and Redmond⁶ as modified by Andrew Saxon (personal communication). The changes in the solvents used in the recrystallization of the penicilloate were found to produce a more consistent result. The most notable changes were the use of acetone, water, and methanol as opposed to only acetone and water to dissolve the crude crystals and the recrystallization with ethyl acetate rather than acetone. There were no significant changes in the penilloate procedure, but if the starting penicilloate had not been hydrated, the crystallization of the penilloate appeared to proceed more reproducibly.

Penicilloate and penilloate assay

The melting points of penicilloate and penilloate were consistent with the published predictions.⁷ The identities of the penicilloate and penilloate were demonstrated by nuclear magnetic resonance and mass spectrometric studies, and the specific data are given in the Methods section. The hydrogen 1 and carbon 13 nuclear magnetic resonance studies of each minor determinant showed only the resonance frequencies expected for the bonds in each molecule, with no evidence of any organic contamination. Mass spectrometry of the penicilloate showed a major peak at 375 d, consistent with the protonated derivative, and several minor bands appropriate for the salt-hydrate complexes. The mass spectrometry of the penilloate showed a major peak at 309 d, consistent with the protonated penilloate; a peak 20% as large corresponding to a mass of 331 d, consistent with the sodium salt; and several bands 5% or less as large with molecular weights of 263 d or less, consistent with fragmentation products from the analysis.

Indirect biologic evidence that neither minor determinant preparation contained significant amounts of either starting penicillin or the other determinant was provided by PenST in that five patients uniquely reacted to penicilloate and six patients uniquely reacted to penilloate.

Clinical testing data

All patients were entered into the study between November 16, 1994 and May 28, 1996. The mean age of the patients who had PenST was 38 ± 23.0 years (range, 0.2 to 80.5 years). A total of 60 of the 348 subjects

(17.2%) had positive responses to at least one reagent. Subjects with positive PenST results compared with those with negative results were younger (mean age, 28.4 ± 22.6 years vs 40.0 ± 22.6 years; $p = 0.0003$) and had a more recent time since index adverse reaction (6.9 ± 12.0 years vs 15.6 ± 16.0 years; $p = 0.0001$). There was no gender difference in prevalence of subjects with positive PenST results, though more women (70.1%) than men (29.9%) were tested.

The distribution of positive PenST reactions is significant in that the newly produced penicilloate and penilloate uniquely accounted for 12 of the 60 (20%) positive test results. PrePen alone accounted for 24 subjects with positive results (40%), native penicillin alone for two (3.3%), and native amoxicillin alone for only one (1.7%). All other combinations (35%) accounted for all of the remaining subjects with positive PenST results (Table I).

There was no apparent association between the time to onset of the index adverse reaction to a penicillin class antibiotic and PenST status (Table II), nor was there any association between the pattern of symptoms reported with the index adverse reaction and PenST results (Table III).

Of the 288 subjects with negative results, 215 agreed to an AC. Subjects who had AC were significantly younger than nonchallenged subjects (mean age, 38.1 ± 23.0 years vs 45.6 ± 20.5 years; $p = 0.0138$) but had a similar time since reaction (15.3 ± 15.9 years vs 16.2 ± 16.3 years; $p = \text{NS}$).

Adverse reactions

Four subjects experienced adverse reactions temporally associated with PenST. All reactions occurred within the first 1/2 hour after the PenST. Fewer reactions occurred at the San Diego site (1 in 264, 0.38%) compared with the other sites (3 in 84, 3.57%; $p = 0.0451$). Two adverse reactions occurred in subjects who had positive PenST results, and two occurred in subjects with negative PenST results. One reaction occurred after a positive intradermal test response to PrePen, which consisted of generalized urticaria, and was treated with epinephrine and an antihistamine. One reaction occurred after a positive intradermal test response to

penicilloate, which consisted of itching without rash, and was treated with an antihistamine. Both index reactions were hives. Both reactions resolved promptly. In two subjects the PenST result was negative, yet the subjects complained of an adverse effect. One subject had itching without rash and was given an antihistamine, and one subject was lightheaded but normotensive and only received assurance. The subject who was lightheaded tolerated a therapeutic course of amoxicillin 3 months after the negative skin test results, the other subject received no antibiotics during a 19-month follow-up period.

AC resulted in a 5.1% (11 in 215) rate of mild acute adverse reactions, all of which completely resolved within 1 hour. Most of the reactions (10 of 11) occurred in women, and the subjects who noted reactions were on average older (mean age, 58 years; range, 35 to 78 years). A possible IgE-mediated reaction was noted in only two subjects (0.9%). One subject had transient hives on the trunk, which resolved without any treatment in less than 1/2 hour. The other subject experienced coughing and was treated with epinephrine and an antihistamine. Neither patient could recall the antibiotic associated with the index reaction. The other, probably nonallergic reactions included itching without rash ($n = 6$, three treated with an antihistamine), coughing ($n = 1$), and gastrointestinal upset ($n = 2$, one treated with an antihistamine). The wide range of treatments given reflects the personal preference of the consulting physician present at the time of the AC. A prospective follow-up of antibiotic use was then undertaken. During a follow-up period ranging from 10 to 25 months, seven of these 11 subjects received at least one therapeutic course of a penicillin class antibiotic, including the patient who had received epinephrine and an antihistamine, and no adverse reactions were noted.

Two delayed reactions, which occurred 24 to 48 hours after AC, were reported (0.9%). Both may have been immunologically mediated but were clearly non-IgE-dependent. They involved nonurticarial skin rashes, one isolated to the face and the other more generalized with associated mild joint discomfort and myalgias. Both resolved over several days without any therapy. These subjects were not re-exposed to penicillins.

DISCUSSION

The literature does not suggest that a major problem exists in the preparation of penicillin minor determinants, but those who have attempted it were often faced with failure (personal communication with Andrew Saxon), and a commercial source does not exist. Although the descriptions of the preparation of these minor determinants by Levine and Redmond⁶ and Mazingo and Folkers⁷ indicate good yields of usable products, this is only sometimes the case. Frequently, the crystallization does not occur. The modified solvent system presented here is the best of a number tried and should

make the crystallization of penicilloate and penilloate more consistent.

Twenty percent of the subjects with positive PenST results were identified solely by use of the minor determinants, penicilloate and penilloate, prepared by this revised method (Table I), an incidence similar (14% to 22%) to that noted by others using these determinants in both adults and children.^{1, 2, 5, 8-10} The present modified method of penicillin minor determinant preparation generates reproducible high yields of pure penicilloate and penilloate. These reagents possess the capacity to identify the requisite prevalence of patients with positive PenST responses solely to minor determinants and as such represent an important advance in minor determinant production. Those wishing to prepare minor determinants of other penicillin analogs may want to refer to the work of Goldschmidt and Levine.¹¹ These investigators prepared the penicilloic acid derivatives of 11 different penicillin analogs, using several different solvent systems.

Only 0.3% of the subjects tested with the PenST battery had an apparent allergic reaction (urticaria), testimony to the relative safety of such testing in subjects with a positive history of penicillin allergy, as noted by others^{1-5, 8-10, 12} when performed in experienced hands and with appropriate concentrations of reagents and starting with prick-puncture testing before intradermal testing. Subjects with positive PenST results were about 30% younger and had experienced the index adverse reaction to penicillin in less than half the elapsed time from testing than subjects with negative PenST results, confirming earlier reports.^{8, 9} Subject recollection of both the time of onset to (Table II) and the symptoms of a reaction (Table III) could not discriminate between those with positive or negative PenST results as noted previously.⁸ This attests to the importance of PenST to safely document true penicillin allergy.

Oral amoxicillin challenge immediately after negative PenST results in these subjects with positive histories led to mild reactions in 5.2% and delayed reactions in 0.9% of subjects, an incidence similar to that noted by others.¹ These findings attest to the safety of penicillin challenge or administration in subjects with positive history and negative PenST results. Moreover, these findings fail to show a significant allergy to the aminopenicillin, amoxicillin, in this population of subjects with negative PenST results.

These findings support the routine PenST of subjects thought to be allergic or intolerant to penicillin and provide a novel method for the preparation of high-quality penicillin minor determinants in high yield to accomplish this task in a more efficient manner than previously reported. An economic and safety analysis of antibiotic use before and after PenST is currently being determined for these subjects.

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