Basophil histamine release to Amoxicilloyl-poly-L-lysine compared to amoxicillin in patients with IgE-mediated allergic reactions to amoxicillin.

RUNNING TITLE: Specific IgE in amoxicillin allergy.

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ABSTRACT

Background: Amoxicillin (AX) is the betalactam most often involved in IgE-mediated reactions and the diagnosis is mainly based on skin testing (ST) although without optimal sensitivity. We have produced a newly AX derivative, amoxicilloyl-poly-L-lysine (APL), and have analysed its IgE recognition by passive histamine release test (pHRT).

Methods: Patients (N=19) with confirmed AX allergy and with specific IgE positive to AX and controls (N=10) with good tolerance to AX were included. pHRT was done using "IgE-stripped" blood from a unique donor, sensitized *in vitro* by patient sera, and incubated with AX or APL. Histamine released was determined and expressed as ng of histamine release/mL blood.

Results: Patients clinical symptoms were anaphylaxis (N=9), urticaria (N=7), erythema (N=2) and not defined immediate reactions (N=1). The median time interval between reaction and study was 90 days (IQR: 60-240) and between drug intake and development of symptoms 24 min (IQR: 10-60). The median sIgE level was 3.37 kUA/L (IQR: 0.95-5.89). Sensitivity of pHRT to APL was 79% and the specificity 100% which were higher than data obtained with pHRT to AX (63% sensitivity and 90% specificity). There was a positive correlation between maximal histamine release levels obtained with AX and APL (r=0.63).

Conclusions: In patients with immediate hypersensitivity reactions to AX, APL showed a higher sensitivity and specificity compared to the culprit drug, AX, when tested *in vitro* by pHRT. This indicates that APL can improve *in vitro* diagnostic accuracy of AX allergic reactions. Further use for ST needs to be done.

KEYWORDS: Allergy, Amoxicillin, Amoxicilloyl-poly-L-lysine, Basophil histamine release, Immediate reactions.

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RESUMEN

Introducción: La amoxicilina (AX) es la betalactama más frecuentemente implicada en reacciones mediadas por IgE y el diagnóstico se basa principalmente en pruebas cutáneas (PC) aunque no tiene una óptima sensibilidad. Hemos diseñado un nuevo derivado de AX, amoxicilloyl-poly-L-lisina (APL), y se ha analizado el reconocimiento frente a la IgE mediante la prueba de liberación de histamina por sensibilización pasiva (pHRT).

Métodos: Se incluyeron pacientes (N = 19) con alergia a AX confirmada y con IgE específica positiva para AX y controles (N = 10) con buena tolerancia a AX. La pHRT se realizó utilizando sangre "libre de IgE" de un único donante, sensibilizada in vitro con el suero de los pacientes e incubada con AX o APL. La histamina liberada se determinó y se expresó como ng de liberación de histamina / ml de sangre.

Resultados: Los síntomas clínicos de los pacientes fueron anafilaxia (N = 9), urticaria (N = 7), eritema (N = 2) y reacciones inmediatas no definidas (N = 1). El tiempo medio del intervalo entre la reacción y el diagnóstico fue de 90 días (IQR: 60-240) y entre la ingesta de fármacos y el desarrollo de los síntomas 24 min (IQR: 10-60). Los valores medios de sIgE fueron de 3.37 kUA/L (IQR: 0.95-5.89). La sensibilidad de pHRT a APL fue de 79% y la especificidad de 100%, siendo superior a la obtenida con pHRT a AX (63% de sensibilidad y 90% de especificidad). Hubo una correlación positiva entre los niveles máximos de liberación de histamina obtenidos con AX y APL (r = 0,63).

Conclusiones: En los pacientes con reacciones de hipersensibilidad inmediata al AX, el APL mostró una mayor sensibilidad y especificidad en comparación con el fármaco causante de la reacción (AX), cuando se analizó por pHRT. Esto indica que APL puede mejorar la precisión diagnóstica in vitro de las reacciones alérgicas AX. Es necesario seguir utilizando PC.

PALABRAS CLAVE: Alergia, Amoxicilina, Amoxiciloyl-poli-L-lisina, Basófilo liberación de histamina, Reacciones inmediatas.

INTRODUCTION

Betalactam (BL) antibiotics are still the most frequent drugs causing IgE-mediated allergic reactions [1-3]. Although all BLs can be responsible, currently amoxicillin (AX) alone or combined with clavulanic acid and, to lesser extent, cephalosporins, are the most frequent culprits [1-3]. AX can induce selective reactions, implying that patients react to this antibiotic while tolerating other BL as benzylpenicillin (BP) [4-6], with immunochemical studies indicating that the side chain structure is important for immunological recognition [7-9].

The diagnosis of immediate allergic reactions to AX is mainly based on skin tests (ST) or drug provocation test (DPT), procedures that are time consuming and not risk free [10-14]. ST is performed using major and minor determinants of BP plus AX, however, it sensitivity is less than 70% [11]. For increasing ST sensitivity, AX minor determinants, amoxicilloyl and diketopiperazine [15], have been used although none of them increased sensitivity when compared with AX itself, indicating that new AX structures need to be investigated. Recently benzylpenicilloyl-Octa-L-lysine has been shown to be a stable compound useful for ST [16], so it is tempting to speculate that amoxicilloyl-Octa-L-lysine (APL) could be synthetized in a similar way.

In vitro tests, such as ImmunoCAP and basophil activation test (BAT), represent a complementary approaches to diagnose allergy to BL [17-20], although the former has low sensitivity [21] and the latter need to be done in few hours after blood extraction and require expertise [19]. Other cellular test, such as passive histamine release test (pHRT), has shown to be useful for diagnosing clavulanic acid allergy [22]. This test is based on the detection of histamine release using “IgE-stripped” donor blood sensitized with patient serum followed by incubation with the antibiotic and is suitable as a routine diagnostic test as the plates can be sent to a reference laboratory for histamine detection and data analysis [23].

In this study a new synthetic product, APL, which it is expected to have greater stability than the parent drug AX, will be produced. The basophil reactivity to both APL and AX will be analysed using pHRT with sera from patients with confirmed immediate allergic reactions to AX. This could be the first step for further evaluation of APL as a reagent useful for ST diagnosis.

MATERIAL AND METHODS

Patient selection

The study group included patients with a confirmed diagnosis of immediate allergic reactions to AX during 2013 in the Allergy Unit of four Spanish Hospitals. For diagnosis, we followed the European Academy of Allergy and Clinical Immunology (EAACI) general guidelines for the evaluation of immediate reactions to BLs [12].

Patients included had an allergic reaction that appeared in less than 90 minutes after AX administration and presence of serum specific IgE (sIgE) to AX (≥0,35 kU_A/L). A control group comprising 10 healthy individuals who had previously received AX without any adverse effects, including allergic reactions, and with negative ST and serum sIgE to AX were included.

Relevant institutional review boards approved the study and informed consent was obtained from all patients and controls.

Skin testing

Skin prick and intradermal tests were carried out as previously described [12], using 0.03 ml of solution prepared daily. The reagents were: benzylpenicilloyl octa-L-lysine (BPOL) 0.04 mg/ml, with a molar concentration of the benzylpenicilloyl (BPO) moiety of $8.64 \cdot 10^{-5}$ M, minor determinant (DM) 0.5 mg/ml, with a molar concentration of the sodium benzylpenilloate of $1.5 \cdot 10^{-3}$ M and AX 20 mg/ml (Diater laboratories, Madrid, Spain). In skin prick testing, a wheal larger than 3 mm surrounded by erythema, with a negative response to the control saline, was considered positive. In intradermal tests, the wheal area was marked initially and 20 minutes after testing, and an increase in diameter greater than 3 mm surrounded by erythema was considered positive. In those cases with a history of severe anaphylaxis we performed the test with increasing dilutions before reaching the maximum concentration as described.

Specific IgE determination

This was performed by CAP-FEIA system (Pharmacia diagnostics, Uppsala, Sweden), using c6 (amoxicilloyl, AXO) following the manufacturer's instructions (21). The results were obtained by direct comparison with standards run in parallel, considering a value greater than 0.35 kUA/L as positive.

Synthesis and characterization of APL

APL a molecule with a molecular mass of 3966.6 Da and a chemical formula $C_{176}H_{250}N_{40}O_{49}S_{8,}$ was obtained through a process of clogging octa L-lysine resulted in a linear chain molecule with 8 recognizable fragments of amoxicilloyl. The scheme of the reaction and the resulting structure is shown in supplementary figure 1A. Briefly, dissolution of sodium AX (in excess) and octa-L-lysine in aqueous basic pH solution were reacted until no traces of octa-L-lysine were detectable. Media acidification with

HCl solution to pH below 4.5 was performed followed precipitation. The obtained product was pre-purified with cycles of re-suspension/centrifugation in cold methanol and the resulting solid mixture was lyophilized. The last purification stage was conducted through semi-preparative chromatography using UV detection, selecting the APL eluting time, and discarding the other residues. The resulting product was characterized by proton and carbon nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) performed in a Bruker AV-300 spectrometer as well as by fourier transform infrared (FT-IR) spectrometry using a Nicolet Avatar 320 FT-IR spectrometer. The purity of the compound was analysed using high-performance liquid chromatography (HPLC) (supplementary figure 1B).

Passive Basophil Histamine Release Test

This has been done as previously described [22]. Briefly, passive sensitization of basophils was performed using heparinized blood from the same single healthy non-allergic donor, with confirmed strong reaction to anti-IgE (histamine released: 45 ± 5 ng/mL) and no response to AX or APL. To remove the IgE bound on the basophils surface, 3 mL of blood sample was treated with 10 mL of stripping buffer (0.14 M NaCl, 0.005 M KCl and 0.0134 M lactic acid) for 10 min at 4˚C. Then, 0.5 mL of sera from patients or controls was incubated with 3 mL of the "IgE-stripped" donor blood for 60 min at 37˚C. Aliquots of 25 µL of passively sensitized blood in the presence of IL-3 (2 ng/mL blood) were incubated in the glass fibre-prepared microtiter plates (RefLab ApS, Copenhagen, Denmark), with 25 µl of AX 5 µg/mL or APL 10 µg/mL for 60 min at 37°C. The AX and APL concentration chosen for this test was the highest value that did not lead to unspecific histamine release (> 10 ng/mL) in a dose-response curve. This level corresponded to the mean plus 3 standard deviation (SD) of the histamine release values measured in 5 non-allergic subjects without a specific stimulus. As a positive control, we used 25 µl of anti-IgE (KPL, USA, MD) in a concentration of 5 µg/mL as well as histamine in concentrations of 0 and 50 ng/mL.

After incubation, 75 µl of NaOH/ortho-phtaldialdehyde (OPA) mixture was added to each well, allowing glass fiber bound histamine to be released and coupled to OPA. After 10 min the coupling reaction was stopped and histamine-OPA complexes stabilised by adding 75 µl of 0.59% $HClO_4$. Histamine was determined fluorometrically in the Histareader™ 501 manufacturing company (RefLab, Copenhagen, Denmark). Results were expressed as ng/mL of histamine released.

Statistical analysis

Description of quantitative variable was performed including the median and the interquartile range (IR). Comparisons for variables without a normal distribution were carried out by the Mann-Whitney test. ROC curve analyses were performed to

calculate the optimal cut-off value corresponding to the best sensitivity and specificity. All reported p-values represent two-tailed tests, with values < 0.05 considered statistically significant.

RESULTS

The synthesis of APL was carried out by acylation of the primary amino groups of octa-L-lysine with the ß-lactam ring of AX molecules. The chemical characterization of the resulting conjugate was based on ^{1}H-NMR and ^{13}C-NMR, which spectra show the signals corresponding to its structure (supplementary figure 1A and B). The characterization was also confirmed by FT-IR spectroscopy that showed the absorption bands consistent with the functional groups included in the structure of the molecule.

The study included 19 patients with immediate hypersensitivity reactions to AX with the presence of serum sIgE to AX. The clinical characteristics and the results of the allergological work-up are shown in Table 1. The median age was 51 years (IR: 33-62), 6 were females, AX was the drug involved in all reactions, 9 developed anaphylaxis, 7 urticaria, 2 erythema accompanied of intense pruritus and in one patient the clinical reaction, although immediate, was not clearly defined. The median time interval between reaction and study was 90 days IQR: 60-240) and between drug intake and development of symptoms 24 min (IQR: 10-60). The median sIgE level to AX was 3.37 kUA/L (IQR: 0.95-5.89).

Considering ST results to AX, 10 patients were positive in immediate reading: 7 had a prick test positive at the maximum concentration of 20 mg/ml and 3 had intradermal test positive also at the maximum concentration of 20 mg/ml. In 2 patients ST were negative to AX although they had positive ID test to BPOL (Patient 10) or to BPOL and DM (patient 11). In five patients (4 anaphylaxis and 1 urticaria) ST were not done due to patient risk factors (Patient 17 and 19) or because of the severity of the reaction (Patients 1, 5 and 14). Finally, in two cases with prick negative to AX, ID was not done due to the high positivity in CAP assay (6,03 kUA/L in patient 8 and 5,26 kUA/L in patient 12). No patient developed systemic symptoms after ST, being negative to BPOL, DM and AX in all 10 controls subjects.

For pHRT standardization, we first performed a dose response study establishing the highest concentration of AX and APL that did not induce unspecific histamine release corresponding to 3+SD of background values measured in 5 non-allergic subjects. We found that 5 µg/ml of either AX or APL were the best concentrations to perform the test and therefore they were used for further studies (supplementary figure 2).

The pHRT results obtained with AX and APL for each patient are shown in Table 1. When using AX results were significantly higher in patients (median: 16.5 ng/ml; IR: 7-

22) compared to controls (median: 12 ng/ml; IR: 8.88-12.5) and the same happened with APL being higher in patients (median: 19,5 ng/ml; IR:13-23.5) compared to controls (median: 8 ng/ml; IR: 5.13-11). Comparison of the median values of histamine released between patients and controls showed differences using either AX (p=0.13) or APL (p=0.0013), being differences greater for APL (11.5 ng/ml) when compared with AX (4.5 ng/ml) (Figure 1). Although the median histamine release with the APL molecule was higher than that obtained with AX, comparisons were not statistically significant (Figure 1). Moreover, a positive correlation between AX and APL induced histamine release in patients was detected (Pearson´s r = 0.631 and p = 0.004) (Figure 2).

In order to determine the optimal cut-off value for considering results as positive, ROC curve analyses were done, with results indicating that values > 12.5 ng/mL of histamine were the optimal cut-off for APL and > 14.5 ng/mL of histamine for AX (supplementary figure 3). Using these cut off, pHRT results with APL we found 15 out of 19 (79 %) positive cases and using AX showed 12 out of 19 (63 %) positive cases indicating a higher sensitivity for APL. The specificity results were also better for APL (100%) compared to AX (80%). There was not any patient only responding to AX.

DISCUSSION

Allergy to BLs is a major health problem with 10% of the population reporting to be allergic although less than 20% being truly allergic [1,2]. The diagnosis is complex and mainly based on ST, with sensitivity less than 70%, that imply that in a non-negligible percentage of cases a DPT need to be done for obtaining an accurate diagnosis [10-12,14]. The sensitivity of *in vitro* tests is also not optimal being especially lower with the CAP immunoassay [21,22]. The BAT which has a higher sensitivity has the disadvantage that need to be done in fresh blood. Recently the pHRT has been shown to be a useful method for diagnosing patients with immediate allergic reactions to CLV with a sensitivity of 55% and specificity of 85% and this is why we have applied this methodology in the present study [23].

All BLs used in clinical practice can induce allergy, but AX, combined or not with CLV, is the most common elicitor [1]. AX is a low molecular weight molecule formed by a ß-lactam ring fused to a thiazolidine ring and a side chain [9]. According to the hapten hypothesis AX needs to bind covalently to a carrier, usually a protein, in order to give an immune response, and this occurs through the opening of the ß-lactam ring by the amino groups of protein lysine residues, forming the AXO antigenic determinant [25-27]. However, other structures such as amoxicilloic acid (result from ß-lactam ring

hydrolysis) and diketopiperazine (resulting from intramolecular acylation by the amino group of AX side chain) can be formed, structures that has shown not to be useful for improving in vitro diagnosis using BAT of patients with immediate hypersensitivity reactions [16].

The major determinant of BP, the BPOL, is an stable product that has been shown in a prospective multicenter clinical trial to be useful for diagnosing patients with immediate BL allergy by ST [17]. Following the same synthetic approach, we have produced APL by acylation of the primary amino groups of octa-L-lysine with the ß-lactam ring of AX molecules. We have used both APL and AX as reagents in pHRT in order to confirm the specific recognition by sIgE from allergic patients bound to basophils. There are different reasons that have made us choosing pHRT instead of BAT: (i) it is a simple method that has been improved using glass microfiber plates to which histamine is specifically bound and that allows performing the tests in any laboratory independently of equipment availability and sending them to a reference laboratory; (ii) up to 10 drugs can be tested on the same plate; (iii) the test can be performed only by sending sera giving the possibility of the in vitro determination to centers where the technology is not available. However, it is important to use a single donor with highly responding basophils, to reduce interindividual variability due to the basophil releasability.

Using this method we have obtained a sensitivity of 63% with AX and higher with APL (79%), with all patients positive to AX being also positive to APL. Also specificity was higher with APL (100%) compared to AX (80%). We also found a good correlation between positive *in vitro* histamine release responses to AX and APL. This indicates that APL is a good reagent for in vitro diagnosis in patients with immediate hypersensitivity reactions to AX, especially if we take into account that we found positive results in two cases with ST negative (Patients 10 and 11) and in three cases where ST were not done (Patients 1, 5 and 19) due to severity of the reactions or high risk patients. In fact in the 9 patients with ST negative to AX 8 (88,9%) were pHRT positive to APL and 5 (55,5%) to AX confirming the utility of this test for diagnosis.

It is of note that all patients were selected based on the presence of a CAP positive and therefore pHRT had lower sensitivity for both AX and APL. However we do not know which is the sensitivity of both methods when compared in a general population with a history of immediate reaction to AX.

In conclusion, we have shown in this study that pHRT using APL is a promising in vitro method for the evaluation of patients with immediate allergic reactions to AX, including patients where ST are negative or cannot be performed. Moreover, it would be interesting to analyze the use of APL as a diagnostic reagent in both BAT and ST.

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CONFLICT OF INTEREST

Sidsel Falkencrone and Per Stahl Skov are researcher consultants for RefLab. Francisca Arribas David Rodríguez and Fernando Pineda, are employees of DIATER laboratories (Madrid, Spain).

All authors had full access to all the data (including statistical reports) and can take responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1. Clinical, demographic characteristics and results of skin tests and specific IgE to AX in the group of patients and controls included.

Pat	Age (years)	Gender	Reaction	IgE-AXO (kUA/L)	IntStu (days)	IntReac (min)	Skin test-AX (mm)		HRT (ng/mL)	
							Prick	ID	AX	APL
1	24	F	Anaphylaxis	1.14	90	5	ND	ND	**15**	**17,5**
2	31	M	Urticaria	3.37	450	5	Pos (6)	ND	**19,5**	**21,5**
3	57	M	Anaphylaxis	2.24	60	10	Pos (8)	ND	**18**	**22**
4	36	F	Anaphylaxis	0.95	90	15	ND	Pos (11)	**27**	**26**
5	33	F	Anaphylaxis	1.28	60	18	ND	ND	**21**	**18,5**
6	22	M	Urticaria	1.65	180	10	Pos (6)	ND	**30**	**23,5**
7	64	M	Erythema	3.57	90	30	Neg	Pos (10)	**27,5**	**25**
8	49	M	Urticaria	6.03	210	60	Neg	ND	**27,5**	**24,5**
9	53	M	Anaphylaxis	5.97	14	90	Pos (5)	ND	**22**	**17,5**
10	66	M	Urticaria	>100	10	55	Neg	Neg*	**21,5**	**19,5**
11	56	M	Urticaria	7.03	15	60	Neg	Neg**	12,5	**17,5**
12	35	M	Anaphylaxis	5.26	45	30	Neg	ND	**17,5**	**22,5**
13	69	M	NK	3.76	10680	NK	Neg	Pos (9)	**15**	**22**
14	47	M	Anaphylaxis	0.85	240	30	ND	ND	8	**13**
15	51	F	Erythema	5.89	90	60	Pos (8)	ND	7	4
16	28	F	Urticaria	0.84	240	60	Pos (6)	ND	4,5	11
17	57	M	Urticaria	0.81	180	10	ND	ND	6	5,5
18	62	M	Anaphylaxis	0.76	60	5	Pos (7)	ND	5,5	3
19	74	F	Anaphylaxis	3.86	240	10	ND	ND	3	**24,5**
1	45	M	-	0.03	-	-	Neg	Neg	5	3
2	48	F	-	0.07	-	-	Neg	Neg	10	9
3	37	F	-	0.01	-	-	ND	ND	10	6
4	32	M	-	0.01	-	-	Neg	ND	7	5
5	23	M	-	0.03	-	-	Neg	Neg	6	6
6	33	F	-	0.10	-	-	ND	ND	2	2
7	47	F	-	0.01	-	-	Neg	Neg	6	4
8	28	M	-	0.01	-	-	Neg	ND	7	7
9	44	F	-	0.03	-	-	Neg	ND	5	1
10	47	F	-	0.04	-	-	Neg	Neg	4	3

F: Female; M: Male; NK: Not known; IntStu: Time interval between reaction occurrence and the study; IntReac: time between drug administration and appearance of the symptoms; ID: Intradermal test; Pos: Positive; Neg: Negative; ND: Not Done; *: ID positive to BPOL; **: ID Test positive to BPOL and DM. Positive results are indicated in bold

FIGURE LEGENDS

Figure1: Comparison of results of the basophil passive histamine release (pHRT) test for AX and APL between patients and controls.

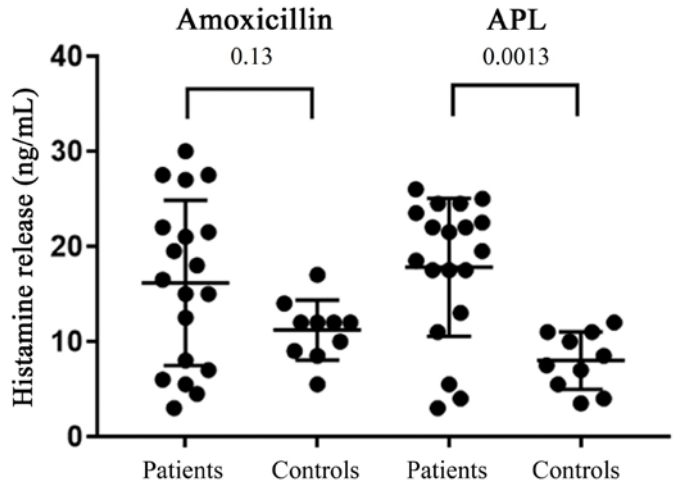
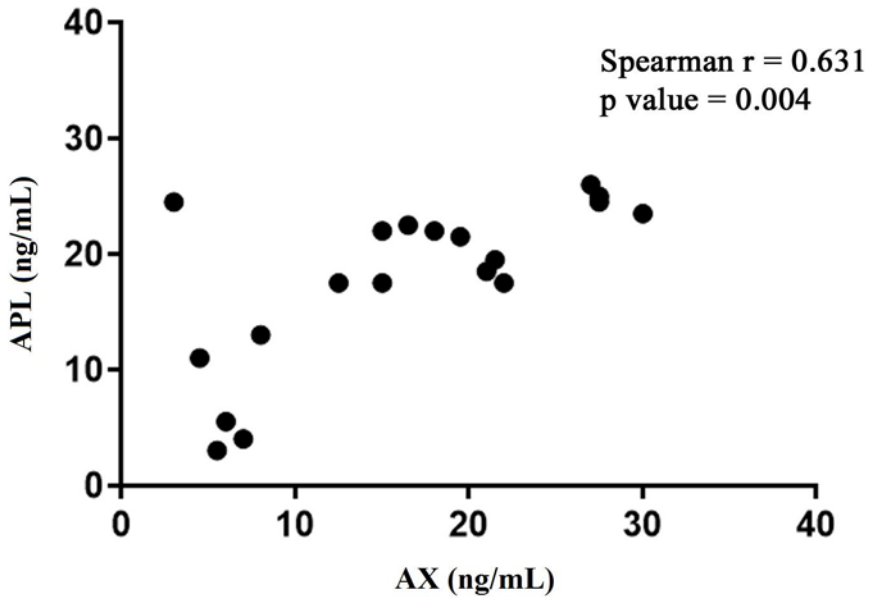


Figure 2. Correlation analysis between amoxicillin and APL in the basophil histamine release technique of allergic patients.



Supplementary Figure 1. A) Chemical synthesis of amoxicilloyl-Octa-L-lysine (APL); B) Chromatogram of amoxicilloyl-octa-L-lysine.

Supplementary Figure 2. Dose-response study for determining the highest concentration of AX and APL that did not induce unspecific histamine release (>10 ng/ml of histamine).

Supplementary Figure 3. ROC curves generated after the comparison of amoxicillin and APL in the basophil histamine release technique of allergic patients vs non-allergic patients