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Note

Thin-layer chromatographic method for screening the hydrolysis of β-lactam esters

In vitro hydrolysis of benzylpenicillin-benzamidomethyl ester by non-specific serum esterases

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Although β-lactam esters are biologically inactive, they are hydrolyzed to active compounds during absorption through the intestinal wall or in the blood and other tissues following absorption and distribution[1]. This pro-drug principle has been successfully applied to avoid gastric cleavage and to improve the oral absorption of penicillins[2–4] and cephalosporin derivatives[5–7].

In mass screening of the multitude of samples during physical purity control of substances, pH stability investigations, *in vitro* enzymatic and *in vivo* hydrolysis studies, simple, rapid and reliable qualitative and quantitative methods of analysis are required. Many combined chromatographic–bioautographic methods have been used[1–10]. In this article we suggest a simplification of the original Thomas[11] method by replacing NaOH alkalization spraying and heat treatment of the hydrolysis of esters on developed chromatograms by their incubation in a concentrated ammonia atmosphere at room temperature. In this way, sensitive detection of the esters and their parent compounds is achieved on the deep blue iodine–starch background. When paper or thin-layer chromatograms are obtained by any of the above methods in duplicate one of them is stained for documentation and for automated scanning reflection photometry in the visible region and the other is used for quantitative bioautography.

We now describe thin-layer chromatographic (TLC) studies on the *in vitro* hydrolysis of benzylpenicillin-benzamidomethyl ester, FI-7303 (CAS-51164-29-5)[12] by non-specific serum esterases of different species.

EXPERIMENTAL

Materials

Samples of benzylpenicillin-benzamidomethyl ester (BP-BAM) and benzylpenicillin (BP) were generously provided by Biogal Pharmaceutical Works (Debrecen, Hungary). All chemicals were of analytical grade and imported by Reanal (Darmstadt, G.F.R.). For TLC, Merck Alurolle-DC Kieselgel 60F254, 0.20 mm was used.

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TLC

Chromatograms were developed in benzene–ethyl acetate–acetone–acetic acid (70:30:30:1) solvent. This system separated the ester BP-BAM from free BP with R_F = 0.6 ± 0.05 and 0.1 ± 0.02, respectively. Stripes 1 cm wide and 17 cm long were scratched on the plates. At about 12 cm long runs were the solvent fronts developed.

The plates were dried for 20 min at room temperature then sprayed with iodine–starch solution (1% soluble starch–0.1 *N* iodine in 4% KI, 50:1). The wet dark blue plates were put immediately in a concentrated ammonia atmosphere in chromatography tanks at room temperature. Development of the white spots of BP was complete within 5 min, depending on the amount of substance. Hydrolysis of the ester in the respective spots and subsequent complete development of the white areas required 20 min. Finally the plates were photographed for documentation.

For bioautography, acetic was omitted from the solvent system because its complete elimination by evaporation required 24 h at room temperature (remaining acetic acid killed bacteria on the plates). The corresponding R_F values were 0.0 for BP and 0.78 ± 0.05 for BP-BAM.

In vitro hydrolysis of BP-BAM

Samples of the ester were hydrolyzed with serum specimens from CFLP mice, cats, white rabbits, Wistar rats and humans known to contain non-specific esterases.

The composition of the enzymatic hydrolysis mixture was as follows: 250 μg of BP-BAM suspended in the appropriate amount of 0.1 *M* sodium phosphate buffer, pH = 6.0 to give a final volume of 300 μl. The serum samples were thermostatted prior to use at 37°C. The following volumes were used for the hydrolysis studies: 200 μl of mouse serum; 150 μl of rabbit, cat or human serum; 100 μl of rat serum. The pH of the serum samples was preadjusted to 6.0 with the minimum amount of 0.1 *M* NaH_2PO_4.

Aliquots (10–20 μl) were applied to the TLC stripes in duplicate. One of the

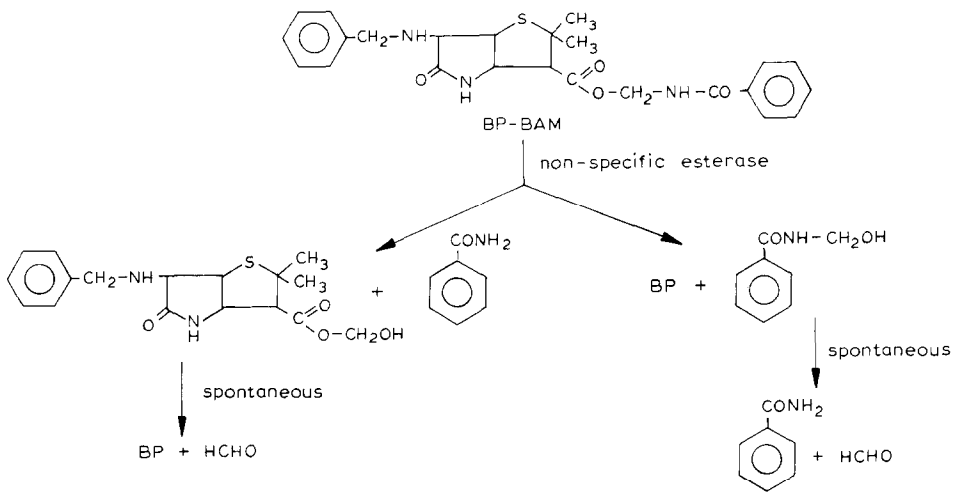

Fig. 1. Alternative hydrolysis pathways of BP-BAM.

plates was developed for qualitative detection and the other for quantitative bioautography.

Standard BP and BP-BAM containing inactivated blanks were run with every serum specimen. In order to activate the non-specific monoesterases, native serum samples were kept at 80°C in a thermostat for 10 min, then adjusted to pH 6.0 with 0.1 *M* NaH_2PO_4. BP (1 mg ml^{-1}) plus BP-BAM (1.26 mg ml^{-1}) were dissolved in the inactivated serum species and 15-μl samples were applied on the plates.

Quantitative bioautography

Standard solutions of BP-BAM and the parent compound of various concentrations were applied to the TLC plates together with samples of mixtures incubated for various times, chromatographed in duplicate, dried and cut into strips 1 cm wide and 12 cm long and finally plated on *Bacillus subtilis* ATCC 6633. The diameters of the zones were measured and the amounts (μg) in BP equivalents were assessed with a simple regression computer program. It was shown separately that equivalent amounts of BP and BP-BAM caused identical exctinction zones either upon direct application of the substances in wells or by plating each substance separately on 1-cm wide chromatograms.

RESULTS AND DISCUSSION

Some alkyl and benzyl esters of BP were prepared by Mayer *et al.*[13] in 1943. However, there was controversy regarding the antibacterial activities of these acid-stable compounds *in vivo*[14,15]. The second generation of acid-stable β-lactam esters

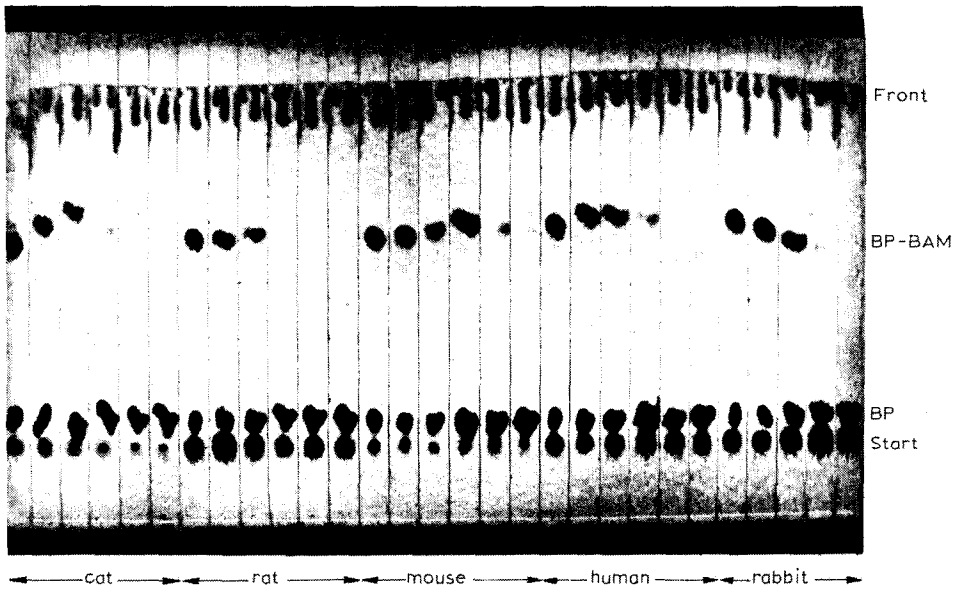

Fig. 2. TLC negative picture of the time course of the hydrolysis of BP-BAM by serum specimens from different species.

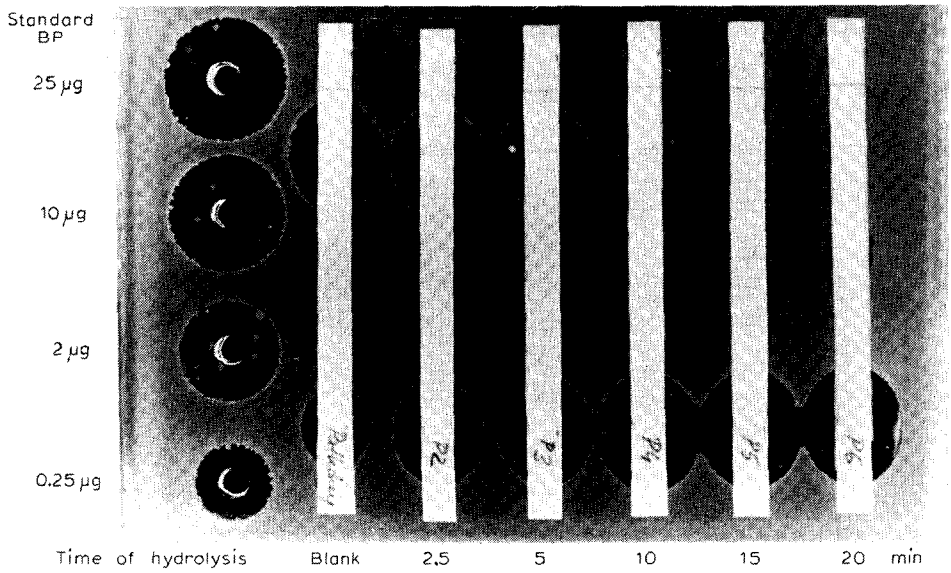

Fig. 3. Time course of hydrolysis of BP-BAM by rat serum: quantitative bioautography on *B. subtilis* ATCC 6633 plate.

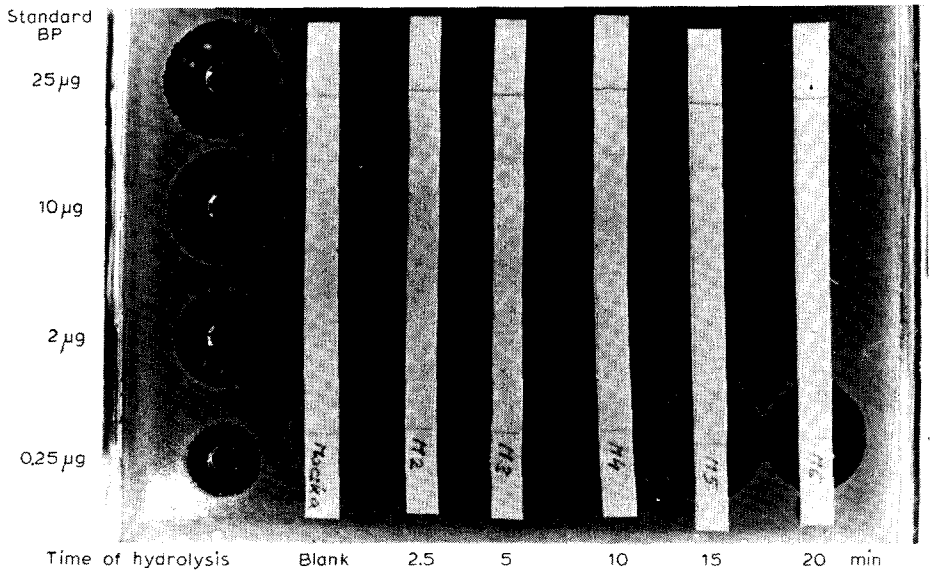

Fig. 4. Time course of hydrolysis of BP-BAM by cat serum: quantitative bioautography on *B. subtilis* ATCC 6633 plate.

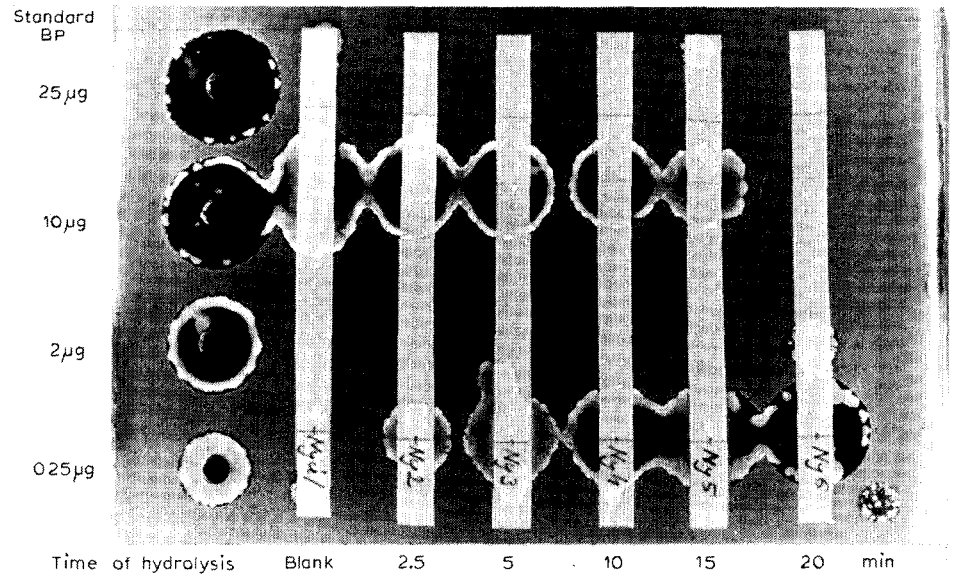

Fig. 5. Time course of hydrolysis of BP-BAM by rabbit serum: quantitative bioautography on *B. subtilis* ATCC 6633 plate.

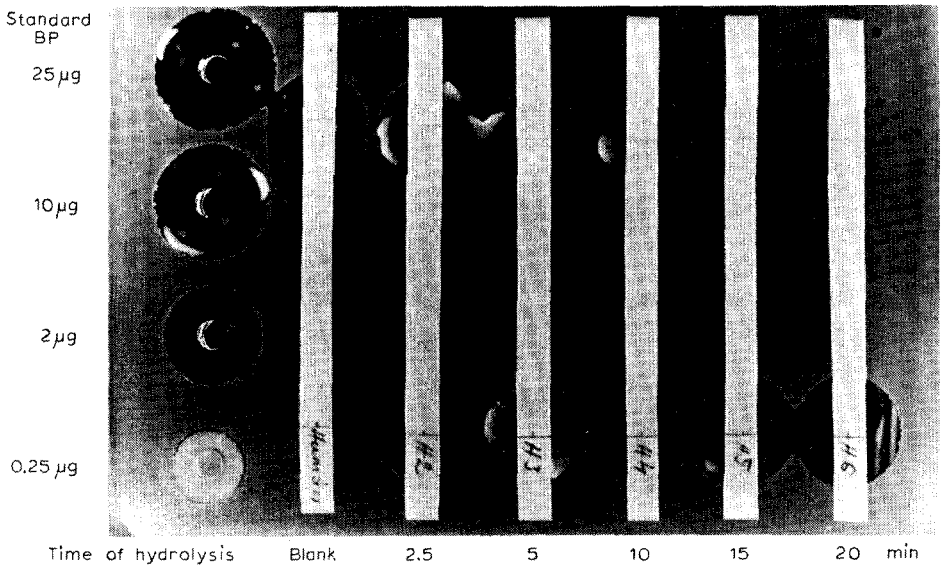

Fig. 6. Time course of hydrolysis of BP-BAM by human serum: quantitative bioautography on *B. subtilis* ATCC 6633 plate.

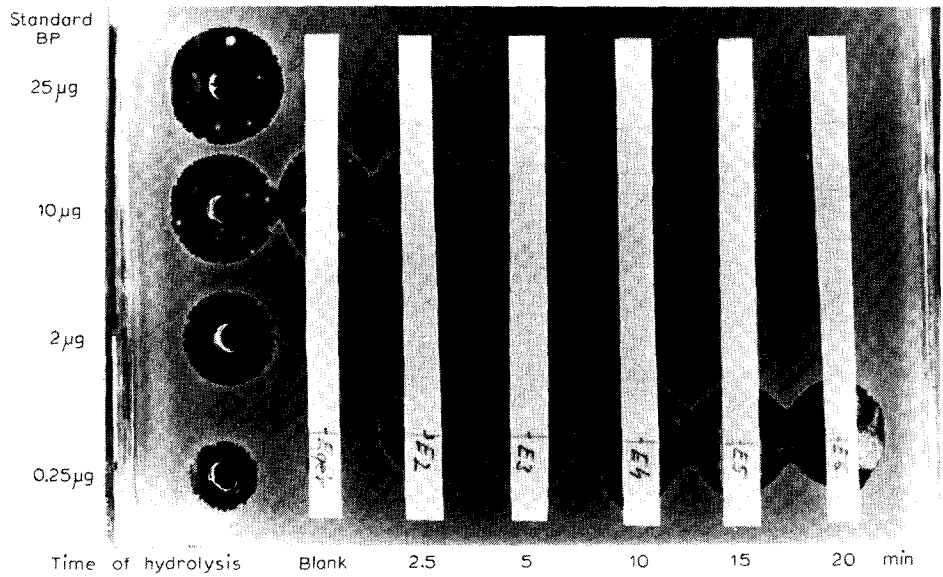

Fig. 7. Time course of hydrolysis of BP-BAM by mouse serum: quantitative bioautography on *B. subtilis* ATCC 6633 plate.

began with acetoxymethyl-BP[16], which showed identical *in vivo* antibacterial activity to BP, but its pharmacokinetics were different[17–19]. This compound and some other oxymethyl esters[20,21] are hydrolyzed by non-specific esterases common in mammalian tissues. According to Jansen and Russel[16], BP-acetoxymethyl ester first yields a labile monoester and acetic acid. The monoester is then spontaneously transformed to BP and formaldehyde. The same mechanism was also adopted by other authors[7,17,20,21].

For BP-BAM we suggest two alternative hydrolysis pathways (Fig. 1). One is similar to that of Jansen and Russel[16], leading to the labile monoester and benzamide, but the other pathway furnishes BP and benzamidomethanol which is regarded as a labile compound and splits to yield formaldehyde and benzamide. As Fig. 2 shows, besides the ester only BP was detected within 2.5 min; BP-CH_2OH should have spontaneously and quantitatively been hydrolyzed at almost the same rate, if not faster, than BP-BAM itself.

Fig. 2 illustrates the qualitative time course of the hydrolysis of BP-BAM by non-specific serum esterases of different species. Spots which remained at the start were unidentified reducing substances of the serum samples, probably reducing sugars, haem, etc. Only BP and BP-PAM could be detected at the corresponding R_F values. Gradual hydrolysis of the ester was observed by its decreasing amounts in the $R_F = 0.6 \pm 0.05$ spots with increasing time of incubation. Complete disappearance of the ester required about 10–15 min in case of cat, rabbit and human sera as sources of non-specific esterases; however, rat serum was able to hydrolyze BP-BAM within 10 min under the experimental conditions. Standard BP- and BP-BAM-containing inactivated blanks were run with every serum species.

The results of quantitative bioautography are shown in Figs. 3–7 and Table I.

TABLE I

QUANTITATIVE BIOAUTOGRAPHY OF *IN VITRO* HYDROLYSIS OF BP-BAM BY NON-SPECIFIC SERUM ESTERASES FROM DIFFERENT SPECIES

Species	*Aliquot of rection mixture* (μl)	*Incubation time* (*min*)	*BP-BAM** *concn.* ($\mu g\ ml^{-1}$)	*BP*** *concn.* ($\mu g\ ml^{-1}$)	*Total**** *concn.* ($\mu g\ ml^{-1}$)	*Hydrolysis* (%)	*Standard BP*§	
							Regression eqn.	*r*
Rat	10	2.5	8.86	0.14	9.00	1.6		
		5.0	5.63	2.5	8.13	30.75		
		10	0.79	8.1	8.89	91.0	$y = 3.30 \ln x + 28.8$	0.9965
		15	0.025	8.35	8.375	99.7		
		20	0.0	9.13	9.13	100.0		
Cat	12	2.5	11.2	0.0	11.2	0.0		
		5.0	8.5	2.0	10.5	19.0		
		10	1.36	9.1	10.46	87.0	$y = 2.90 \ln x + 27.6$	0.9941
		15	0.25	12.0	12.25	98.0		
		20	0.11	11.6	11.76	98.6		
Rabbit	12	2.5	11.42	0.0	11.42	0.0		
		5.0	10.45	0.042	10.492	4.0		
		10	7.65	3.92	11.57	33.9	$y = 2.24 \ln x + 28.93$	0.8026
		15	0.53	8.74	9.27	94.3		
		20	0.28	10.0	10.28	97.3		
Human	15	2.5	12.12	0.11	12.23	0.9		
		5.0	9.99	2.94	12.93	22.7		
		10	1.8	10.32	12.12	85.1	$y = 3.10 \ln x + 26.75$	0.9974
		15	0.8	11.37	12.17	93.4		
		20	0.0	12.52	12.52	100.0		
Mouse	20	2.5	18.85	0.0	18.85	0.0		
		5.0	17.3	1.95	19.25	10.13		
		10	10.64	7.13	17.77	40.12	$y = 3.50 \ln x + 23.73$	0.8544
		15	1.61	16.8	18.41	91.25		
		20	0.0	18.6	18.6	100.0		

* BP equivalent of the unhydrolyzed BP-BAM concentration.

** BP concentration freed from BP-BAM during hydrolysis.

*** Sum of BP-BAM and BP.

§ The concentrations of unhydrolyzed BP-BAM and the liberated BP were computed from area diameter data of BP standards, simultaneously run on every

The data in Table I illustrate the time dependence of the hydrolysis of the ester, taking into consideration both the amounts of serum used and the aliquots applied to the chromatograms. The rate of ester hydrolysis was highest with rat serum, intermediate with cat, rabbit and human sera in decreasing order and lowest with mouse serum. The rate of hydrolysis of BP-BAM in the blood, therefore, is different in different species. This can be related to the philogenetic order, but mouse serum still had a lower activity than expected.

Similar trends were reported by Agersborg *et al.*[17] for the benzylpenicillin-acetoxymethyl ester, but the differences were not so pronounced. Our *in vitro* hydrolysis results with non-specific serum esterases suggest that the bioavailability of the sparingly soluble BP-BAM must be limited in the gastrointestinal tract prior to absorption.

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