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Visible spectrophotometric determination of cephalosporins and penicillins by indophenol derivatization with and without alkaline degradation to ammonia

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Abstract: Cephalosporins and penicillins give reproducible yields of ammonia on degradation in 0.5 M sodium hydroxide solution at 100°C: the ammonia formed was determined in the degraded solutions using the indophenol reaction. In another approach the ammonia driven off on refluxing alkaline solutions of the cephalosporin or penicillin was collected in dilute hydrochloric acid solution and determined using the indophenol reaction. For eight of the fourteen cephalosporins and penicillins studied identical yields were recorded using the two procedures: these varied from 29% for penicillin G to 137% for cephalonium based on the production of one ammonia molecule per beta-lactam molecule. For six other cephalosporins the distillation method gave substantially higher yields of ammonia than did the direct determination. Eight cephalosporins and penicillins were found to give substantial indophenol-type reactions without prior hydrolysis of the beta-lactam, but the sensitivities were usually lower than for the hydrolysis method. Manual spectrophotometric procedures for the determination of cephalosporins and penicillins based on these reactions have been developed.

Keywords: *Cephalosporin determination; penicillin determination; alkaline degradation of β-lactams; ammonia formation; indophenol reactions.*

Introduction

Polarographic studies have shown that sulphide is formed during the degradation of cephalosporins in neutral solution [1]. Manual and automated visible spectrophotometric methods of determining cephalosporins were developed based on hydrolysis in sodium hydroxide solution and conversion of the sulphide formed to methylene blue [2–4]. The methods are selective for cephalosporins, as penicillins do not form sulphide under these conditions. Indeed cephalosporins can be determined in penicillin samples, the detection limit being 1–2 μg of cephalosporin per g of penicillin [4].

Ammonia was considered to be another probable degradation product of penicillins and cephalosporins, although previous workers do not appear to have attempted to

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detect or determine it. For example, 3-aminomethylene-6-phenyl-piperazine-2,5-dione, a known degradation product of cephalexin [5], must produce ammonia on degrading further to the known 3-hydroxy derivative. In the present work all fourteen cephalosporins and penicillins studied were shown to give appreciable yields of ammonia during alkaline degradation. The yields of ammonia were determined by visible spectrophotometry based on the formation of indophenol, and manual visible spectrophotometric methods of determining cephalosporins and penicillins have been developed based on an extension of this method. Visible spectrophotometric methods based on the formation of indophenol derivatives have previously been developed in this laboratory for the determination of paracetamol [6, 7], sulphanilamide [8] and sulphaguanidine [9]: polarographic methods involving indophenol derivatization have also been developed for the determination of ammonia, *p*-aminophenol and sulphanilamide [10], and of sulphaguanidine [9].

Experimental

A Pye Unicam SP8-100 UV and visible spectrophotometer was used in the present work. The recommended procedures for the determination of cephalosporins and penicillins are as follows:

Reagents

Sodium hydroxide solution, 0.5 M. Dissolve 10 g of sodium hydroxide in water and dilute to 500 ml.

Phenol solution, 3% m/v. Dissolve 3 g of phenol in water and dilute to 100 ml. (Care must be taken in handling phenol and phenol solutions.)

Alkaline phenol solution, 3% m/v. Dissolve 3 g of phenol in 100 ml of 0.5 M sodium hydroxide solution. (Care must be taken in handling phenol and phenol solutions.)

Sodium nitroprusside solution, 0.1 mg ml^{-1}. Dissolve 10 mg of sodium nitroprusside dihydrate in 100 ml of distilled water.

Sodium hypochlorite solution. Dilute 20 ml of commercial sodium hypochlorite solution (10–14% available chlorine) to 1 l with water. This solution should be prepared freshly and should give an apparent molar absorptivity of 2.35×10^4 $lmol^{-1}$ cm^{-1} for the determination of ammonium ion.

Standard penicillin and cephalosporin solutions, 0.1% m/V. Dissolve 0.1 g (accurately weighed) of penicillin or cephalosporin (sodium or potassium salts) in water and dilute to 100 ml in a calibrated flask. In the case of 7-aminocephalosporanic acid (7-ACA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA) dissolve the solid in a small amount of dilute hydrochloric acid before diluting to 100 ml.

Prepare more dilute standard solutions by dilution of these solutions.

Procedure 1. *Determination of penicillins and cephalosporins by hydrolysis in* 0.5 M *sodium hydroxide solution and formation of indophenol without distillation*

Transfer by means of a pipette aliquots (< 10 ml) of a standard or sample solution

containing a suitable amount (see Table 1) of penicillin or cephalosporin into screw-capped, autoclavable bottles and add 0.5 M sodium hydroxide solution from a burette to make the total volume 100 ml. Screw on the tops firmly and heat the bottles in a boiling water bath for a length of time sufficient to give a full yield of ammonia (see Table 1).

Table 1
Details of recommended procedures

	Recommended minimum hydrolysis time (Procedure 1) (min)	Recommended amount of determinand* (mg)		
		Proc. 1	Proc. 2	Proc. 3
Cephalexin	60	<15	<35	<15
Cephradine	50	<15	<35	<15
Cephaloglycin	60	<15	<35	<3
Cefaclor	50	<25	<60	<25
Cephalothin	65	<60	<70	<5
Cephaloridine	90	<90	<75	<3
Cephalonium	55	<10	<25	<3
Cephoxazole	50	<100	<200	<15
Cefazolin	70	<25	<35	<4
7-ACA	70	<20	<35	<10
7-ADCA	50	<10	<15	<10
Penicillin V	50	<25	<60	<5
Penicillin G	50	<40	<100	<6
Ampicillin	40	<30	<70	<4

*Maximum amount gives an absorbance of *ca* 0.7.

Cool the solution to room temperature and after mixing carefully transfer by pipette 5 ml of each solution to 50 ml calibrated flasks and add in turn with mixing, 3 ml of phenol solution, 5 ml of sodium nitroprusside solution and 2 ml of sodium hypochlorite solution. Heat the flasks in a boiling water bath for 3–4 min. Cool the flasks to room temperature, dilute to volume with water, mix and read the absorbance of the solution at 625 nm in a 10 mm cell against water.

Procedure 2. Determination of penicillins and cephalosporins by degradative distillation from alkali and formation of indophenol from the ammonia produced

Transfer by means of a pipette aliquots of a standard or sample solution containing a suitable amount (see Table 1) of penicillin or cephalosporin to a 250 ml ammonia distillation flask. Add some porous porcelain. Place 100 ml of 0.1 M hydrochloric acid solution in the receiver and adjust its position so that the end of the condenser just dips into the acid. Run 100 ml of 20% m/V sodium hydroxide solution into the flask and boil the contents of the flask gently for 40 min, collecting the ammonia that distils over. Transfer the contents of the receiver to a 250 ml calibrated flask and dilute to volume.

Transfer by pipette 5 ml of this solution to a 50 ml calibrated flask and add in turn with mixing, 3 ml of alkaline phenol solution, 5 ml of sodium nitroprusside solution, and 2 ml of sodium hypochlorite solution. Cool the flask to room temperature, dilute to volume with water, mix and read the absorbance of the solution at 625 nm in a 10 mm cell against water.

Procedure 3. *Determination of penicillins and cephalosporins by means of an indophenol reaction without pre-hydrolysis*

Transfer by means of a pipette aliquots (< 10 ml) of a standard or sample solution containing a suitable amount (see Table 1) of penicillin or cephalosporin into 50 ml calibrated flasks. Add in turn, with mixing, 3 ml of alkaline phenol solution, 5 ml of sodium nitroprusside solution and 2 ml of sodium hypochlorite solution. Heat the flasks in a boiling water bath for 3–4 min, cool to room temperature, dilute to volume with water, mix and read the absorbance of the solution at 625 nm in a 10 mm cell against water.

Results

All the penicillins and cephalosporins studied gave ammonia on hydrolysis in 0.5 M sodium hydroxide solution. Many compounds other than ammonia give indophenol-type reactions [6–10] and the presence of ammonia was confirmed here by means of the distillation experiments. For indophenol derivatizations carried out on the hydrolysed solutions without distillation, the change in absorbance with length of time of hydrolysis before the indophenol derivatization was carried out for all the β-lactam antibiotics studied is shown in Table 2. Three types of behaviour can be identified: (a) appreciable indophenol formation occurs without hydrolysis, but with increasing hydrolysis time the absorbance first falls markedly before increasing again (cephaloridine and cephalothin), (b) very little indophenol formation occurs before hydrolysis, i.e. absorbance before hydrolysis is < 10% that after hydrolysis (cephalexin, cephradine, cefaclor, 7-ACA and 7-ADCA) and (c) appreciable indophenol formation occurs before hydrolysis and there is a steady increase in sensitivity with hydrolysis time. In all cases a constant yield of ammonia (and hence indophenol) is obtained after a suitable hydrolysis time which depends on the particular β-lactam. Most probably cephaloridine and cephalothin themselves give strong indophenol-type reactions and the initial decrease in absorbance occurs because of the more rapid loss of the cephalosporin compared with the rate of formation of ammonia.

In Table 3 the yields of ammonia after hydrolysis with and without distillation are compared. For cephalexin, cephradine, cephaloglycin, cefaclor, cephalonium, penicillin V, penicillin G and ampicillin the yield of ammonia was the same in both cases. For cephalothin, cephaloridine, cephoxazole, cefazolin, 7-ACA and 7-ADCA, there was a significant increase in yield when the ammonia was distilled. The apparent molar absorptivities for the formation of the indophenol-type compounds (Procedure 3) are also given in Table 3. For ease of comparison of the sensitivities of the three methods, the apparent molar absorptivities are also given for Procedures 1 and 2 in which ammonia is measured.

In obtaining the results shown in Table 3 six completely independent determinations were made for each β-lactam using Procedures 1 and 3, and three independent distillations were carried out for Procedure 2. In all cases the coefficient of variation was less than 2%: calibration graphs were rectilinear in the ranges indicated in Table 1.

Discussion

Ammonia has been shown to be a major degradation product of the alkaline degradation of penicillins and cephalosporins. The yield of ammonia from a range of

Table 2
Effect of hydrolysis time at 100°C in 0.5 M sodium hydroxide solution on the absorbance obtained subsequently in the indophenol method using Procedure 1. Values given are absorbance at 625 nm*

Hydrolysis time (min)	Compound													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	0.210	0.028	0.021	0.092	0.022	0.526	0.240	0.124	0.203	0.523	0.412	0.024	0.031	0.145
5	—	—	—	—	—	0.742	0.079	—	—	0.184	—	—	—	—
10	0.301	0.124	0.101	0.104	0.095	—	0.061	0.201	0.297	0.053	0.490	0.123	0.204	0.187
15	—	—	—	—	—	0.943	0.120	—	—	0.047	—	—	—	—
20	0.422	0.185	0.202	0.255	0.142	—	—	0.254	0.317	—	0.519	0.292	0.397	0.201
25	—	—	—	—	—	0.997	0.201	—	—	0.062	—	—	—	—
30	0.539	0.307	0.315	0.300	0.195	—	—	0.299	0.359	—	0.602	0.450	0.502	0.292
35	—	—	—	—	—	1.099	0.264	—	—	0.084	—	—	—	—
40	0.547	0.413	0.421	0.322	0.252	—	—	0.315	0.399	—	0.720	0.603	0.699	0.373
45	—	—	—	—	—	1.099	0.355	—	—	0.097	—	—	—	—
50	0.548	0.530	0.430	0.350	0.320	—	—	0.331	0.404	—	0.797	0.779	0.841	0.401
55	—	—	—	—	—	1.112	0.428	—	—	0.174	—	—	—	—
60	0.549	0.540	0.429	0.389	0.319	—	—	0.330	0.405	0.250	0.892	0.899	0.842	0.409
65	—	—	—	—	—	1.116	0.473	—	—	—	—	—	—	—
70	—	0.545	—	0.397	0.321	—	—	—	—	0.295	0.942	0.901	0.843	0.410
75	—	—	—	—	—	1.115	0.474	—	—	—	—	—	—	—
80	—	0.540	—	0.399	—	—	—	—	—	0.345	0.941	—	—	—
85	—	—	—	—	—	—	0.473	—	—	—	—	—	—	—
90	—	—	—	—	—	—	—	—	—	0.483	—	—	—	—
110	—	—	—	—	—	—	—	—	—	0.485	—	—	—	—

*Compounds and amounts taken (mg): 1, ampicillin (21); 2, cephalexin (9.8); 3, cephradine (8.2); 4, cephaloglycin (7.4); 5, cefaclor (11); 6, cephalonium (43); 7, cephalothin (30); 8, penicillin G (18.8); 9, penicillin V (28); 10, cephaloridine (60); 11, cefazolin (46); 12, 7-ACA 13; 7-ADCA (8.2); 14, cephoxazole (14).

Table 3
Apparent molar absorptivities of cephalosporins and penicillins based on the indophenol compounds formed and yields of ammonia in hydrolysis procedures

	Apparent molar absorptivity, 10^4 l mol^{-1} cm^{-1}			Molar yield of ammonia, %	
	Proc. 1	Proc. 2	Proc. 3	Proc. 1	Proc. 2
Ammonium chloride	2.35	2.35	2.35	100	100
Cephalexin	1.95	1.95	0.09	83	83
Cephradine	1.76	1.76	0.08	75	75
Cephaloglycin	2.19	2.21	0.50	93	94
Cefaclor	1.08	1.08	0.06	46	46
Cephalothin	0.56	1.06	0.35	24	45
Cephaloridine	0.49	1.01	0.56	21	43
Cephalonium	3.22	3.22	0.56	137	137
Cephoxazole	0.28	0.42	0.13	12	18
Cefazolin	1.32	2.37	0.41	56	101
7-ACA	0.96	1.50	0.05	41	64
7-ADCA	1.69	2.47	0.08	72	105
Penicillin V	1.06	1.06	0.24	45	45
Penicillin G	0.68	0.66	0.24	29	28
Ampicillin	1.06	1.06	0.40	45	45

penicillins and cephalosporins has been determined under conditions in which (i) the hydrolysis is effected at 100°C in a closed vessel and (ii) hydrolysis occurs during refluxing and distillation of the ammonia. In some cases the yield is significantly greater when the solution is refluxed. Some of the β-lactams also give significant indophenol reactions without additional hydrolysis, and the possibility of using this method to determine these β-lactams has been shown to be feasible also.

Procedure 3, involving direct indophenol reaction of the undegraded cephalosporin or penicillin, is the simplest, and might be used with advantage, particularly for determining cephaloridine, but also for determining cephaloglycin, cephalothin, cephalonium, cefazolin, penicillin V, penicillin G and ampicillin. Procedure 1, involving prior hydrolysis in a sealed vessel, is more generally applicable, and the use of this hydrolysis procedure would appear to be essential for the determination of cephalexin, cephradine, cefaclor, cephoxazole, 7-ACA and 7-ADCA. Procedure 2, involving distillation of the ammonia, is more involved and for eight of the compounds studied gives no increase in sensitivity over Procedure 1.

Cephalosporins can be determined by an Autoanalyzer method involving alkaline hydrolysis to sulphide followed by conversion of this to methylene blue which is determined using a visible spectrophotometric detector [4]. Preliminary studies have shown that the construction of a similar manifold to determine cephalosporins and penicillins by alkaline hydrolysis to ammonia and determination of the ammonia formed is feasible.

These indophenol methods are proposed as additional methods of determining penicillins and cephalosporins which may have useful applications in routine or research laboratories. Combinations of the indophenol procedures and the indophenol procedures and the sulphide procedure having different sensitivity ratios could be used to determine mixtures of cephalosporins and/or penicillins, or for use as a check on identity during determination. In the automated sulphide system [4] re-sampling is carried out

after the hydrolysis step. The hydrolysate could be re-sampled twice to enable both sulphide and ammonia to be determined.

In addition to utilizing the alkaline degradation to ammonia for the determination of cephalosporins and penicillins, the procedures developed should prove useful in elucidating their degradation mechanisms. Information on the final yield of ammonia during alkaline degradation has been given in Table 3.

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