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New stability-indicating high performance liquid chromatography assay and proposed hydrolytic pathways of chlorhexidine[1]

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Abstract

Chlorhexidine (CHD) is an antiseptic agent widely used in mouth rinses and ophthalmic solutions. Its assay in these solutions has been commonly performed with high performance liquid chromatography (HPLC). Published HPLC assays were unable to detect the many possible hydrolysis products of CHD, with the exception of *p*-chloroaniline. This raises concerns regarding the stability-indicating capabilities of the HPLC assays. In addition, the reliability of these assays was affected by the irreversible absorption of CHD on silica-based reversed phase (RP) columns. Special attention must be paid to the mobile phase or sample matrices in order to obtain reliable assay results. This paper presents a polymer-based RP-HPLC method that is stability-indicating. The method detected six UV-absorbing hydrolysis products of CHD. The identities of the products, which were elucidated by UV and mass spectral analyses, are consistent with the proposed hydrolytic pathways of CHD. Quantitation problems observed with silica-based RP-HPLC assays do not arise with the new method. Preliminary data indicate that the new HPLC method can be developed into a reliable, stability-indicating assay for CHD in ophthalmic solutions.

Keywords: Chlorhexidine; HPLC; Hydrolysis products and pathways; Stability indication

1. Introduction

Chlorhexidine (CHD), 1,6-bis[N^5-(*p*-chlorophenyl)-N^1-biguanido]hexane, is an effective antibacterial agent. Due to its low toxicity and broad antimicrobial activity, it is widely used as an antiseptic agent in mouth rinses [1,2] and as a disinfectant in ophthalmic solutions [3,4]. The CHD concentrations in these solutions are usually below 100 ppm [4–6]. Many high performance liquid chromatography (HPLC) methods have been reported for CHD analysis [7–15]. In these reports, *p*-chloroaniline, *p*-chlorophenylbiguani-dine and phenylbiguanidine are the only degradation products or impurities detected. The latter is a photolysis product of CHD.

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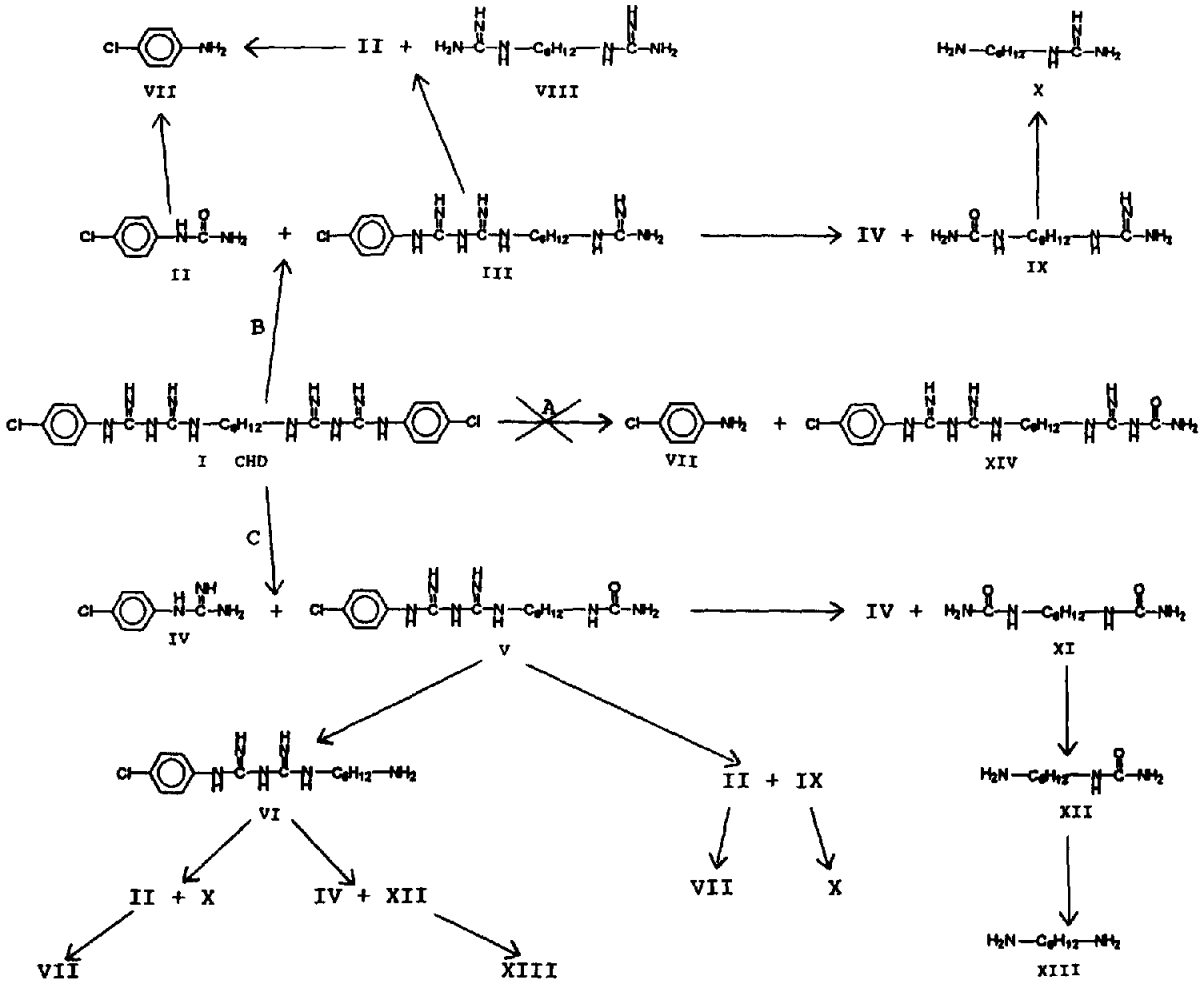

Fig. 1. Proposed hydrolysis pathways of CHD in aqueous solutions.

The biguanido moiety is the hydrolytically sensitive group in CHD. Elpern [16] proposed that biguanide, in acidic solution, was hydrolyzed to guanylurea and an amine (Path A, Fig. 1). In a solution containing ammonia, two guanidine units can be formed from a biguanide [17]. The structure of biguanide is traditionally represented by A in Fig. 2, which is in tautomeric equilibrium with structure B. Indeed, in dilute solutions, Nandi [18] suggested that structure B was more consistent with the UV data. Thus, hydrolysis of biguanide probably involves the hydration and subsequent cleavage of the C(2)=N(3) double bond in structure B to form a guanidine and a urea. The urea is further hydrolyzed to its amine whereas the guanidine is presumed to be relatively stable [17]. Therefore, the hydrolysis of CHD is likely to proceed via Paths B and C, Fig. 1, generating a total of 12 hydrolysis products (**II–XIII**). Six of these, **II–VII**, possess the chlorophenylbiguanido or chlorophenyl chromophore and should be detected by UV during HPLC. The inability to detect all but **VII** (*p*-chloroaniline, a secondary product) by existing HPLC assays casts doubt on their stability-indicating capability.

Published HPLC assays also suffer from quantitation problems caused by irreversible adsorption of CHD onto the silica-based reversed phase (RP)-HPLC columns [7,8]. Although the problems can be overcome by adding NaCl to the

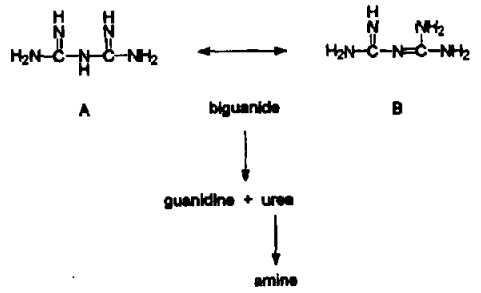

Fig. 2. Tautomeric structures of biguanide and its proposed hydrolysis scheme.

mobile phase [8] or by matching the solution matrices of the reference and the sample [7], the need for a new stability-indicating assay for dilute solutions of CHD is evident.

This paper presents a new stability-indicating HPLC method that separates CHD from its UV-absorbing hydrolysis products. The identities of these products have been confirmed by the presented UV and mass spectral data. The hydrolysis products are consistent with the proposed hydrolytic pathways of CHD (Paths B and C). The suitability of this new method as a reliable, stability-indicating HPLC assay for CHD in an ophthalmic solution has been demonstrated.

2. Experimental

2.1. Reagents and materials

Ammonium dihydrogen phosphate, ammonium hydroxide, and glacial acetic acid were purchased

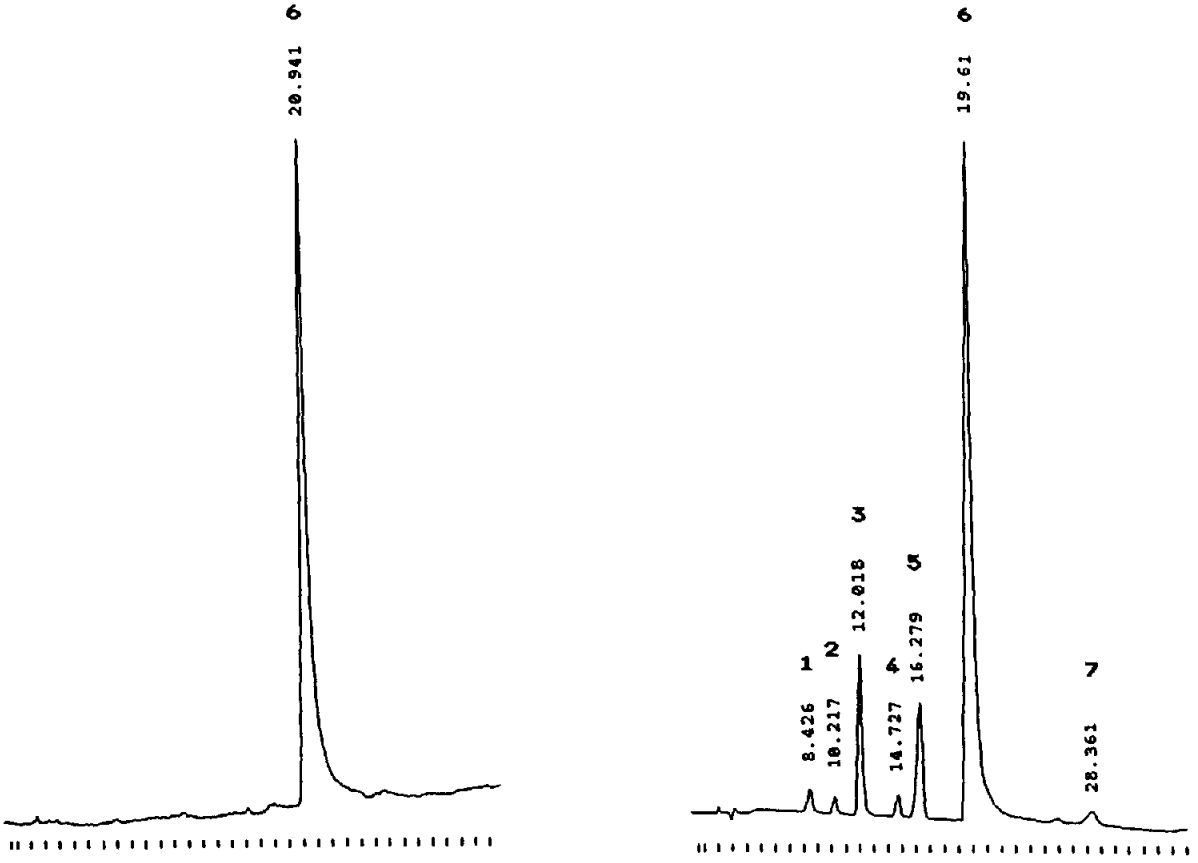

Fig. 3. HPL chromatograms of a 800 ppm CHD solution in H_2O: freshly prepared (left) and heated at 80°C for 5 days (right). See Section 2 for HPLC conditions.

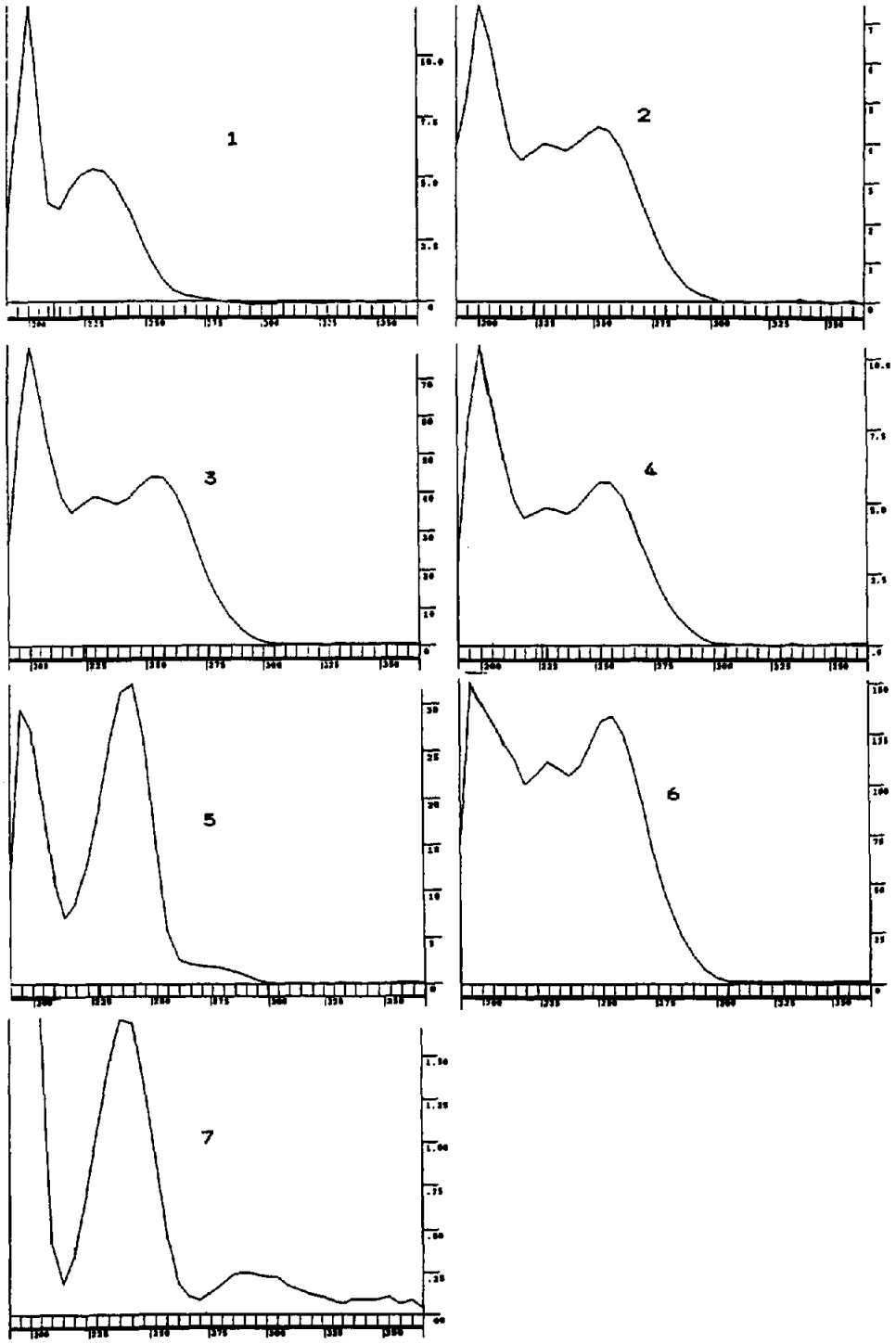

Fig. 4. UV profiles of CHD and hydrolysis products, obtained by HPLC–PAD. Spectral identification corresponds to peak assignments in Fig. 3. Wavelength (nm) on horizontal scale and absorption units (mAu) on vertical axis.

from Mallinckrodt (Paris, KY). Ammonium acetate and chlorhexidine digluconate (20%) were obtained from Sigma (St. Louis, MO). The chemicals were reagent grade. HPLC-grade acetonitrile (ACN) was from Burdick & Jackson Division, Baxter Healthcare Corp. (Muskegon, MI). Buffer solutions were prepared with water purified through a Millipore Super-Q Pure Water System (Waltham, MA).

2.2. HPLC

The HPLC system consisted of a Varian (Walnut Creek, CA) 9010 pump, a Varian 9050 UV–Vis detector, a Varian 9065 photodiode array detector (PAD), and a Rheodyne (Cotati, CA) 7125 manual injector. Data were collected and analyzed with a Varian LC STAR workstation. LC–MS was performed with a Vestec 201 XL mass spectrometer equipped with an LC thermospray interface (Houston, TX).

Sample solutions (100 μl) were manually injected onto a Hamilton PRP-1, 10 μm, 4.1mm × 250 mm stainless-steel column (Alltech Assoc., Inc., Deerfield, IL). Chromatographic analyses were carried out by linear gradient elution from 10% ACN in pH 5.0, 0.02 M ammonium acetate or phosphate buffer to 65% ACN in buffer over 40 min at 1.0 ml min^{-1}. Detection was by UV at 235 nm, PAD or LC–MS.

Table 1
LC–MS data for CHD and hydrolysis products shown in Fig. 3

LC peaks	Major mass fragments	Identity[a]
1	211 (M+H+ACN), 170 (M+H)	IV
2	311 (M+H), 184[b], 159[c]	VI
3	353 (M+H), 201[d], 184, 159	III
4	354 (M+H), 311[e], 202[f], 185[g], 159	V
5	212 (M+H+ACN), 171 (M+H)	II
6	505 (M+H), 353, 311, 201, 170	I, CHD
7	127 (M)	VII

[a] Refer to Fig. 1 for chemical structures. [b] $HN{\equiv}C-NH-C(=NH)-NH-C_6H_{12}-NH_2$. [c] $H_2N-C(=NH)-NH-C_6H_{12}-NH_2+H$. [d] $H_2N-C(=NH)-NH-C_6H_{12}-NH-C(=NH)-NH_2+H$. [e] (M−CONH+2H). [f] $H_2N-C(=NH)-NH-C_6H_{12}-NH-C(=O)-NH_2+H$. [g] $O{\equiv}C-NH-C(=NH)-NH-C_6H_{12}-NH_2$.

3. Results and discussion

3.1. Detection and identification of hydrolysis products

Fig. 3 presents LC chromatograms, obtained with the new polymer-based RP-HPLC system, of a fresh and a heat-stressed solution of 800 ppm CHD in H_2O. The heat-stressed solution generated six hydrolysis products: two major (**3**, **5**) and four minor ones (**1**, **2**, **4**, **7**). Peak **7** has been identified as **VII** by spiking with reference material. UV profiles of CHD and the six hydrolysis products are shown in Fig. 4. Three of the products (**2**, **3**, **4**) have UV profiles nearly identical to that of CHD (**6**), suggesting that a chlorophenylbiguanidino (CPBG) moiety is still present in them. The UV profiles of **1** and **5**, with a single absorption maximum between 230 and 245 nm, are similar to that of **7** (**VII**) and are indicative of the absence of the more conjugated CPBG system. Although alkyl-substituted biguanides and phenylbiguanide have a single absorption maximum between 230 and 245 nm [18], their presence is not predicted from CHD hydrolysis. The number of products observed and their UV data are consistent with the hydrolysis Paths B and C proposed in Fig. 1. The proposed hydrolysis pathways would generate three UV-absorbing products containing CPBG (**III**, **V**, and **VI**) and three others containing no CPBG (**II**, **IV**, and **VII**) from CHD. Thus, it is probable that **2**–**4** are the CPBG-containing **III**, **V** and **VI**, while **1**, **5** and **7** account for the non-CPBG-containing **II**, **IV** and **VII**. The exact identities of these hydrolysis products are deliberated over using mass spectral analysis in the next paragraph.

Mass spectral data from LC–MS for the peaks observed in Fig. 3 are summarized in Table 1. They are consistent with the UV data and confirm that **2**, **3** and **4** are **VI**, **III** and **V** respectively. Similarly, **1**, **5** and **7** are respectively identified as **IV**, **II** and **VII**. Thus, all six UV-detectable hydrolysis products of CHD, predicted by Paths B and C (Fig. 1), are separated and detected by the new HPLC method. In a recent paper, Revelle et al. [19] described the detection and identification of hydrolysis products in a heat-stressed concen-

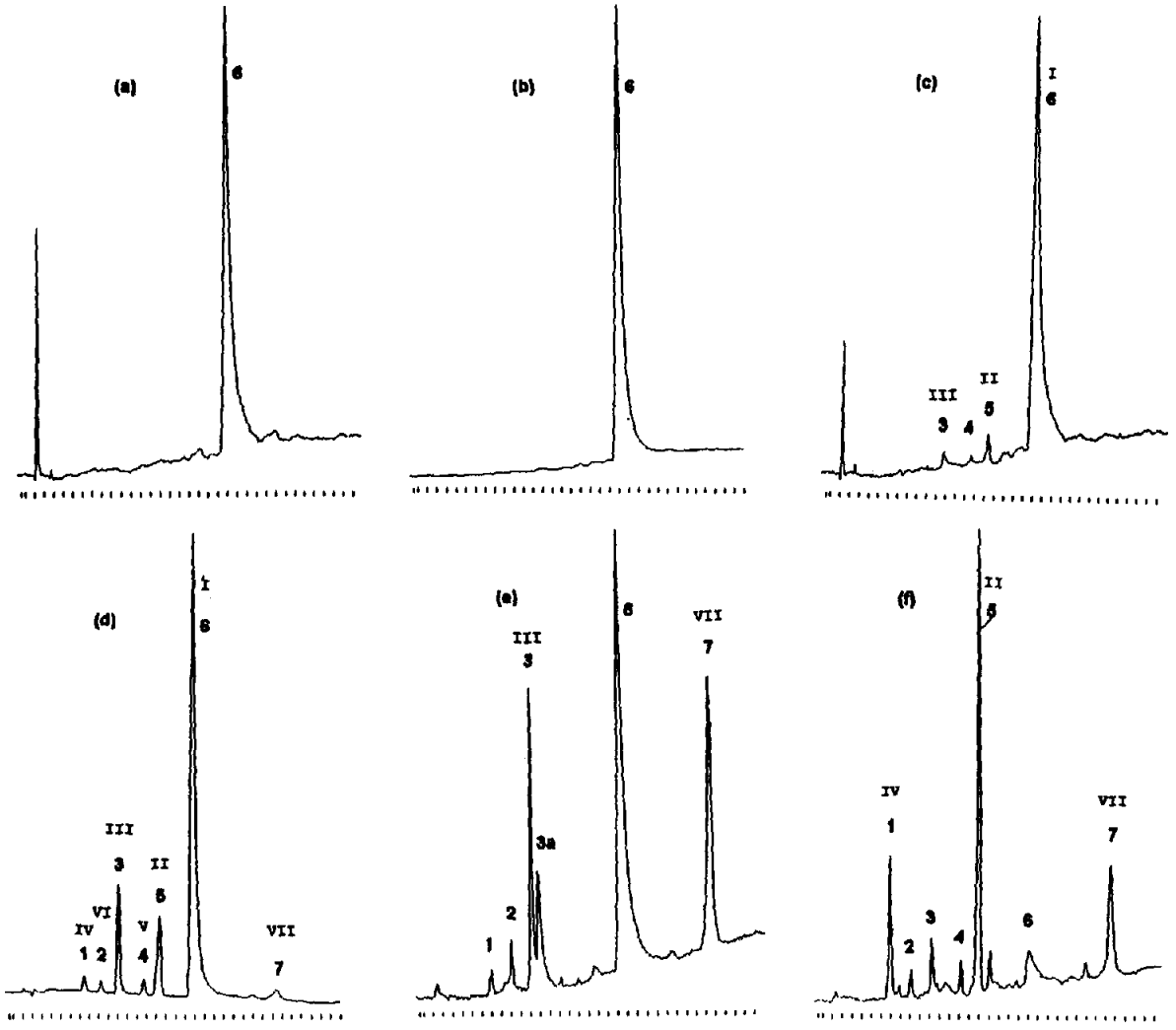

Fig. 5. HPL chromatograms of dilute CHD solutions: (a) fresh, 96 ppm ml^{-1} in H_2O; (b) solution (a) stored for 1 year at room temperature; (c) solution (a) heated at 50°C for 21 days; (d) 800 ppm ml^{-1} in H_2O heated at 80°C for 5 days; (e) 800 ppm ml^{-1} in 0.01 N HCl, heated at 80 °C for 2 days; and (f) 800 ppm ml^{-1} in 0.01 N NaOH, heated at 80 °C for 2 days. HPLC conditions identical to those in Fig. 3, see Section 2 for details. Peak assignment: **1**, **IV**; **2**, **VI**; **3**, **III**; **4**, **V**; **5**, **II**; **6**, CHD; **7**, **VII**.

trated (20%) solution of CHD in H_2O. The separation was achieved with several isocratic elutions in succession and was not suitable for assay purposes. In addition to products **II**–**VII**, they detected the non-UV-absorbing products **VIII** and **XIII** with LC–MS. Similar to the current findings, **III** was a major hydrolysis product. **XIV**, a primary product from Path A (Fig. 1), was not detected in the study of Revell et al.

3.2. Stability of dilute CHD solutions

Dilute aqueous solutions of CHD are stable at room temperature. The chromatogram of a one-year-old solution (Fig. 5b) is identical to that of a fresh solution (Fig. 5a). Heating the solution to 50°C for 21 days (Fig. 5c) created a small amount of **II** and **III**. The other products, **IV**, **V**, **VI** and **VII**, were observed only under more vigorous

heating (Fig. 5d). Heating an acidic solution of CHD (Fig. 5e) resulted in **III** and **VII** as the major products, although another significant product, **3a**, was also present. The identity of **3a**, which has m/z values of 365 and 170 and a UV spectrum similar to that of CHD, has not yet been confirmed. Its spectral data are consistent with the structure of CHD minus the equivalent of a chlorophenyldiimide molecule (ClBzN=NH). Heating a basic solution of CHD (Fig. 5f) resulted in the near disappearance of biguanide-containing materials (**I**, **III**, **V** and **VI**). Similar to the observations of Revelle et al. [19], compound **XIV** (m/z 395), the product complement to **VIII** in Path A (Fig. 1), was also not detected in these hydrolysis experiments. Therefore, contrary to Elphern's hypothesis [16], hydrolysis of CHD in aqueous solution probably does not involve Path A (Fig. 1). Identification of the hydrolysis products in Fig. 5 indicates that hydrolysis of biguanides generally follows the scheme in Fig. 2. In H_2O, hydrolysis of CHD predominantly follows Path B to give urea (**II**) and guanidine (**III**), although formation of small amounts of **IV** and **V** via Path C is also evident (Fig. 5d). In acidic solution (Fig. 5e), the guanidine (**III**) appears fairly stable while urea (**II**) is further hydrolyzed to the amine (**VII**). In basic solution (Fig. 5f), only small amounts of biguanide-containing materials (**I**, **III**, **V**, **VI**) survive; in their place are the non-biguanide-containing guanidine (**IV**), urea (**II**) and amine (**VIII**).

Table 2
HPLC detection response (peak area) of 25 ppm CHD solutions containing various salts[a]

CHD solution medium	Peak area	RSD (%)	n
Deionized H_2O	435011	3.1	3
0.9% NaCl	484822	5.4	3
0.5% $Na_2S_2O_3$	418379	1.9	3
0.1% Boric acid	433548	2.4	3
0.1% $Na_2B_4O_7$	465043	1.9	3

[a] See Section 2 for HPLC conditions.

3.3. HPLC assay

The stability-indicating ability of the new HPLC method has been validated by the detection of all UV-detectable hydrolysis products. In an earlier silica-based RP-HPLC assay of CHD [7], it was observed that the detection response (peak area) of the CHD peak was affected by the sample solution media. The detection response of the CHD peak from water solutions containing 0.9% sodium chloride, 0.5% sodium thiosulfate, or 0.1% boric acid was about twice that of the water or water solution containing 0.1% sodium borate. With the current organic polymer-based RP-HPLC method, the detection responses of CHD are similar among these solutions (Table 2). Thus the new HPLC assay eliminates the need to match the matrices of the sample and reference solutions. The peak area precision (RSD) of 50 μl injections of a 25 ppm CHD solution in H_2O is $< 3\%$ ($n = 3$). A $< 6\%$ RSD in peak area ($n = 9$) was obtained for a viscous ophthalmic solution containing 30 ppm CHD, 1% of wetting agent and/or viscosity modifier, and 1% inorganic salts, among other additives [4]. The RSD for the retention time is 0.3%. The lowest limit of quantitation for CHD is < 0.3 ppm when 50 μl sample solution is injected. The assay precision of this HPLC method may be significantly improved with the use of an internal standard. However, due to the interference of the many potential hydrolysis products, an internal standard for the assay is not yet available.

4. Conclusion

This paper presents a hydrolysis scheme for CHD in aqueous solutions. The scheme is consistent with hydrolysis products detected and identified by LC–UV and LC–MS. This paper also presents a new HPLC method for the analysis of aqueous solutions of CHD. The method is capable of detecting all UV-absorbing hydrolysis products of CHD. Preliminary data suggest that the method is capable of being an accurate, reproducible, reliable, and stability-indicating assay

of CHD in dilute aqueous solutions such as ophthalmic solutions.

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