Journal of Chromatography, 594 (1992) 209–216
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 820

Development of stability indicating assay methods for the determination of hydroxamic acids in topical formulations☆

James E. DiNunzio* and R. Rao Gadde

Bristol-Myers Squibb Company, Pharmaceutical Research Institute, 100 Forest Avenue, Buffalo, NY 14213 (USA)

(First received May 23rd, 1991; revised manuscript received October 22nd, 1991)

ABSTRACT

This paper reports on the development of high-performance liquid chromatographic methods for the determination of hydroxamic acids in topical formulations. A poly(styrene–divinylbenzene) copolymer stationary phase with a mobile phase of acetonitrile and pH 6 phosphate buffer at a flow-rate of 1.0 ml/min was used. Analyte detection was at 254 nm. This system is capable of the separation of phenylalkylhydroxamic acids and benzohydroxamic acids.

Excellent quantitative results were obtained. The recoveries from both cream and gel formulations were >99% with <1% relative standard deviation. Linearity of over one order of magnitude was obtained.

The selectivity of the method using the polymeric stationary phase was investigated. Variation of the mobile phase composition and pH showed that the selectivity of the method could be easily changed by changing mobile phase pH. The method selectivity was demonstrated by forced degradation of selected acids and separation of the analyte from the products. Decomposition to the corresponding carboxylic acid was found to be a major pathway for degradation of these compounds.

The separation of the hydroxamic acids on silica bonded phases was also investigated. It was found that the poor chromatography observed on these phases resulted from the presence of metal impurities in the silica support.

INTRODUCTION

Hydroxamic acids are known to possess biological activity [1]. They are capable of both *in vitro* and *in vivo* enzyme inhibition, and have been shown to possess anti-inflammatory and analgesic properties. The bioactivity of hydroxamic acids makes them potential candidates for drug product development, and there are a few products on the market containing hydroxamic acids.

Hydroxamic acids also possess properties which make them difficult to separate. They are thermally unstable and their strong chelate formation makes many separation schemes unsuitable. However, a few methods for their separation have been reported [2–7]. Desferoxamine, a naturally occurring trihydroxamic acid was determined by reversed-phase chromatography [2]. Purging the column with the analyte along with the addition of EDTA to the mobile phase was required in order to eliminate the interference of traces of iron in the chromatographic system. Aromatic hydroxamic acids were separated by reversed-phase chromatography employing a methanol phosphate buffer eluent system [3]. While it was found that peak symmetry was improved by the use of the buffer, severe tailing was still observed for some of the hydroxamic acids. A procedure for the extraction and quantitation of cyclic hydroxamic acids in grains employed reversed-phase chromatography with a methanol–1% acetic acid eluent and gradient elution [4]. Good peak symmetry was obtained for the compounds determined since the cyclic hydroxamic acid group is not available for interactions with metals or residual silanol groups

☆ Presented at the *Fifth Annual Meeting and Exposition of the American Association of Pharmaceutical Scientists, Las Vegas, NV, 1990.*

on the stationay phase. Recoveries ranged from 78 to 106%. The separation of N-arylhydroxamic acids was performed by reversed-phase chromatography in a methanolic eluent containing 0.01% desferal mesylate [5]. This trihydroxamate was used to improve peak symmetry of the analytes. This reagent was also used for an improved assay of desferoxamine [6]. In a similar manner ferric chloride was added to the eluent for the separation of hydroxamic acids [7].

Recently, the biochemical and anti-inflammatory activity of a new topical anti-inflammatory hydroxamic acid was described [8,9]. It was reported that this compound was capable of inhibition of cyclooxygenase and lipoxygenase, and thus could be a potent anti-inflammatory agent. This compound was identified as a result of the investigation of the bioacitvity of a variety of hydroxamic acids. The data presented here describes the analytical method development work performed to support this investigation.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 1090 high-performance liquid chromatography (HPLC) apparatus with a diode array detector was used for method development. Routine analyses were performed on a Waters Assoc. Model 204 HPLC system with a Model M6000 pump, a Model 440 absorbance detector at 254 nm, and a Model 712B WISP autoinjector. Data collection was performed on a Hewlett-Packard HP-3350A LAS computer. A Waters μBondapak C_{18} column (30 cm × 3.9 mm I.D., 10 μm packing), a Whatman 10 ODS-3 column (25 cm × 4.6 mm I.D., 10 μm packing) and a Hamilton PRP-1 column (15 cm × 4.6 mm, 5 μm packing) were used.

Reagents

Series of phenylalkylhydroxamic acids and benzohydroxamic acids, the structures of which are shown in Fig. 1, were prepared in-house [9] for use in this study. The following phenylalkylhydroxamic acids were used: 6-phenylhexanohydroxamic acid, 8-phenyloctanohydroxamic acid, 9-phenylnonanohydroxamic acid, and 10-phenyldecanohydroxamic acid. The following benzohydroxamic acids were used: 3-chlorobenzohydroxamic acid, 2,4-dichlorobenzohydroxamic acid, 4-methylbenzohydroxamic acid and 4-methoxybenzohydroxamic acid.

For development and validation of the topical product assays two compounds were selected: 3-chlorobenzohydroxamic acid (3CBHA) and 9-phenylnonanohydroxamic acid (9PNHA). For product analysis 2,4-dichlorobenzohydroxamic acid and 8-phenyloctanohydroxamic acid were used as internal standards for 3CBHA and 9PNHA, respectively.

The carboxylic acids corresponding to the individual hydroxamic acids were obtained from Aldrich (Milwaukee, WI, USA). HPCL grade acetonitrile, HPLC grade tetrahydrofuran (THF) and reagent grade sodium phosphate salts and phosphoric acid were obtained from Fisher Scientific (Springfield, NJ, USA). Solvents and samples were filtered through type HVLP membrane filters (0.45 μm) (Millipore, Bedford, MA, USA).

Procedure

Effect of eluent pH. The effect of eluent pH on the selectivity of the method was determined by measuring the change in retention time with the change in the apparent pH of the eluent. The apparent pH was determined by measuring the pH of the eluent, which was collected at the detector outlet, using a

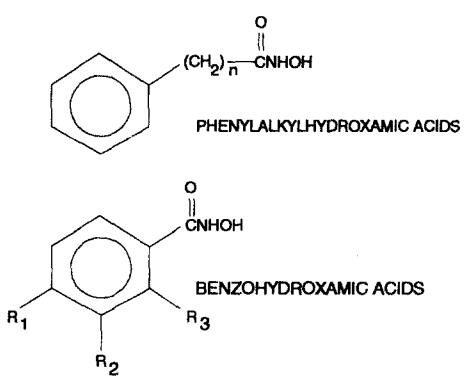

Fig. 1. Structures of test hydroxamic acids. 6-Phenylhexanohydroxamic acid, n = 6; 8-phenyloctanohydroxamic acid, n = 8; 9-phenylnonanohydroxamic acid, n = 9; 10-phenyldecanohydroxamic acid, n = 10; 3-chlorobenzohydroxamic acid, R_1 = R_3 = H, R_2 = Cl; 2,4-dichlorobenzohydroxamic acid, R_2 = H, R_1 = R_3 = Cl; 4-methylbenzohydroxamic acid, R_2 = R_3 = H, R_1 = CH_3; and 4-methoxybenzohydroxamic acid, R_2 = R_3 = H, R_1 = OCH_3.

glass electrode conditioned and calibrated with pure aqueous buffers.

The pK_a values of 9-phenylnonanoic acid and 3-chlorobenzoic acid were determined by potentiometric titration of the acids in acetonitrile–water. The volume ratios for the acids were 40:60 and 15:85, respectively.

Sample preparation. Standard and internal standard stock solutions were prepared by accurately weighing known amounts of the reagents and dissolving in acetonitrile. Working standard solutions were prepared for analysis by pipetting aliquots of these stock solutions into volumetric flasks and adding the appropriate amounts of acetonitrile and aqueous sodium phosphate solution so that the final solution was identical to the mobile phase.

A variety of sodium phosphate solutions at different pH were used in this study. Unless stated otherwise they were prepared as follows. The appropriate weight of either dibasic or tribasic sodium phosphate equivalent to 0.1 moles was accurately weighed into a 1-l beaker. This was dissolved in 900 ml of water and adjusted to the required pH with phosphoric acid. The solution was quantitatively transferred to a 1-l volumetric flask and diluted to volume with water.

Both cream and gel formulations were assayed. Gel samples were prepared by accurately weighing 2 g of gel product into a 50-ml volumetric flask. This was dispersed in 10 ml of THF and about 25 ml of acetonitrile was added. The sample was mixed well and diluted to volume with acetonitrile. Aliquots of 5 ml of this solution and 5 ml of the internal standard stock solution were pipetted into a 50-ml volumetric flask. Acetonitrile (12 ml) was added and the sample diluted to volume with aqueous sodium phosphate solution.

Cream samples were prepared by accurately weighing 1 g of product into a 100-ml volumetric flask. This was dispersed in 15 ml of aqueous sodium phosphate solution and diluted to volume with acetonitrile. After mixing an aliquot was centrifuged, 5 ml of the clear solution and 5 ml of the internal standard stock solution were pipetted into a 50 ml volumetric flask. This was then diluted to volume with aqueous sodium phosphate solution.

Both the cream and gel sample final solutions were filtered through 0.45-μm membrane filters prior to injection. Between 30 and 50 μl of sample were injected.

RESULTS AND DISCUSSION

Method development

The major obstacle in the development of separation methods for hydroxamic acids is the strong chelating ability of these compounds. This leads to broad asymmetric bands in the chromatograms of

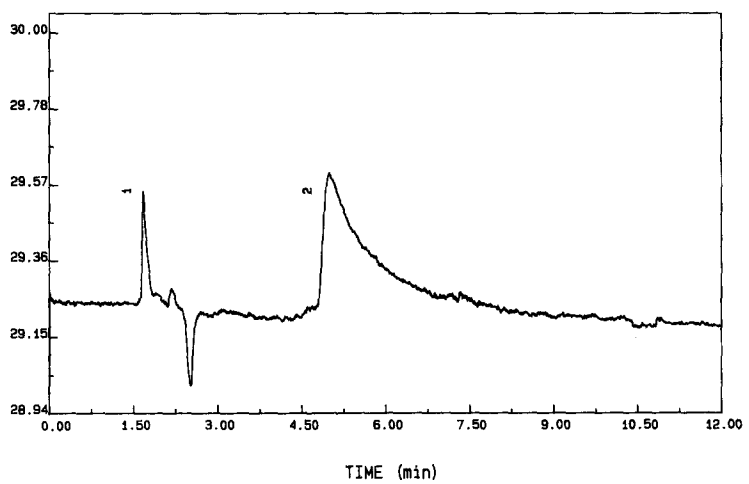

Fig. 2. Chromatogram of 9-phenylnonanohydroxamic acid on a silica bonded phase. Column, μBondapak C_{18}; eluent, acetonitrile–aqueous sodium phosphate (pH 6) (60:40, v/v). Identification: 1 = solvent; 2 = 9-phenylnonanohydroxamic acid. The values on the y-axis are arbitrary units.

hydroxamic acids [2–6]. Fig. 2 shows a typical chromatogram obtained for the separation of 9PNHA on a reversed-phase system. The broad asymmetric peak is typical of those obtained for the hydroxamic acids investigated in this work.

The severity of the band broadening problem depends on several factors including the degree of surface coverage of the stationary phase, the amount of free metal ion in the chromatographic system, and the substitution of the hydroxamic acid functional group. Based on our results band broadening appeared to be more severe for the unsubstituted hydroxamic acids than that reported previously [4,5] for substituted acids.

Several different chromatographic systems were tested in order to improve the peak shape of the hydroxamic acids. These results are summarized in Table I which shows a comparison of the peak tailing factor [10] for 9 PNHA under the different chromatographic conditions.

Comparison of the first two entries in Table I shows the effect of addition of EDTA to the eluent. The addition of competing chelating agents has been shown to improve the peak symmetry of hydroxamic acids [2,6]. The addition of 0.01 M EDTA for the seaparation of 9PNHA reduced the tailing factor on the Waters C_{18} column from 8.7 to 5.7. However, the peak symmetry is not improved enough to use this system for analysis.

In order to determine the effect of unreacted silanol groups in the stationary phase on the chromatography of these hydroxamic acids, a comparison of end-capped *vs.* non end-capped phases (Whatman 10 ODS-3 *vs.* Waters μBondapak C_{18} was made. Some improvement with the end-capped phase was observed, but the peak symmetry was still poor.

TABLE I

PEAK TAILING ON SELECTED STATIONARY PHASES

Eluent: acetonitrile–aqueous sodium phosphate solution pH 6.

Column type	Tailing factor
Waters μBondapak	8.7
Waters μBondapak	5.7[a]
Whatman 10 ODS-3	4.3
Hamilton PRP-1	1.3

[a] Eluent contained 0.01 *M* EDTA.

The use of the polymeric stationary phase (Hamilton PRP-1) resulted in excellent peak shape, as indicated by the tailing factor listed in Table I. The nearly symmetrical peaks obtained with the polymeric phase produces good resolution and improved sensitivity. This makes this system suitable for analytical purposes.

Peak asymmetry of hydroxamic acids could result from chelation of the hydroxamic acids with metal impurities in the stationary phase [5], trace metals, particularly Fe(III), in the chromatographic system, or residual silanol groups on the bonded stationary phase. No studies have attempted to differentiate between these causes. The data presented here supports the theory that metal impurities in the silica support cause band broadening of the acids. The facts that EDTA and the use of a stationary phase with low residual silanol groups resulted in only a marginal improvement in chromatography, along with the fact that replacing the silica-based column with the polymeric column on the same chromatographic system resulted in very good peak shape, indicate that neither trace metals in the chromatographic system nor residual silanol interactions are the main cause of peak asymmetry. Therefore, the loss in efficiency when using the silica-based stationary phases must be due to metals in the silica support. Since the polymeric stationary phase is essentially free of trace metals band broadening is significantly reduced compared to the silica supports.

The influence of metal impurities in the bonded stationary phase on chromatographic retention processes has been investigated [11–13] and has been shown to be important for some classes of compounds. Chromatographic grade silica gel has been found to contain more than 1000 ppm of metal impurities (not including Group I metals) [14]. This high content has been shown to produce broad asymmetric peaks, similar to those observed here for the hydroxamic acids. The use of the polymeric stationary phase results in superior chromatography for the hydroxamic acids because of the much lower level of metal impurities. Analysis of this material found no detectable heavy metals [15]. Since this stationary phase has a significantly lower level of metal impurities which can influence the chromatographic retention process, its use results in the improved peak shapes observed.

TABLE II

EFFECT OF MOBILE PHASE COMPOSITION ON RETENTION TIME

Mobile phase: acetonitrile–aqueous sodium phosphate solution pH 6. Column: Hamilton PRP-1.

Acetonitrile (%, v/v)	Retention time (min)	
	3CBHA	9PNHA
46	–	5.8
40	1.8	12.2
35	–	23.1
30	–	55.9
20	4.1	–
15	7.2	–
13.5	9.1	–
12	11.5	–

Initial experiments to optimize the eluent showed that the test compounds behave as would be expected in a reversed-phase system. As the amount of acetonitrile increased the retention time decreased. This is shown by the data in Table II. These data reflect the difference in polarity of the two classes of acids in that the more hydrophobic phenylalkylhydroxamic acids required more organic solvent to be eluted than the hydrophilic benzohydroxamic acids.

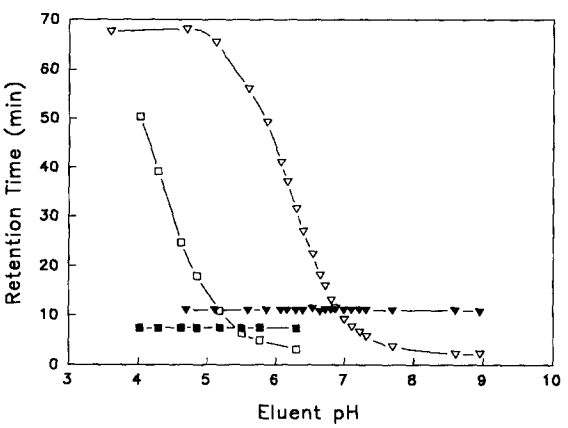

Fig. 3. Effect of eluent pH on retention time of hydroxamic and carboxylic acids. Column, Hamilton PRP-1; eluent, acetonitrile–aqueous sodium phosphate (40:60, v/v for ▽ and ▼), (15:85, v/v for □ and ■). Identification: ▽ = 9-phenylnonanoic acid; □ = 3-chlorobenzoic acid; ▼ = 9-phenylnonanohydroxamic acid; ■ = 3-chlorobenzohydroxamic acid.

In order to develop a stability indicating assay method which can be used to support pharmaceutical product development it is necessary to be able to separate the analyte from its degradation products. Although two classes of hydroxamic acids were investigated they can be expected to form analogous degradation products. Our work demonstrated that hydrolysis to the parent carboxylic acid is a main mechanism for degradation of these compounds in solution. Therefore, any stability indicating method must be able to separate both the hydroxamic and carboxylic acids.

The effect of pH on the separation of test hydroxamic acids and their corresponding carboxylic acids is shown in Fig. 3. These data show that the separation of the hydroxamic acids and their main degradation product, the carboxylic acid, can be controlled by controlling the pH of the eluent. The data is consistent with the ionization of these acids. The carboxylic acids, which are stronger than the hydroxamic acids ionize at a lower pH. Therefore, their retention times begin to decrease at a lower pH. At very high pH both acids can be completely dissociated. Experiments performed at pH 11.5 showed that both the hydroxamic acid and the corresponding carboxylic acid co-eluted due to their like charge.

From the data in Fig. 3 an interesting observation can be made regarding the effect of acetonitrile on the dissociation of the carboxylic acids. The data for these acids is essentially the titration curve for the acids in the mixed solvent system. At the inflection point then the pH of the eluent is equal to the pK_a of the acid. For 9-phenylnonanoic acid this occurs at a pH of about 6.2. The pK_a of this carboxylic acid in water is about 4.8. This difference is due to the suppressed dissociation of the acid in the mixed solvent. In a similar, but less dramatic manner the pK_a of 3-chlorobenzoic acid shifted from 3.82 [16] in aqueous solution to about 4.2 in a mixed solvent containing 15% acetonitrile. These values are in excellent agreement with the pK_a values determined in our laboratory for these compounds by potentiometric titration in mixed acetonitrile and water. Clearly, the suppression of dissociation of ionizable species due to the presence of organic modifiers in the eluent must be considered when developing an HPLC separation for these types of compounds.

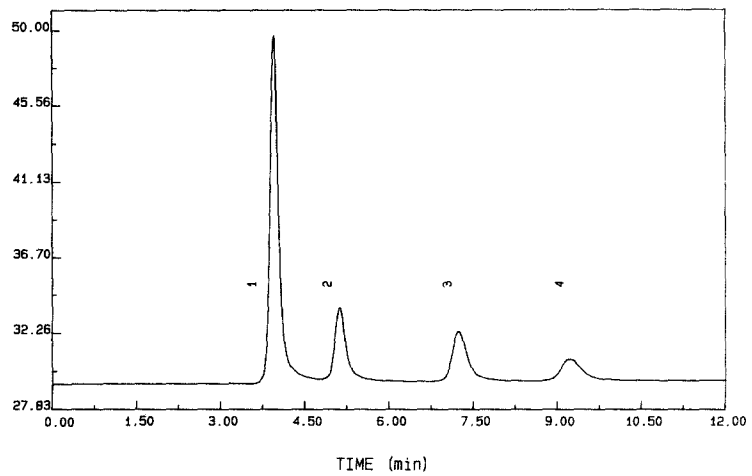

Fig. 4. Separation of benzohydroxamic acids. Column, Hamilton PRP-1; eluent, acetonitrile–aqueous sodium phosphate (pH 6) (15:85, v/v). Peaks 1 = 4-methoxybenzohydroxamic acid; 2 = 4-methylbenzohydroxamic acid; 3 = 3-chlorobenzohydroxamic acid; 4 = 2,4-dichlorobenzohydroxamic acid. *y*-Axis in arbitrary units.

Figs. 4 and 5 show chromatograms for the series of benzohydroxamic and phenylalkylhydroxamic acids, respectively. Good peak shape and resolution were obtained for each class of hydroxamic acid. Based on these separations an eluent of acetonitrile–aqueous sodium phosphate solution (pH 6) (15:85) was used for analysis of the benzohydroxamic acids, and acetonitrile–aqueous sodium phosphate solution (pH 6) (43:57) was used for the phenylalkylhydroxamic acids.

Method validation

In order to demonstrate that an assay method is stability indicating, it is necessary to demonstrate

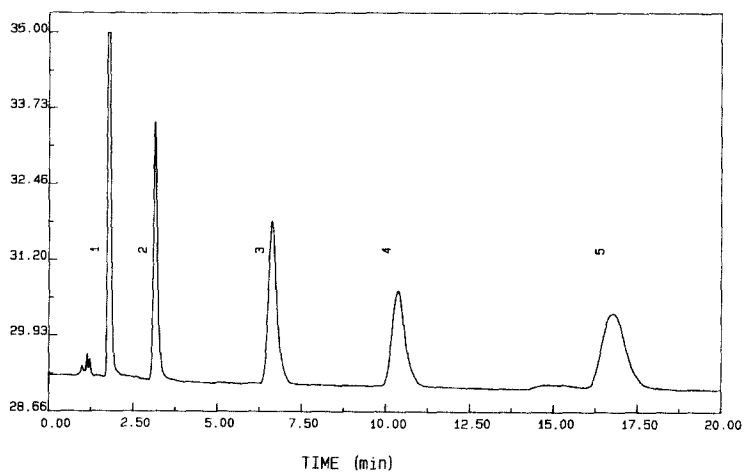

Fig. 5. Separation of phenylalkylhydroxamic acids. Column, Hamilton PRP-1; eluent, acetonitrile–aqueous sodium phosphate (pH 6) (40:60, v/v). Peaks: 1 = 3-Chlorobenzohydroxamic acid; 2 = 6-phenylhexanohydroxamic acid; 3 = 8-phenyloctanohydroxamic acid; 4 = 9-phenylnonanohydroxamic acid; 5 = 10-phenyldecanohydroxamic acid. *y*-Axis in arbitrary units.

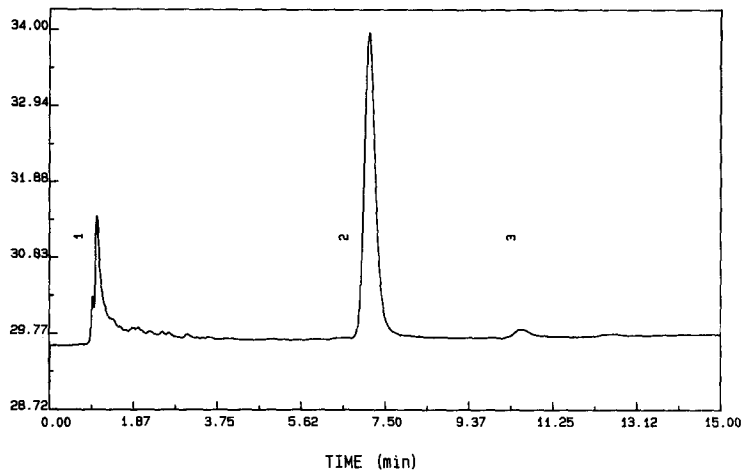

Fig. 6. Chromatogram of light-degraded 9-phenylnonanohydroxamic acid. Column, Hamilton PRP-1; eluent, acetonitrile–aqueous sodium phosphate (pH 6) (43:57, v/v). Peaks: 1 = solvent; 2 = 9-phenylnonanohydroxamic acid; 3 = 9-phenylnonanoic acid. *y*-Axis in arbitrary units.

that the active and an internal standard, if used, can be separated from potential degradation products which could form during the life of the product. This was demonstrated by separating forced degraded solutions of the test compounds. These were degraded by exposure to light or by hydrolysis in basic solution. Absorbance spectra were collected using a diode array detector. From these data absorbance ratio plots of the active from the degraded samples were made to verify that no co-eluting degradation products were present in the chromatograms.

Fig. 6 shows the separation obtained for 9PNHA which had been forced degraded by exposure to UV radiation. A number of small peaks are seen from the degradation of the active. These include the parent carboxylic acid at a retention time of about 10.5 min, and several unidentified products eluting before the active. Absorbance ratio plots showed that no detectable co-eluting products could be ob-

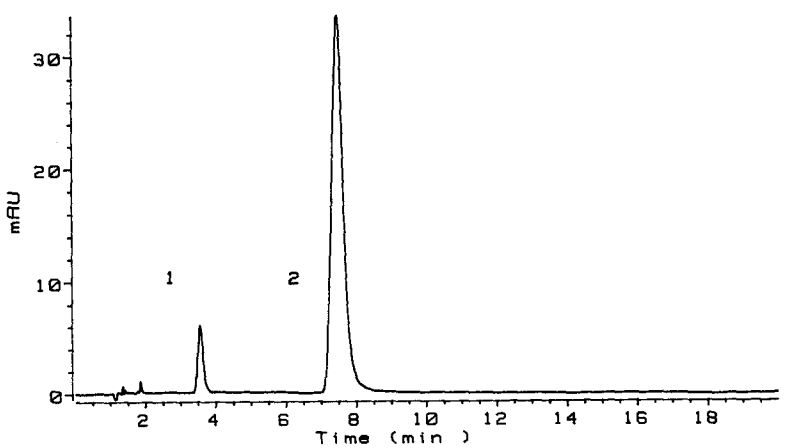

Fig. 7. Chromatogram of base-degraded 3-chlorobenzohydroxamic acid. Column, Hamilton PRP-1; eluent, acetonitrile–aqueous sodium phosphate (pH 6) (15:85, v/v). Peaks: 1 = 3-chlorobenzoic acid; 2 = 3-chlorobenzohydroxamic acid.

served. Similar results were obtained for the separation of 3CBHA and its photodegradation products.

Fig. 7 shows the separation obtained for 3CBHA which has been forced degraded in basic solution at elevated temperature. The parent carboxylic acid is seen as the major degradation product. Absorbance ratio plots showed that no co-eluting products could be observed. Similar results were obtained for the separation of 9PHHA and its base degradation products.

The method was validated for assay of topical creams and gels by determining the response linearity, precision and accuracy for the determination of 9PNHA and 3CBHA. For both analytes excellent linearity ($r > 0.999$) of over one order of magnitude was obtained. The linear range tested was from 0.3% (w/w) to 13.5% (w/w) of the drug in product.

The precision of the method was determined by the analysis of replicate samples of the products. The precision for the determination of 3CBHA in a cream formulation determined on 11 replicate assays was ± 0.5% relative standard deviation. The precision for the determination of 9PNHA in a gel formulation determined on 6 replicate assays was ± 0.4% relative standard deviation.

The accuracy for the determination of hydroxamic acids in topical formulations was determined by the analysis of synthetic samples prepared by spiking product placebos with solutions of the analytes. The recovery of 3CBHA for 9 replicate assays of the cream formulation was 100.0 ± 0.2%. The recovery of 9PNHA for 18 replicate assays of the gel formulation was 99.4 ± 0.5%.

CONCLUSIONS

The method described is precise and accurate when used to determine hydroxamic acids in topical formulations. This method has been used for routine analysis with good success.

The use of the polymeric stationary phase allows the separation of these hydroxamic acids which could not be achieved using the silica-bonded phases.

ACKNOWLEDGEMENT

The authors would like to thank Dr. S. Marathe for supplying the hydroxamic acids used in this work. The excellent technical support of Ms. Janice Mayer is also gratefully acknowledged.

REFERENCES

1 H. Kehl (Editor), *Chemistry and Biology of Hydroxamic Acids*, S. Karger, Farmington, CT, 1982.
2 S. Cramer, B. Nathanael and Cs. Horváth, *J. Chromatogr.*, 295 (1984) 405.
3 E. Lipczynska-Kochany, *J. Chromatogr.*, 260 (1983) 493.
4 P. C. Lyons, J. D. Hipskind, K. V. Wood and R. L. Nicholson, *J. Agric. Food Chem.*, 36 (1988) 57.
5 M. D. Corbett and B. R. Chipko, *Anal. Biochem.*, 98 (1979) 169.
6 J. D. Glennon, M. R. Woulfe, A. T. Senior and N. NiChoileain, *Anal. Chem.*, 61 (1989) 1474.
7 J. A. Hinson, L. R. Pohl and J. R. Gilette, *Anal. Biochem.*, 101 (1989) 462.
8 K. M. Tramposch, F. C. Zusi, S. Marathe, S. Stanley, S. Steiner, X. Nair and J. W. Quigley, *J. Invest. Dermatol.*, 92 (1989) 533.
9 K. M. Tramposch, F. C. Zusi, S. Marathe, S. Stanley, S. Steiner, X. Nair and J. W. Quigley, *Agents and Actions*, 30 (1990) 443.
10 The United States Pharmacopeia, United States Pharmacopeial Convention, Rockville, MD, 1990, p. 1567.
11 Y. Ohitsu, Y. Shiojima, T. Okumura, J. Koyama, K. Nakamura and O. Nakata, *J. Chromatogr.*, 481 (1989) 147.
12 J. Koyama, J. Nomura, Y. Ohtsu, O. Nakata and M. Takahashi, *Chem. Lett.*, 1990 (4) 687.
13 P. Sadek, C. Koester and L. Bowers, *J. Chromatogr. Sci.*, 352 (1987) 489.
14 M. Verzele, M. De Potter and J. Ghysels, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 151.
15 Hamilton Company, Reno, NV, personal communication.
16 R. C. Weast, (Editor), *CRC Handbook of Chemistry and Physics*, CRC Press, Boca Raton, FL, 65th ed., 1984; p. D165.