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Note

Purification of hydroxamic acids by the use of ferric SP-Sephadex

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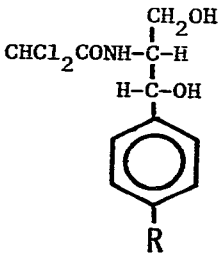
Until recent years the hydroxamic acid functional group had received relatively little interest by organic chemists. Following the discovery that hydroxamic acids are significant and toxic metabolites of certain amines and amides, interest in the chemistry of these compounds increased dramatically. The carcinogenic properties of many aromatic hydroxamic acids are now well recognized. It is generally accepted that the carcinogenic properties of many aromatic nitrogenous compounds are the result of their conversion to hydroxamic acids *in vivo*[1]. The most extensively investigated biochemical pathway for hydroxamic acid production is N-oxidation of amides by microsomal oxidases[1]. More recently we have demonstrated the existence of a new pathway to these toxic metabolites that involves the interaction of aromatic nitroso compounds with thiamine-dependent enzymes[2–4].



I: R = $-NO_2$
II: R = $-N(OH)COCH_3$
III: R = $-NHCOCH_3$

During the course of an investigation on the toxicological properties of chloramphenicol (I), we synthesized the hydroxamic acid analog (II) of this antibiotic. Unlike our previous extensive experience with hydroxamic acids[5,6], the chloramphenicol-derived hydroxamic acid, II, tenaciously resisted purification. Extensive chromatography of II on silica gel and Sephadex LH-20 gave a product which appeared to be a single compound by thin-layer chromatographic (TLC) analysis. However, this product contained trace impurities as indicated by NMR analysis, and existed as an extremely hygroscopic, amorphous solid, unlike most hydroxamic acids which are generally highly crystalline compounds. We now report a method that enabled us to obtain II in pure form and which is based on the strong chelate properties of the hydroxamic acid grouping[7,8]. This method should be applicable for the purification of any hydroxamic acid.

EXPERIMENTAL

Materials

All chemicals and solvents were ACS reagent grade quality. Ferric chloride ($FeCl_3 \cdot 6H_2O$) and sodium dithionite were obtained from Fisher Scientific. SP-Sephadex C-25 (40–120 μm) was obtained from Pharmacia. The hydroxamic acid II was prepared by reduction of chloramphenicol (I) with Zn dust and NH_4Cl, followed by acetylation according to previously described methods[6]. The resulting hydroxamic acid (II) was chromatographed on silica gel employing 10% methanol in chloroform. Those fractions that gave a single $FeCl_3$-positive spot on TLC analysis were combined to give a yellow oil, which was employed in the final purification process.

Preparation of ferric SP-Sephadex column

The SP-Sephadex (10 g, 23 mequiv.) was allowed to swell in 150 ml of 25% aqueous methanol, then the fines were decanted from the gel. The gel was then stirred briefly with 100 ml of 1% $FeCl_3$ in 25% aqueous methanol, and the supernatant discarded. This treatment was repeated twice to fully convert the sulfopropyl exchange groups to the ferric form. The ferric Sephadex gel was washed with 25% aqueous methanol until the supernatant was colorless. The gel was then slurry-packed into a 33 mm I.D. chromatography column to give a bed height of 65 mm. A glass wool plug and washed sea sand was employed as a support for the gel, and a glass wool plug was placed on top of the gel to prevent disruption.

Ferric Sephadex procedure for hydroxamic acid purification

The impure hydroxamic acid (II: 1.4 g, 4 mmoles theoretical) was dissolved in 50 ml of 25% aqueous methanol and passed throught the SP-Sephadex (Fe^{3+}) column at a flow-rate of approximately 5 ml/min. Adsorption of the hydroxamic acid via chelation of Fe^{3+} was most evident by the deep purple coloration of the gel. The gel with the retained hydroxamic acid was then washed with 50 ml of 25% aqueous methanol to remove non-hydroxamic acid impurities. The original and wash effluents were combined and saved for the isolation and identification of the impurities.

The hydroxamic acid was eluted from the gel in its ferric chelate form by passing a solution of 25% aqueous methanol saturated with NaCl through the column. This elution required about 30 ml of NaCl-saturated aqueous methanol, and was easily monitored by following the intense purple color of the chelate complex.

The ferric hydroxamate complex was decomposed by titration with a freshly prepared solution of sodium dithionite (10% in water) until the chelate color was discharged, a process requiring approximately 10 ml of reductant. The aqueous solution of the free hydroxamic acid and ferrous salts was saturated with NaCl and extracted three times with 50 ml of ethyl acetate. The ethyl acetate extracts were combined, dried (Na_2SO_4) and evaporated *in vacuo* to give a residue which crystallized from diethyl ether to give 0.90 g (64% of theoretical yield) of II as a white, microcrystalline powder (m.p. 133–134.5°). NMR and elemental analyses indicated that the product had a purity in excess of 99%.

A partial identification of the impurities that had contaminated II was achieved by analysis of the combined effluents from the gel prior to elution of the hydroxamic acid with NaCl solution. Extraction of the combined washings with ethyl acetate,

drying (Na_2SO_4) the organic extract and evaporation gave an oil. TLC analysis indicated that the major contaminant had an R_F value identical with that of II (0.15 in 10% methanol in chloroform) but, unlike II, did not give a color reaction with $FeCl_3$ spray. Crystallization of this oil from diethyl ether gave 0.15 g (11% of theoretical yield) of a compound consisting of pale yellow needles (m.p. 164–165°) and which was identified as the amide compound III[9]. The identification of other trace and colored impurities was not possible.

RESULTS AND DISCUSSION

The hydroxamic acid, II, was readily purified by application of a newly developed method based upon the ability of hydroxamic acids to strongly chelate ferric ions. Also critical to the success of this method was our development of a procedure to reduce ferric to ferrous ions, which do not form chelate complexes with hydroxamic acids. A solution of the impure hydroxamic acid was passed through a bed of ferric ions immobilized on a Sephadex cation-exchange gel, which resulted in a highly selective retention of the hydroxamic acid. All contaminants were readily washed from the gel. The immobilized hydroxamic acid was then eluted from the gel by displacing the ferric ions from the cation exchanger with a swamping quantity of sodium ions. The free hydroxamic acid was recovered by reducing the ferric chloride solution to the ferrous state with sodium dithionite.

Extensive attempts to obtain II in pure form by employing the usual liquid–solid adsorption and liquid–liquid partition forms of chromatography failed. Thus, our new method offers a very effective process for the purification of hydroxamic acids that resist the usual methods of purification. Although simple monofunctional hydroxamic acids are generally purified with little trouble[6], other, more complex hydroxamic acids, such as II and others reported in the literature[10] are not as readily purified.

Our experience with hydroxamic acids has consistently demonstrated that the corresponding amide has very similar chromatographic properties in the liquid–solid and liquid–liquid modes of separation. In the case of the chloramphenicol-derived hydroxamic acid (II) and amide (III) these properties are identical, resulting in a nearly impossible purification problem. The use of acid–base partitioning methods to separate II and III also proved to be useless because of the appreciable water solubility of both II and III at all pH values.

The repetition of this purification process on impure II consistently resulted in the recovery of pure II in yields ranging from 60 to 65% of the impure hydroxamic acid. This recovery is considered to be quite good in view of the fact that nearly 20% of the starting material was non-hydroxamate material, in particular, the amide III. The appreciable water solubility of II also results in some loss of hydroxamic acid. Separate studies of our method that employed pure II demonstrated the expected recovery of this hydroxamic acid to be about 90%. Further studies on less water-soluble hydroxamic acids have been conducted and indicate that the recovery of hydroxamic acid approaches being quantitative when more lipophilic hydroxamates are employed. To apply the method to more lipophilic hydroxamates, it is only necessary to increase the methanol content of the solvent. We have successfully applied the technique with a solvent of 80% methanol.

A further advantage of this chelation method for hydroxamic acid isolation is the fact that the recovery of hydroxamates from very dilute solutions is quite high. In the method described in Experimental, the hydroxamic acid was initially dissolved in 50 ml of solvent; however, virtually identical recoveries were obtained when the same amount of hydroxamic acid was dissolved in 1 l of solvent. This demonstrates the very strong chelation that occurs between the hydroxamic acid and the immobilized ferric ions. Unfortunately, the presence of salts, such as might be found in seawater, causes the leaching of ferric ions from the column, and failure of the method. Thus in its present form, this method could not be employed to isolate hydroxamic acid siderophores[11,12] from culture media or seawater.

In a modification of our method designed to circumvent the effects of salts, we investigated the use of Chelex 100 in place of SP-Sephadex. Chelex-100 has been employed in its cupric form for the isolation of amino acids from dilute solutions, including seawater[13]. Although we observed the immobilization of hydroxamates onto the Chelex resin (Fe^{3+}), it was not possible to recover hydroxamic acids from the resin in reasonable yields.

This ferric SP-Sephadex method for the purification of hydroxamic acids closely parallels the use of other chromatographic techniques employing a bed of immobilized metal cations which serve to effect separation based upon selective chelation. Included among these new techniques are the metal chelate affinity chromatography of proteins[14], silver-sulfoethyl cellulose for the purification of olefinic and alkynyl compounds[15] and the common use of cupric Chelex for amino acid isolations[13,16]. The ferric SP-Sephadex technique appears to be especially valuable for the final separation of intractable mixtures of a hydroxamic acid and its analogous amide.

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