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Derivatization approaches for a fermentation derived, cyclic peptide analog

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Abstract

Several derivatization approaches for a cyclic, fermentation derived peptide analog were investigated. The most viable derivatization reagent, 5-[2-(and-3)-*S*-(acetylmercapto)succinoyl)-amino]fluorescein (SAMSA-FL), derivatized the analyte through a Michael addition mechanism. SAMSA-FL was activated under basic conditions to produce a free thiol functionality, which in turn added across the double bonds of available α,β-unsaturated amide sites present on the substrate. Derivatizations performed at 50°C with a 200-fold molar ratio of SAMSA-FL to peptide analog produced several fluorescently active products as seen by HPLC. These products were effectively quantitated by coeluting all of the derivative species into a single peak via the use of a step gradient condition. Quantitation of the derivatized peptide analog was achieved on actual samples with prederivatization concentrations ranging from 25.0–500 μ*M* (31.1–622 ppm). The method was validated by performing an analysis on three single blind spiked samples.

Keywords: Derivatization, LC; Michael addition; Peptide analogue; 5-((*S*-(Acetylmercapto)succinoyl)-amino)fluorescein

1. Introduction

Derivatizations in chromatography are often applied to improve detection limits and sensitivity for the most common detection methods, including ultraviolet (UV), fluorescent (FL), electrochemical (EC), or even mass spectrometric (MS) [1–19] detection. Derivatization schemes involving small molecules can usually be tailored so that a single site of reaction or attachment is targeted and a single product can often be formed in high yield. Thus, amino acids, amines, amino alcohols, phenols and other organic groups/compounds, are often derivatized to a single, homogeneous product with one or more tags in high yield and purity.

The derivatization of larger, biologically relevant species is not generally as straightforward. The basic problem comes down to the chemical fact that biological species (polypeptides, proteins, antibodies, etc.) often have several sites of reactivity, amino groups, thiols, phenols, disulfides, etc. As a result, mixtures of products will arise, for almost any protein. For a relatively simple protein that can have several different amino groups, e.g. insulin, this will lead to several different tagged products, singly tagged at different amino acid sites, ditagged, tritagged, etc. [20–24] These are usually formed at different rates of reaction, with different efficiencies

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of conversion, and in different yields, although there have been some notable efforts to control the selectivity of these reactions [25]. It is also likely that different products may not have identical physical and detection properties.

Ideally, one would like to control where the reaction occurs, the efficiency and time, and the conversion yield of the starting analyte. One would also like to realize complete tagging of all possible sites, to a single, homogeneous product, which will provide maximal detector response. However, the successful conversion of a starting analyte to a single, fully tagged, homogeneous product is much hindered by the vast differences in reactivities of different taggable sites.

The formation of multiple products is not necessarily a bad result if these products can be made to coelute. In that case, a single peak will result, hopefully resolved from other sample components, and identification and quantitation can result [21]. To achieve this, the chromatographic conditions must be optimized to produce a single peak from what is really a mixture of derivatives. This scenario is not all that different from strategies in which on-line, post-column derivatization are used in that the exact nature of the derivatives formed in the flow stream may not be relevant, nor necessary for quantitation or validation.

Additionally, problems arise in derivatizing at very low levels, because the rates of these reactions become slower and slower, to the point of impracticality. In reality, it is extremely difficult to efficiently and rapidly tag at trace levels, with chemical selectivity and uniformity, particularly when other matrix components present in the sample may act as inhibitors or simultaneously react.

This report details our efforts to derivatize the major component of a fermentation reaction product, PNU-82127. The major component of this fermentation product is a cyclic peptide analog, referred to as sulfomycin, which promotes growth in animals and possesses α,β-unsaturated amide moieties, Fig. 1. Successful derivatization of sulfomycin was accomplished through the use of 5-[(2-(and-3)-*S*-(acetylmercapto)succinoyl)amino]fluorescein (SAMSA-FL), Fig. 2, which is a fluorescein analog that can be activated under basic conditions to yield a free thiol group. The reaction between SAMSA-FL and sulfomycin produced several derivative products, which were then chromatographically coeluted into a single HPLC peak and fluorescently detected.

2. Experimental

2.1. Materials and reagents

All chemicals were of reagent grade or better and used as received. Acetonitrile (ACN) was donated by EM Science (Gibbstown, NJ, USA), as their Omnisolv grade HPLC solvents. Concentrated HCl, triethylamine (TEA), N,N-dimethylformamide (DMF), 9-fluorenylmethylchloroformate (FMOC-Cl) and dansyl chloride (DNS-Cl) were from Fisher Scientific (Pittsburgh, PA, USA). Pyridine and dimethylsulfoxide (DMSO) were from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA), 1-naphthylchloride (1-NC) and 1-naphthylisocyanate (1-NIC) were from Aldrich (Milwaukee, WI, USA). SAMSA-FL, SAMSA fluorescein was sold as a mixture of isomers from Molecular Probes (Eugene, OR, USA). The molecular mass of the free thiolate generated upon base cleavage of SAMSA-FL is 478. 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (6-AQC) was obtained from Waters (Milford, MA, USA). Water used for chromatography was obtained from a Corning Glass Works (Corning, NY, USA) Megapure MG-1 water purification system.

2.2. Instrumentation

Waters Models 6000A and M-45 solvent delivery systems coupled to a Waters Model 660 solvent programmer were used for gradient HPLC capabilities. Post-column pH adjustment was achieved with a Waters Model 590 solvent delivery system and a mixing tee. Sample injection was achieved with Gilson Medical Electronics (Middleton, WI, USA) Models 231 autosampler, 401 dilutor and a 20-μl injection loop. The dilutor solvent was a mixture of ACN–water (10:90, v/v). A 150×3.9 mm Waters Delta Pak (C_{18}, 5 μm, 100 Å) column was used for all the separations.

Ultraviolet detection was performed with a Waters Lambda-Max Model 480 LC spectrophotometer operating at 230 nm. Fluorescence detection was

performed with a Waters Model 420-AC fluorescence detector (excitation with a 450 nm bandpass filter, and emission with a 495 nm longpass filter). Chromatograms were collected with a Macintosh Plus 1 Mb computer (Apple Computer, Cupertino, CA, USA) and analyzed with Dynamax software from Rainin (Woburn, MA, USA).

2.3. HPLC conditions

Chromatographic conditions for the analysis of underivatized sulfomycin and the monitoring of the crude reaction mixture upon derivatization of sulfomycin with SAMSA-FL were achieved with a linear gradient from 22:78 to 42:58 of ACN–water (0.1% TFA) over 30 min at a flow-rate of 1.5 ml/min. In all cases, samples were diluted with ACN–water (10:90, v/v) prior to injection.

HPLC conditions for coeluting all of the derivative products into a single peak were achieved with a step gradient. The initial mobile phase conditions consisted of ACN–water (39:61, v/v, 0.1% TFA) run isocratically for 5 min at 1.5 ml/min, then stepped to ACN–water (90:10, v/v, 0.1% TFA).

2.4. Derivatization procedure

The technical literature from Molecular Probes, which accompanied the SAMSA-FL reagent, was used to design the conditions for the derivatization reaction. Briefly, 1 mg of SAMSA-FL was dissolved in 100 μl of 0.1 *M* NaOH and incubated at room temperature in the dark for 15–30 min to remove the acetyl protecting group. The reaction mixture was then buffered with the addition of 22 μl of 0.5 *M* Na_2HPO_4 (pH 9.0) buffer. The final concentration of SAMSA-FL was 15.7 m*M*.

A 5 m*M* stock solution of HPLC purified sulfomycin dissolved in DMF was kept at −10°C. This solution was periodically evaluated with HPLC to verify the stability of sulfomycin during storage. Working solutions were made by dissolving the stock solution with additional DMF to a desired concentration. A 200-fold molar excess of the activated SAMSA-FL reagent solution was then added. The resulting reaction mixtures were then incubated at 50°C for 2 h in a Reacti-Therm heating module (Pierce, Rockford, IL, USA).

2.5. Validation study

Calibration standards of 0 (derivatization blank), 50, 200, 350 and 500 μ*M* sulfomycin in DMF were prepared by diluting the 5 m*M* sulfomycin stock solution with DMF. These calibration standards, as well as three single blind spiked samples prepared in a similar manner, were derivatized with SAMSA-FL, diluted 50-fold with ACN–water (10:90, v/v) and analyzed in triplicate using HPLC. A calibration plot of peak area (mV/s) versus concentration of sulfomycin (μ*M*) was used to determine the concentrations of sulfomycin in the blind samples.

2.6. MS analysis

Matrix assisted, laser desorption ionization, time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was performed on a PerSeptive Biosystems (Framingham, MA, USA) Voyager RP Biospectrometry Workstation using α-cyano-4-hydroxycinnamic acid as the matrix. Samples were mixed on the analysis plate with equal volumes of a saturated matrix solution and allowed to air dry before analysis. A nitrogen laser operating at 337 nm was used to ionize the samples. The accelerating voltage, grid voltage and guide wire voltages were 15 kV, 94.5% and 0.010%, respectively. Delayed extraction was performed 75 ns after each laser pulse.

Electrospray ionization mass spectrometry (ESI-MS) was performed on a TSQ model 700 triple quadrupole mass spectrometer (Finnigan, San Jose, CA, USA). Samples were dissolved in water–methanol–acetic acid (20:80:0.2, v/v/v). The electrospray ionization source was set at 3.7 kV, 150°C and 866 mtorr with syringe pumping at 5.00 μl/min. The triple quadrupole was set at successive potentials of 68 V, −2.6 V and −15.0 kV for the analysis of positive ions.

3. Results and discussion

3.1. Derivatization of alcohol functionalities

PNU-82127 was a mixture of more than 40 components produced by a beer fermentation. The major component, sulfomycin, is illustrated in Fig. 1

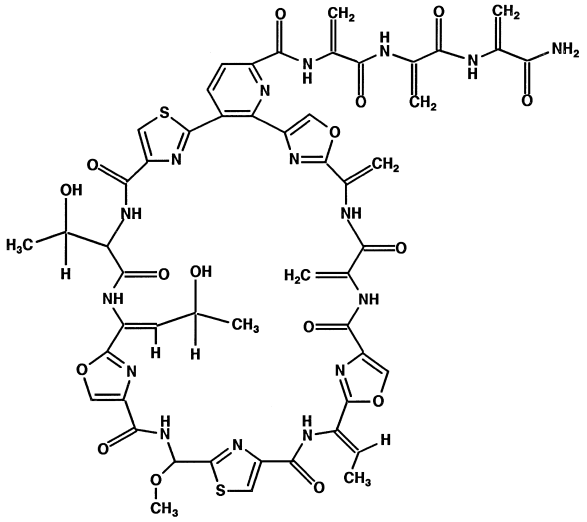

Fig. 1. Structure of the major component of the fermentation beer, sulfomycin.

and was isolated in bulk with the use of preparative scale HPLC. Since sulfomycin had been determined to be an animal growth promoter, our original goal was to develop a derivatization protocol that would allow detection within an animal biofluid. Our strategy was to incorporate a fluorescent moiety into the analyte via derivatization, separate the various species present in the sample with HPLC and perform detection and quantitation by fluorimetry.

Initial inspection of the structure of the analyte revealed certain features as being potential sites for derivatization. Firstly, the analyte possessed two 2° alcohol functionalities, which appeared to be reasonable reactive sites for derivatization. As such, we attempted to derivatize with reagents known to react at alcohol centers, such as acyl chlorides (FMOC-Cl, DNS-Cl, 1-NC), isocyanates (1-NIC), and activated carbamates (6-AQC). Derivatization attempts were made in dry, polar aprotic solvents (DMSO, DMF), since sulfomycin was not soluble in nonpolar solvents like hexanes or dichloromethane. All attempts performed at various temperatures, with and without the use of catalytic organic bases (pyridine, TEA, DMAP), failed to reveal any fluorescent reaction product. As all attempts at derivatization of sulfomycin at the alcohol centers were unsuccessful, derivatization was attempted at the various α,β-unsaturated amide sites present on the substrate.

3.2. Derivatization of α,β-unsaturated amide functionalities via Michael addition

Sulfomycin contained several α,β-unsaturated amide sites situated in different locations in the molecule. However, the different local environments and the possible steric hindrance that a tagged site would have on the subsequent derivatization of a neighboring site made the derivatization rate unpredictable. Considering these factors, it was anticipated that multiple derivative species would most likely result from the derivatization of sulfomycin with an active hydrogen compound via a Michael addition mechanism. SAMSA-FL, illustrated in Fig. 2, is a commercially available derivatization reagent that produces an active hydrogen upon activation with base, i.e. a thiol, and served as the derivatization reagent [26]. Since SAMSA-FL possesses a fluorescein moiety, it appeared to be an ideal candidate for derivatization enabling FL detection.

Analytical scale HPLC analysis revealed that the sulfomycin sample was relatively pure; however, we have already shown with high-performance capillary electrophoresis (HPCE) and MALDI-TOF-MS analysis, evidence suggesting that sulfomycin may actually be comprised of several different, closely related species [23]. Considering this factor and the multiple

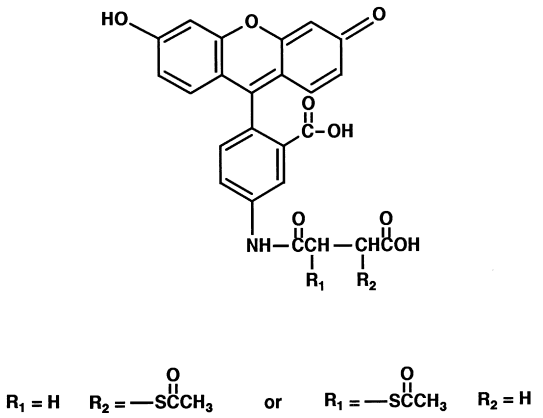

Fig. 2. Structure of SAMSA-FL.

reactive sites, it was certain that derivatization would have produced multiple derivatization products.

3.3. Optimization of the Michael addition reaction conditions

The molar excess of SAMSA-FL versus sulfomycin and the reaction temperature were influential experimental parameters on the rate of derivatization. The reaction temperature not only affected the time required to achieve reaction completion, but also the amount of a background product(s) formed that coeluted with the derivative species under the step gradient conditions. All attempts at chromatographically resolving this background signal from the derivative product were unsuccessful.

Holding all other parameters constant, it was determined chromatographically, vide infra, that a 200-fold molar ratio of SAMSA-FL to sulfomycin produced the best signal-to-background (S/B) ratio. The data (not shown) suggested that too little derivatization reagent failed to fully tag the majority of available reactive sites of sulfomycin, while too much reagent increased the amount of a background product, which coeluted with the derivative peak and decreased the S/B. Therefore, a 200-fold molar excess of SAMSA-FL to sulfomycin was adopted for all future derivatizations.

The temperature at which the derivatization was performed had a profound effect on the rate of both derivative and background product formation and, hence, on the overall S/B. Several experiments were conducted in which sulfomycin was derivatized with SAMSA-FL over a range of temperatures (ambient and 35, 50 and 80°C). The amount of derivative products formed over time was compared to the background signal generated under the same conditions with a blank reaction mixture (no sulfomycin present). At each of the temperatures examined, it was found that the amount of background products generated in the initial stages of the derivatization was minimal in comparison to the amount of derivative products formed; however, the rate at which the background products formed was faster than that of the derivative products. A point in all derivatizations was eventually reached at which the levels of both the derivative products and the background products remained unchanged for a finite amount of time, the duration of which was related inversely to the reaction temperature. After this point, the levels of both the derivative and background products decreased rapidly indicating that neither species were stable over time. Under the most favorable storage conditions (−10°C), the derivative products contained within the crude reaction mixture survived no longer than 36 h. The stability of the derivatives was reduced even further upon HPLC purification. This instability was detrimental to our efforts to characterize the derivative product species.

In general, the S/B for the derivatization of sulfomycin with SAMSA-FL was greater at lower temperatures. The disadvantage of performing the derivatizations at lower temperatures was longer reaction times, which was on the order of 16 h for derivatizations performed at ambient temperatures. Derivatizations were too rapid at the highest temperature studied as minor differences in the overall reaction times between identical samples adversely affected the reproducibility of the results. As matter of convenience and robustness, a reaction temperature of 50°C and a 2-h reaction time was selected for all future derivatizations.

3.4. Optimization of HPLC conditions

SAMSA-FL, which did not detectably fluoresce under the FL detection conditions employed, was activated under basic conditions, added as a 200-fold molar excess to a sample of sulfomycin and allowed to react at 50°C. The progress of the resulting reaction between sulfomycin and activated SAMSA-FL was monitored via HPLC. Both UV and FL detection were used in order to observe both the disappearance of the UV active sulfomycin and the formation of fluorescent products. The formation of multiple derivative species was observed via both FL and UV detection having retention times slightly longer than that exhibited by native sulfomycin under these HPLC conditions. Chromatographic analysis of the final derivative reaction mixture after 120 min, Fig. 3, revealed a broad, ill-defined peak suggestive that the final reaction product consisted of several different derivative species. An analogous chromatogram for the derivatization blank (no sulfomycin) showed no eluting species after 23 min. In all cases, no additional UV or FL active species

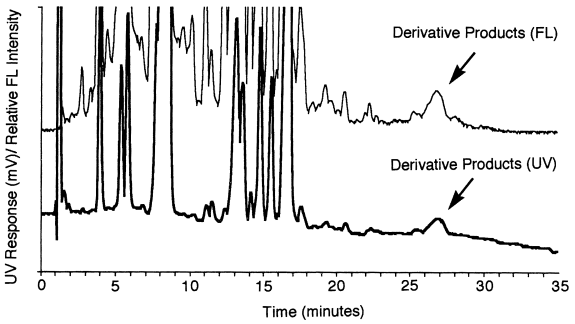

Fig. 3. HPLC monitoring of the reaction between sulfomycin and SAMSA-FL conducted at 50°C after 120 min. Linear gradient from ACN–water (22:78, v/v, 0.1% TFA) to a ACN–water (42:58, v/v, 0.1% TFA) over 30 min at 1.5 ml/min with 230 nm UV detection. The upper trace is the FL signal while the lower, bold trace is the UV signal. Note: detector sensitivities are not equal.

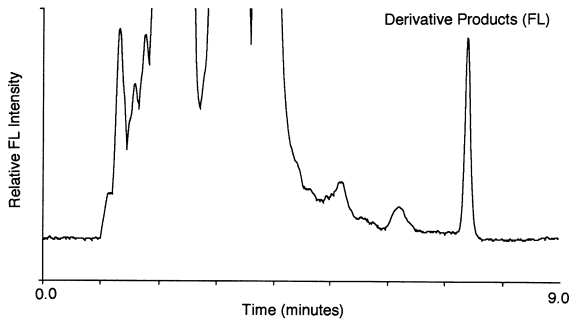

Fig. 4. Chromatographic manipulation to coelute the various derivatives into a quantifiable peak via step-gradient. Conditions were isocratic at ACN–water (39:61, v/v, 0.1% TFA) for the first 5 min of the analysis and then stepped to ACN–water (90:10, v/v, 0.1% TFA). The flow-rate was 1.5 ml/min. Only the FL signal is shown.

eluted when the column was flushed with ACN following a typical chromatographic analysis of the derivatization reaction mixture.

Attempts to chromatographically resolve the various species from one another for quantitative purposes using either linear or curved profile gradients failed. However, it was possible to elute all of the species derived from the SAMSA-FL reagent under isocratic conditions and then coelute all of the various derivative products into one peak with a step gradient, Fig. 4. The peak shape obtained with the step gradient approach was suitable for quantitative purposes. It is worthwhile to note that the efficiency of the column was extremely important in separating SAMSA-FL residue from the derivative product peak. Small losses in column efficiency affected the peak widths of the various components to such an extent that SAMSA-FL derived background products sometimes 'tailed' into the step gradient portion of the chromatographic method and eluted with the derivative products. This led to increases in the background signal and an increase in the detection limit of the method.

3.5. Limit of derivatization

Derivatizations were performed on sulfomycin samples of decreasing concentration in order to determine the effective limit of derivatization, i.e. the lowest concentration of sulfomycin for which a derivative product could be detected. For each of these derivatizations, the amount of SAMSA-FL used was identical in order to produce a consistent amount of background signal from sample-to-sample.

Under these derivatization conditions, the concentration range of sulfomycin that yielded a detectable derivative product upon reaction with SAMSA-FL was 25.0–500 μM (31.1 to 622 ppm). Apparently, the lower concentration level represented the limit of the derivatization, below which a signal greater than the background could not be detected for any derivatization products formed. It was unclear if the inability to observe any signal from the derivative products was due to rapid decomposition of any products formed, or if the formation of derivative products proceeded at a rate insufficient to produce an observable difference from the background at these low concentrations. The problems associated with interferences due to excess reagent and potential solutions to these problems have been discussed in the past [27]. Although not attempted as part of this study, approaches such as solid-phase extraction followed by derivatization may prove suitable for concentrating sulfomycin from dilute samples and allowing for the analysis of sulfomycin at lower concentration levels [28].

3.6. Method validation

A set of calibration standards (0, 50.0, 200, 350 and 500 μ*M* sulfomycin in DMF) as well as three single blind spiked samples were prepared. The samples were derivatized with SAMSA-FL and analyzed in triplicate ($n=3$) via HPLC. The coefficients of variation for the calibration standards ranged from ±1.7 to ±6.4% for the peak areas, in which the largest imprecision came from the standard at 0 μ*M*. A calibration curve of peak area (mV/s) versus concentration (μ*M*) was linear, $y=1.48x+93.8$, $R^2=1.00$. The standard deviation of the slope and intercept were ±0.01 and ±9.35, respectively. The results found for the single blind spiked samples analyzed in triplicate, 96±4.2%, 317±4.7%, and 404±1.0% μ*M*, were representative of the actual levels spiked, 100, 300, 400 μ*M*, indicating that there was reasonable agreement between the theoretical and determined values. Additionally, the step gradient protocol was reproducible as the coefficient of variation for the retention times of all the derivative products was ±0.26% ($n=24$).

3.7. Characterization of the derivative product

Mass spectrometry was utilized in an attempt to characterize the derivative products formed upon reaction between SAMSA-FL and sulfomycin. The mass spectra of sulfomycin was obtained with both MALDI-TOF-MS, Fig. 5, and ESI-MS, Fig. 6. Each set of data shows intact sulfomycin ($m/z=1245$) as well as sodium and potassium adducts, m/z of 1268 and 1284, respectively. Other signals that appear in the spectra provided evidence that other species in addition to sulfomycin were present in the sample prior to derivatization. Attempts to characterize the derivative products present in the crude reaction mixture and after HPLC purification under similar MS conditions failed to yield any signal above background noise for the scanned region m/z 100–10 000. The inability to visualize derivative products may have been due to the instability of the products, or to the formation of multiple products that had a wide range of m/z values, each with a total mass below the detection limit of the technique. It was unlikely, though not entirely impossible, that the

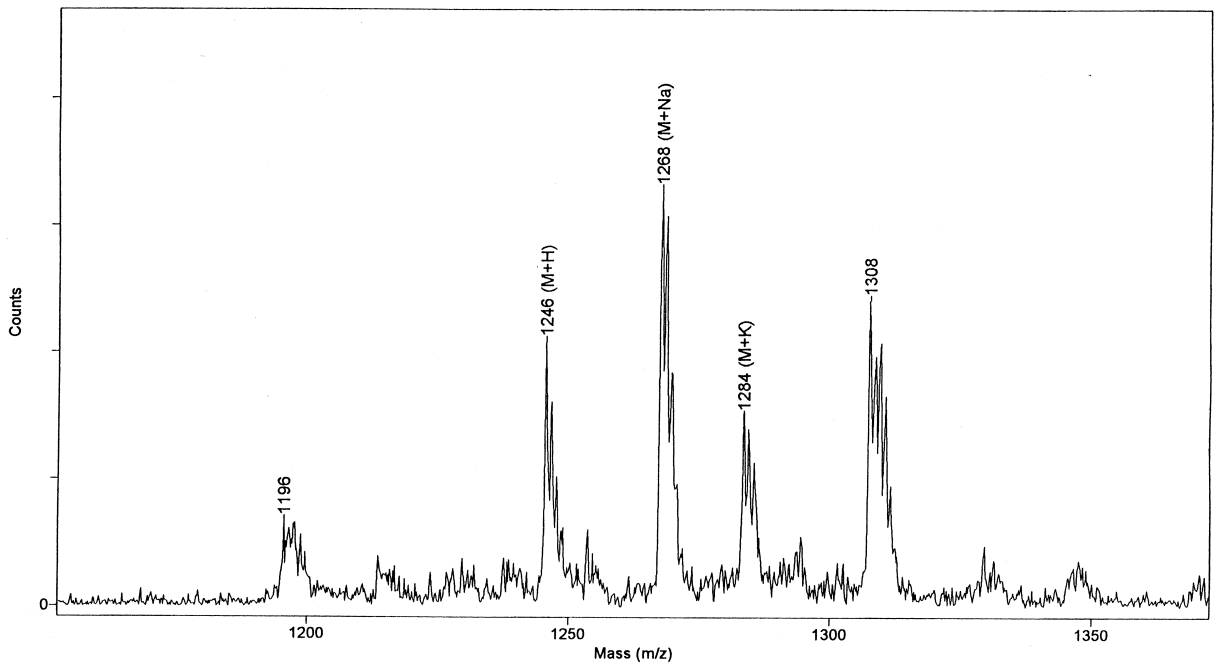

Fig. 5. MALDI-TOF-MS analysis of sulfomycin. Specific conditions listed in Section 2.

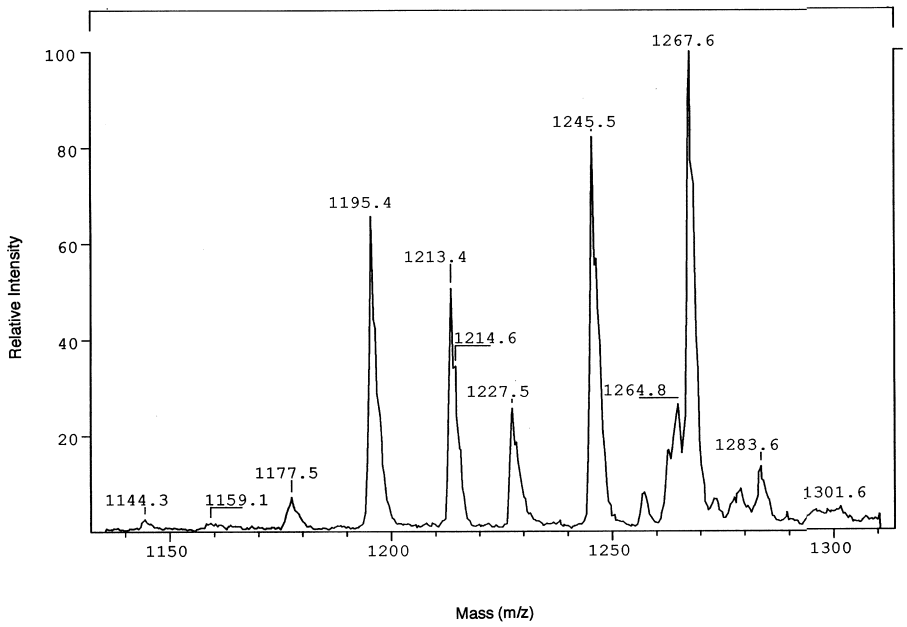

Fig. 6. ESI-MS analysis of sulfomycin. Specific conditions listed in Section 2.

derivative species just simply failed to ionize under the conditions employed, even though two different matrices were used in the MALDI-TOF-MS analysis.

4. Conclusions

This report demonstrates that a potential solution to the problem of forming multiple derivatization products is to chromatographically coelute the various species into one quantifiable peak. While such an approach does not overcome any inherent limitations of a derivatization reaction, it may be effectively used to compensate for instances where incomplete derivatization occurs or where an array of derivative products are formed. This approach should prove suitable with virtually any derivatization reagent, any substrate leading to multiple products, and with other separation approaches.

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