COLORIMETRIC DETECTION AND SPECTROPHOTOMETRIC DETERMINATION OF THE AMINOTHIAZOLYL-ALKOXYIMINO β-LACTAMS

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The 2-aminothiazol-4-yl-2-alkoxyiminoacetamido substituent-containing β-lactam antibiotics (cephalosporins and monobactams) develop a stable, concentration-dependent purple or cherry-red color after reaction with sodium nitrite in acidic condition. The color-formation is highly specific; it requires certain defined structural features such as the simultaneous presence of the intact aminothiazole-ring and an alkoxyimino substituent in the *syn* configuration. Other substituents on the β-lactam nucleus have effect only on the intensity of the color. This simple and fast colorimetric procedure was found to be useful not only for the detection of this class of β-lactam antibiotics but also for their quantitative spectrophotometric determination (λ_{max} 500 nm). A linear relationship exists between the intensity of the color plotted on a logarithmic scale and the concentration (12.5~200 μg/ml) of the compounds on an arithmetic scale. The β-lactams studied in this class with definitely positive purple-red color reaction are; cefotaxime, ceftizoxime, ceftazidime, ceftriaxone, cefmenoxime, cefodizime, ceftiolene, cefpirome, aztreonam, HR 109, FK 027 (cefixime), FR 19346, SK&F 88070, FR 13300, carumonam, YM 13115, BMY 28142, DN 9550, deacetylcefotaxime, deacetoxycefotaxime and deacetylcefotaxime lactone.

Most of the new semisynthetic cephalosporins and the monobactam, aztreonam, belong to the class of compounds containing the 2-aminothiazoleacetamido group. Those which contain a 2-alkoxyimino substituent at the *syn*-configuration are of considerable therapeutic interest. They have broad antibacterial spectra, high levels of activity especially against Gram-negative bacteria and excellent β-lactamase stability[1]. These antibiotics are currently assayed using biological methods. A simple colorimetric procedure was developed based on the structural similarity between these β-lactams and the sulfonamides, procaine (novocain) and other *p*-aminobenzoic acid derivatives. It was reasoned that the aromatic amino group of the aminothiazole ring of the β-lactams similar to that of the sulfanilamides and procaine should form a colored compound after diazotization with sodium nitrite followed by coupling with *N*-1-naphthylethylenediamine. However, when this reaction was carried out as described in the literature[2~7], there was apparently no coupling reaction, as evidenced by the lack of characteristic color. A concentration-dependent purple color did develop, however, when the sodium nitrite-treated solutions of the alkoxyiminoaminothiazolyl cephalosporins were acidified. This observation served as a basis for the development of the colorimetric detection and the spectrophotometric determination of these β-lactam antibiotics, as described herewith. Part of these data were previously reported[8].

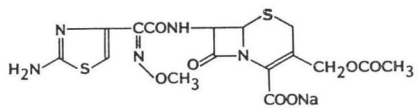
Materials and Methods

Antibiotics and Other Compounds

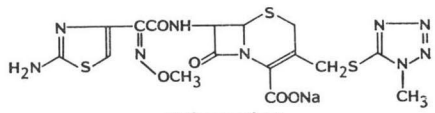
The aminothiazolyl β-lactam antibiotics and the aminothiazole-containing non-antibiotic control

compounds were kindly donated as dry powders from their respective manufacturers: Cefotaxime, cefodizime, cefpirome, HR 109, and the *anti*-isomer of cefotaxime from Hoechst A.G.; ceftizoxime, FK 027, FR 13300, FR 13773, FR 14060 (the *anti*-isomer of ceftizoxime) and FR 19346 from Fujisawa

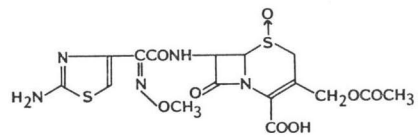
Fig. 1. Chemical formulae of 21 aminothiazolyl-*syn*-alkoxyimino β-lactams tested which gave stable bright purple color with sodium nitrite after acidification.



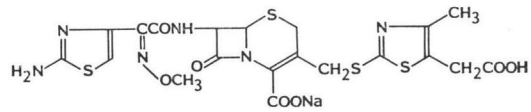
Cefotaxime



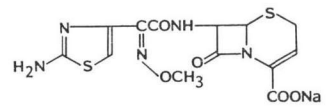
Cefmenoxime



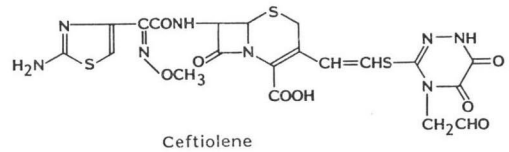
HR 109



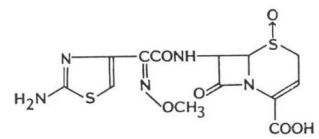
Cefodizime



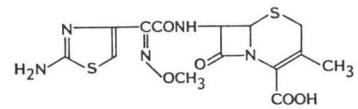
Ceftizoxime



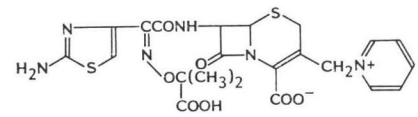
Ceftiolene



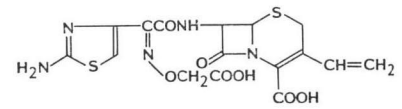
FR 19346



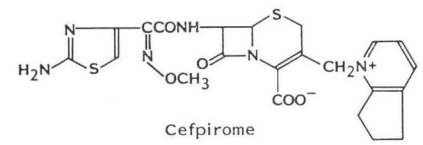
FR 13300



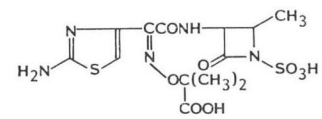
Ceftazidime



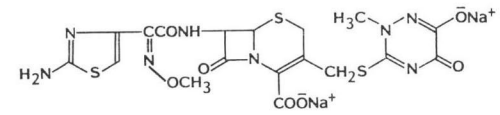
FK 027



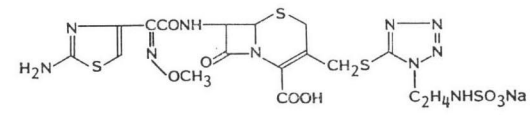
Cefpirome



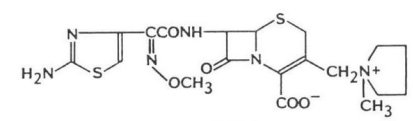
Aztreonam



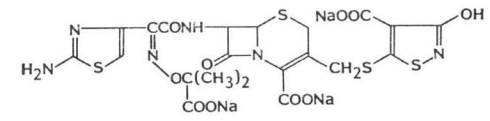
Ceftriaxone



SK&F 88070

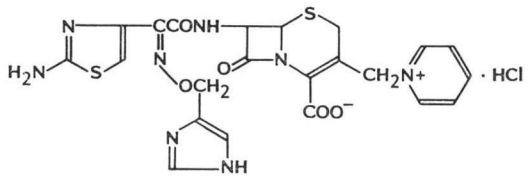


BMY 28142

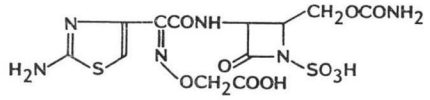


YM 13115

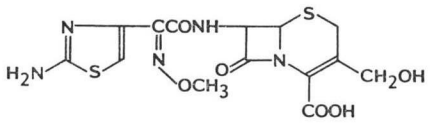
Fig. 1. (Continued)



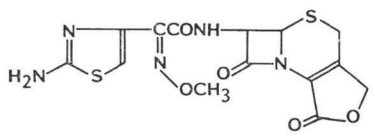
DN 9550



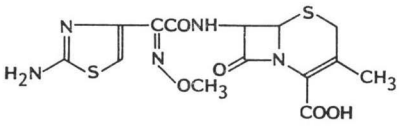
Carumonam
(AMA 1080, Ro 17-2301)



Deacetylcefotaxime



Deacetylcefotaxime lactone



Deacetoxycefotaxime

Pharmaceutical Co., Ltd.; cefmenoxime, cefotiam and carumonam from Takeda Chemical Industries Ltd.; aztreonam, SQ 80940 and SQ 80983 from Squibb Institute for Medical Research; ceftazidime from Glaxo Group Research; ceftriaxone from Hoffmann-La Roche; ceftiolene from Rhône-Poulenc; famotidine and YM 13115 from Yamanouchi Pharmaceutical Co., Ltd.; BMY 28142 from Bristol-Myers Co.; DN 9550 from Daiichi Seiyaku Co., Ltd.; deacetyl and deacetoxycefotaxime as well as deacetylcefotaxime lactone from Biochemie; CGP 31523A from Ciba-Geigy Limited; amiphenazole from Nicholas Gesellschaft; and BHT 920 from Carol Thomai. Other compounds mentioned in the text or for which chemical structures are given in the Figs., but which are not listed here, are either commercial preparations or synthesized by the medicinal chemistry staff of SK&F Laboratories.

Quantitative Measurement

The quantitative colorimetric determination of these β-lactams was carried out as follows; to 3 ml of the aqueous (deionized water) solutions containing 200, 100, 50, 25 or 12.5 μg/ml of these β-lactams were added 0.2 ml of 1% citric acid and also 0.2 ml of 0.5% sodium nitrite solutions at room temperature. No acidification was needed for aztreonam, since it contains a strongly acidic sulfonic acid group with a large electro-negative charge. The color started to develop within a few minutes and was fully developed after 1 hour, when the absorbance (optimally at 500 nm) was measured by using a spectrophotometer (Bausch and Lomb Spectronic 70). The changes in the absorption spectra against the control blank solution were measured and recorded. Compounds can be measured at a minimal level of approximately 10 μg/ml. In addition to citric acid, several other organic and diluted mineral acids were used, but 1% citric acid solution was found to be convenient and was therefore used in the studies reported herewith.

Qualitative Detection

All of the *syn*-alkoxyiminoaminothiazolyl cephalosporins (β-lactams) can be easily and rapidly detected by adding a few crystals of citric acid and sodium nitrite to their dilute solutions in test tubes. The purple color quickly develops. It can also be used qualitatively as a spot-test reaction.

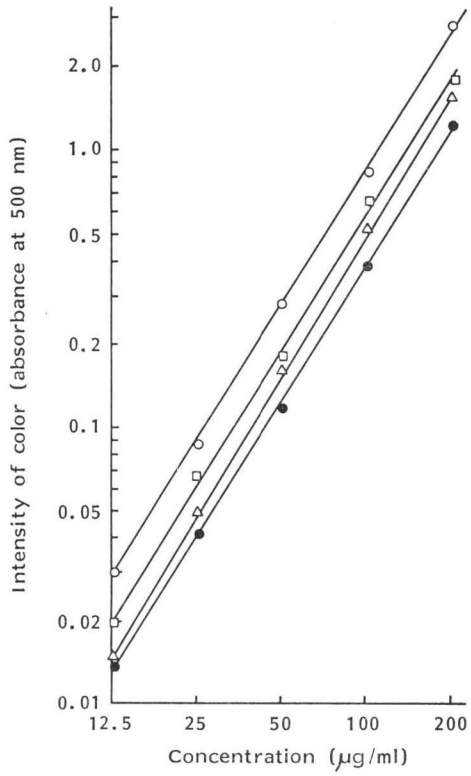
Results

Fig. 1 depicts the chemical structures of 19 cephalosporins and two monobactams tested which

Fig. 2. Spectrophotometric absorption curves of the colored reaction products of some 2-aminothiazolylalkoxyimino β-lactams.

Curves are linear at concentrations between 12.5 and 200 μg/ml of compounds.

○ Ceftizoxime, □ cefotaxime, ceftazidime, ceftriaxone, △ aztreonam, ● SK&F 88070.



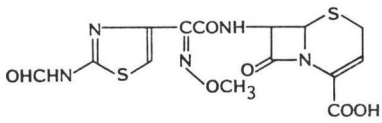
gave positive color reactions. They have the common feature of containing a 2-aminothiazolyl group attached to the acetylamino chain of the β-lactam ring at the 7-position of cephalosporins and the 3-position of the monobactams, aztreonam and carumonam. The other common characteristic of these β-lactams is that they contain an (alk)oxyimino group in the *syn*-configuration attached to the C-2 of the acetamino chain. This is a carboxypropoxyimino with ceftazidime, YM 13115 and aztreonam; a carboxymethoxyimino with FK 027 and carumonam; and a methoxyimino with the rest of the cephalosporins. All of these compounds produce bright purple color when their acidified solutions are treated with sodium nitrite. The intensity of the purple color is proportional to the concentration of these β-lactams. Acidification is necessary for the development of the purple color. Aztreonam, carumonam and SK&F 88070-acid develop color without acidification because of their sulfonic acid groups.

The color reaction can be used for the quantitative spectrophotometric determination of these aminothiazolyl-*syn*-alkoxyimino β-lactam antibiotics. The plot of the logarithm of the absorbance (intensity of purple color) *versus* concentration (12.5 ~ 200 μg/ml) for these compounds (arithmetic scale) is linear. So far, the absorption curves for ceftizoxime, cefotaxime, ceftriaxone, ceftazidime, aztreonam, cefmenoxime, FR 13300, FR 19346, HR 109 and SK&F 88070 have been determined. Fig. 2 demonstrates the linearity of the absorption curves and shows that approximately 10 μg/ml of compounds is the lowest concentration measurable.

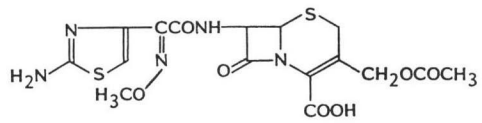
The substituents at the 3-position of the cephalosporins may influence, to a certain degree, the onset and the intensity of the purple color. With a bulky substituent, the onset is somewhat slower and the color may be less intense. The rate of the reaction and the intensity of the color was found to be maximal with ceftizoxime which contains only an H-atom at the 3-position. This was followed by cefotaxime which contains an acetoxy group at the 3-position, and by the other compounds in order of the bulkiness of their substituents at the 3-position. This is, however, not an absolute rule, since Fig. 2 shows that the spectrophotometric absorption curves of the colored reaction products for cefotaxime, ceftazidime and ceftriaxone are superimposable.

The development of the purple color of the reaction product obtained is highly structure specific. It requires the simultaneous presence in the molecule of the free amino group at the 2-position of the thiazole ring and the alkoxyimino (methoxyimino, carboxymethoxyimino, carboxypropoxyimino)

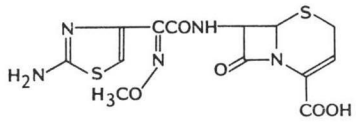
Fig. 3. Chemical formulae of 6 cephalosporins which do not have the structural requirements (aminothiazole ring with free amino group and alkoxyimino chain in *syn*-configuration) and do not develop the purple color with sodium nitrite in acidic environment.



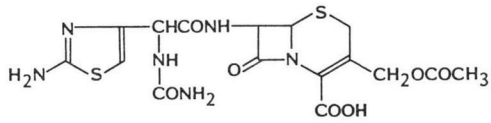
FR 13773



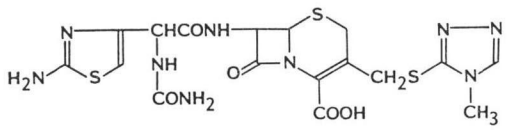
anti-Isomer of cefotaxime



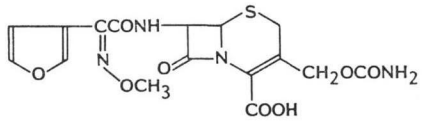
anti-Isomer of ceftizoxime (FR 14060)



SQ 80983



SQ 80940



Cefuroxime

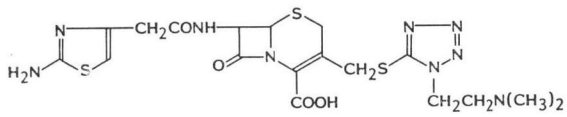
substituent, in *syn*-configuration at the C-2-atom of the acylamino chain of these β-lactam antibiotics. If either of these structural features is missing, no color will develop. Fig. 3 lists six β-lactam structures in which the structural requirements for the color reaction are not met; the amino group of the thiazole ring is blocked in FR 13773; the methoxyimino group is in the *anti*-configuration in the *anti*-isomers of ceftizoxime and cefotaxime; the 2-aminothiazole is intact but the substituent at the C-2-position is a ureido and not an alkoxyimino group in SQ 80983 and 80940; there is a *syn*-methoxyimino group but the ring to which the acylamino group is attached is a furan and not an aminothiazole ring in cefuroxime. Such extreme specificity is striking. As expected, compounds with only remotely similar structural features were found to yield a negative colorimetric result. These compounds included niridazole, procaine, *p*-aminobenzoic acid, *p*-aminosalicylic acid, metronidazole, several sulfonamides and various other cephalosporins. It should, however, be mentioned that the 2-aminothiazole group itself can be considered as a chromogenic group. Fig. 4 demonstrates eight compounds (three cephalosporins and five other types of molecules) which contain a free or substituted (famotidine) aminothiazole ring unit without a *syn*-alkoxyimino chain. Compounds with the free amino group produce a yellowish-pinkish color with sodium nitrite after acidification. In addition, the yellowish product precipitates from the dilute solution at room temperature. Famotidine, in contrast, produces a light violet color which fades gradually.

Discussion

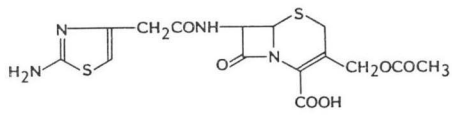
Guided by the structural similarities among the *p*-aminobenzene sulfonamides, procaine (novocain) and the aminothiazole-containing β-lactam antibiotics, we undertook to develop a colorimetric assay which could be used reliably and rapidly for quantitative as well as qualitative determination of these β-lactam antibiotics, in place of the time-requiring microbiological techniques. The BRATTON-MARSHALL[2] method failed to yield any result. Instead, we have found a new colorimetric method

Fig. 4. Chemical formulae of 8 aminothiazole compounds without an alkoxyimino substituent.

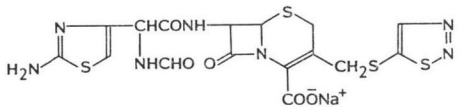
Seven develop a yellowish color with sodium nitrite in acidic solution followed by precipitation of the yellowish product. Famotidine with a substituted 2-amino group on the thiazole ring develops a transient violet color.



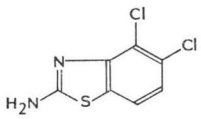
Cefotiam



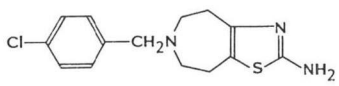
SK&F 86138



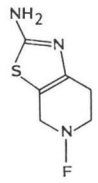
CGP 31523A



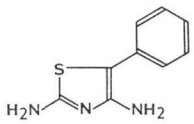
SK&F 60528
(2-Amino-4,5-dichlorobenzothiazole)



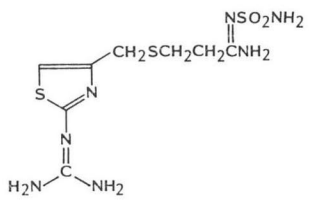
BHT 958



BHT 920



Amiphenazole



Famotidine (YM 11170)

which can be used not only for the qualitative detection but also for the quick quantitative determination of the 2-aminothiazolyl-2-alkoxyimino β-lactam antibiotics. This technique originated from an unexpected observation when the known procedure was applied to the diazotization with sodium nitrite of the primary aromatic amino group of the aminothiazole ring, and its subsequent coupling with *N*-1-naphthylethylenediamine dihydrochloride, as applied to the colorimetry of sulfonamides and procaine[5,6]. The negative result prompted us to investigate further. During these experiments it was observed that the solutions of the 2-aminothiazolylalkoxyiminoacetamido cephalosporins and monobactams developed a cherry red color with sodium nitrite upon acidification. This color development suggests the presence of an internal coupling reaction between the starting β-lactam and its diazo product, *i.e.*, formation of "self-associating dimers". This is probably a facile reaction as indicated by the intensity of the color of the reaction product which develops within 1 or 2 minutes. Regardless of the mechanism of the reaction or the nature of the reaction product, the colorimetric assay reported herein is simple, highly specific, sensitive, reproducible, quick, accurate and universal for the analytical determination and quality control of the oxyimino-*syn*-2-aminothiazole β-lactam antibiotics.

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