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Impurity profiling of clarithromycin using high-performance liquid chromatography with ultraviolet detection

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SUMMARY

Clarithromycin is a semi-synthetic antibiotic. High-performance liquid chromatography is used to identify and estimate both manufacturing and degradation impurities. The sample is chromatographed on a $YMCC_{18}$ column using an eluent of acetonitrile–0.033 *M* KH_2PO_4 (48:52) at an apparent pH of 5.4 and ultraviolet detection at 205 nm. Due to the limited availability of impurities, all related substances are referenced to a single impurity standard. A computing integrator is programmed to compensate for differences in detector responses and to identify and calculate the % (w/w) of all known impurities. The majority of all currently identified impurities are detectable at the 0.10% (w/w) level.

INTRODUCTION

During the synthesis of today's complex pharmaceutical drugs, many potential impurities can be produced. Some sources of these impurities are solvents, reagents, starting materials, inorganics, catalysts, reaction byproducts, reaction intermediates and degradation products. The latter three sources generally produce compounds which are structurally related to the drug substance and are commonly referred to as related substances. The identification and quantification of these related substances have become more important with the concern for the overall chemical purity of the drug substance[1–5]. Due to the similarity of these related substances, high-performance liquid chromatography (HPLC) is typically the method of choice.

Clarithromycin is a semi-synthetic antibiotic which was discovered by Taisho Co., Japan. A HPLC method has been developed for clarithromycin to monitor the levels of related substances. Preliminary identification is made based on retention time. Bulk drug purity has been estimated by many different methods[6–8]. The best approach is a direct identification and quantitation *versus* a reference standard of each impurity. However, for complex molecules like clarithromycin, it is not practical to produce and characterize reference standard quantities of all possible related substances. Therefore,

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most of the existing methods are based on the assumption that the detector response is identical for all compounds of interest. In some cases, the differences in detector response might be within the scientific error; however, using ultraviolet detection at 205 nm for compounds such as clarithromycin, this assumption can lead to errors of an order of magnitude. Thus, our approach is based on an indirect quantitation which compensates for differences in the molar extinction coefficient for each compound. This method should eliminate errors due to the variable detector response.

EXPERIMENTAL

Apparatus

Method development was performed on two different systems. The first system consisted of a ConstaMetric IIIG pump (LDC/Milton Roy, Riviera Beach, FL, U.S.A.), a Shimadzu SIL-6A autosampler, a CTO-6A column oven, and a CR-3A integrator (Shimadzu, Kyoto, Japan). The second system consisted of a SP-8700 solvent delivery system, a SP-4270 computing integrator (Spectra-Physics, San Jose, CA, U.S.A.), a CTO-6A column oven (Shimadzu, Kyoto, Japan) and a IBM LC/9505 autosampler (IBM, Danbury, CT, U.S.A.). Kratos Model 783 variable-wavelength detectors (ABI, Ramsey, NJ, U.S.A.) were used on both systems. The HPLC column used was a YMC A-303 5-μm 120-Å C_{18} column (YMC, Japan) 250 × 4.6 mm I.D.

Reagents and solvents

HPLC-grade acetonitrile and methanol were either purchased from Mallinckrodt (Paris, KY, U.S.A.) or J. T. Baker (Phillipsburgh, NJ, U.S.A.). The potassium phosphate, monobasic, reagent grade was also supplied by Mallinckrodt. The 6-O-methylerythromycin A and 6,11-di-O-methylerythromycin A reference standards were synthesized by Abbott Labs. (N. Chicago, IL, U.S.A.). All other related substances were either synthesized and/or isolated by either Abbott Labs. or Taisho (Tokyo, Japan).

Optimum chromatographic conditions

The mobile phase consisted of acetonitrile–0.033 *M* KH_2PO_4 (48:52, v/v). The apparent pH of the mobile phase was maintained between 5.3 and 5.5. A flow-rate of 1.0 ml/min was used at a column temperature of 50°C. A constant sample injection volume of 50 μl was maintained. The detector was operated at 205 nm with a rise time of 2.0 s and a range of 0.03 a.u./10 mV. The chromatograms were graphed at 1.0 cm/min with an attenuation of 4 mV full scale.

Standard and sample preparation

About 105 mg of clarithromycin reference standard was added to a 50-ml volumetric flask containing 25.0 ml of acetonitrile. After dissolution the solution was diluted with water to volume. This was the stock clarithromycin solution. A bulk 6,11-di-O-methylerythromycin A solution was prepared from 45 mg of 6,11-di-O--methylerythromycin A diluted with methanol to 100.0 ml. A 10-ml aliquot of this solution was further diluted with acetonitrile–water (1:3) to 100.0 ml to give a stock 6,11-di-O-methylerythromycin A solution. The working standard solution was prepared from 5.0 ml of the stock clarithromycin solution and 10.0 ml of the stock

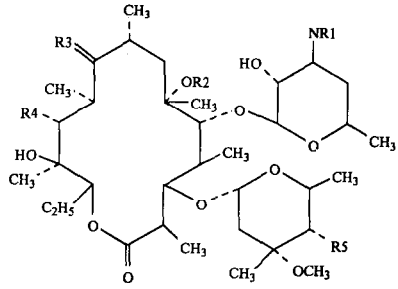
6,11-di-O-methylerythromycin A solution diluted with acetonitrile–water (1:3) to 50.0 ml.

The sample solution was prepared from 105 mg of clarithromycin dissolved in 25.0 ml of acetonitrile and diluted with water to 50.0 ml. A 10-ml aliquot of this solution is further diluted with acetonitrile–water (1:3) to 50.0 ml.

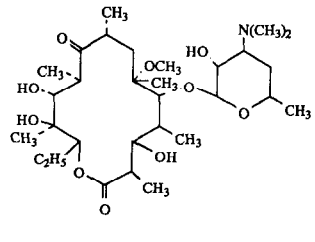
RESULTS AND DISCUSSION

Clarithromycin is a semi-synthetic antibiotic which is a derivative of erythromycin A. The structures of clarithromycin and its related substances are given in Fig. 1. From the similarities in the structures it was obvious that column selectivity was an extremely important parameter in the column selection. Using a test mixture of clarithromycin and seven related substances, several different columns were investigated: (1) Nucleosil, C_{18} 5 μm, 150 × 4.6 mm I.D. and 250 × 4.6 mm I.D. (2) Zorbax Golden Series, C_8 3 μm, 80 × 6.2 mm I.D. (3) IBM, C_8 3 μm, 100 × 4.5 mm I.D. (4) YMC, C_{18} 5 μm, 150 × 4.6 mm I.D. and 250 × 4.6 mm I.D.

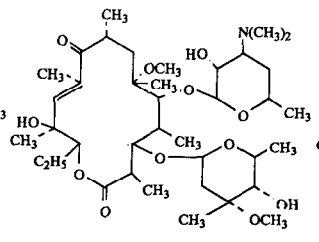
Of these columns both columns 2 and 3 did not have high enough efficiencies to provide the needed separation. The low efficiencies are due to tailing. Column 1 in the 250 × 4.6 mm I.D. configuration did provide a usable separation; however, the column life-time was unacceptable. Also comparing columns 1 and 4 for peak symmetry, the YMC offered the best performance. The 250 mm length was selected for a sight additional efficiency over the 150 mm. Since selection of the YMC column, several additional columns have been tested for equality. These columns were all 250 × 4.6 mm I.D. 5 μm with the following stationary phases: Phenomenex Carbosphere, Whatman ODS, TSK Gel ODS, and Regis ODS II. Of theses columns only the



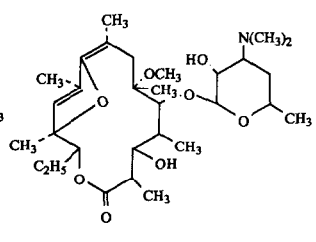
NAME	R1	R2	R3	R4	R5
Clarithromycin	$(CH_3)_2$	CH_3	O	OH	OH
EM=NOH	$(CH_3)_2$	H	NOH	OH	OH
=NOH	$(CH_3)_2$	CH_3	NOH	OH	OH
=NOH,NH	$H(CH_3)$	CH_3	NOH	OH	OH
-NH	$H(CH_3)$	CH_3	O	OH	OH
6,11-Di	$(CH_3)_2$	CH_3	O	CH_3	OH
6,4"-Di	$(CH_3)_2$	CH_3	O	OH	CH_3
=NOMe	$(CH_3)_2$	CH_3	$NOCH_3$	OH	OH
N-Formyl	$(COH)(CH_3)$	CH_3	O	OH	OH



DECLAD



10,11-ANHYDRO



9,12-HEMIKETAL

Fig. 1. Structure of clarithromycin and related compounds. Definitions of abbreviations are found in Table

Phenomenex Carbosphere gave promising results; however, it was not totally equivalent.

During the column screening process, the effects of mobile phase variations were investigated. The effect of mobile phase pH was studied and found to be minimal; thus, the apparent pH is monitored only to verify the proper value. Also, the column temperature does not greatly affect the retention, but is necessary to maintain good peak symmetry and resolution. The organic–aqueous ratio however, has a dramatic effect on the separation. Fig. 2 illustrates this critical relationship. The test compounds in Fig. 2 include those related substances which most closely elute with clarithromycin. At the lower organic concentrations, the separation between most compounds increases; however, 6-O-methylerythromycin A-N-oxide and 6,11-di-O-methylerythromycin A coelute. These two compounds are separated at the higher organic concentration at the sacrifice of resolution of early eluting compounds. To compromise between detection limits and resolution, a mobile phase consisting of 0.033 *M* KH_2PO_4–acetonitrile (52:48) was selected. Typical chromatograms of the reference standard clarithromycin and the reference standard spiked with various different related substances at both the 0.2 and 1.0% (w/w) levels are presented in Figs. 3 and 4.

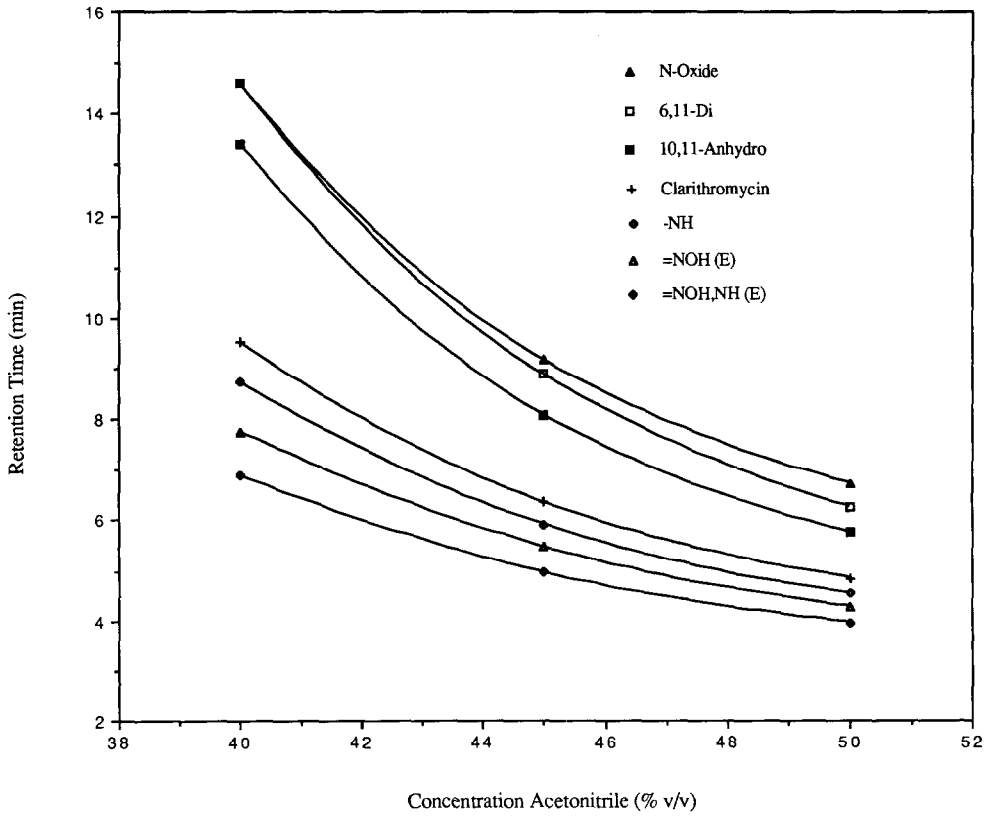


Fig. 2. Effect of acetonitrile concentration on retention time. 0.067 *M* KH_2PO_4 buffer was used to maintain a constant apparent pH of 5.4. Definitions of abbreviations are found in Table I.

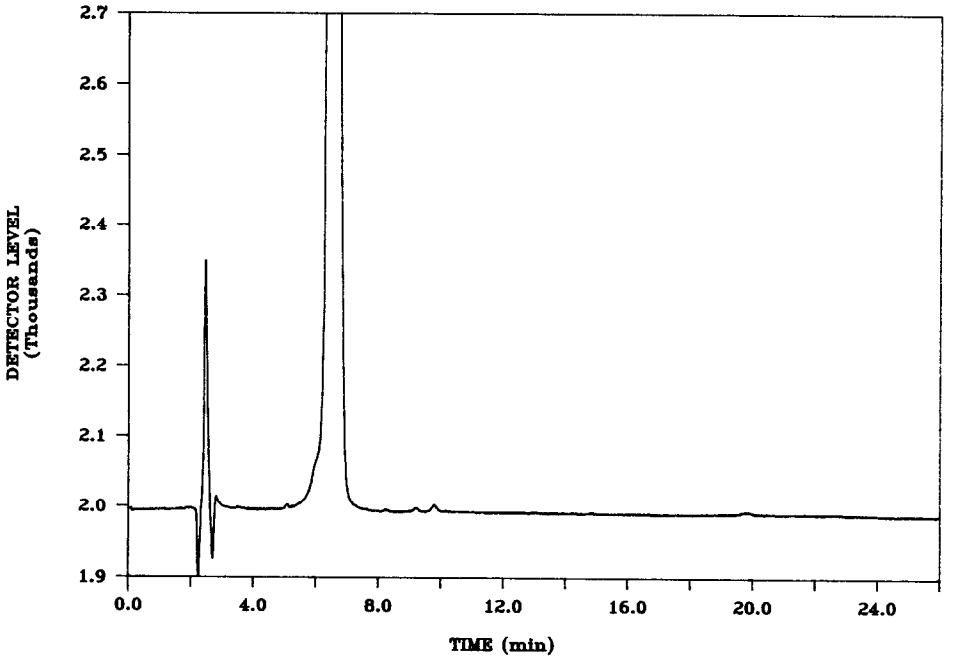


Fig. 3. Typical chromatogram of clarithromycin reference standard.

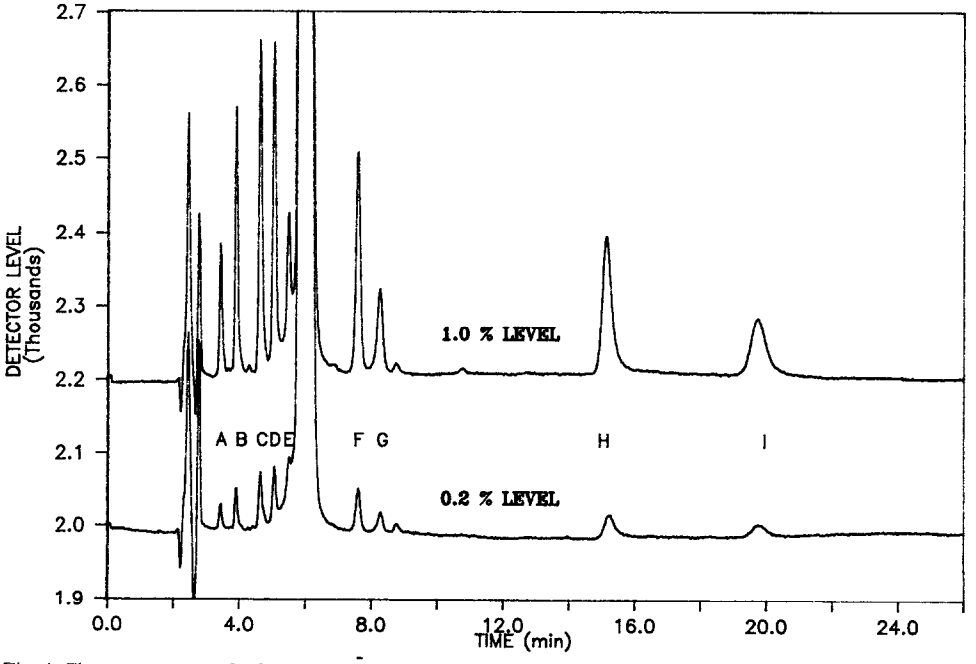


Fig. 4. Chromatograms of reference standard spiked with related substances at the 1.0 and 0.2% (w/w) level. A = Decladinosyl-6-O-Me-ery A; B = Ery A-9-oxime (E); C = N-De-Me-6-O-Me-ery A-9-oxime (E); D = 6-O-Me-ery A-9-oxime (E); E = N-De-Me-6-O-Me-ery A; F = 10,11-anhydro-6-O-Me-ery A; G = 6,11-Di-O-Me-ery A; H = 6-O-Me-ery A-9-Me-oxime (E); I = N-De-Me-N-formyl-6-O-Me-ery A.

Fig. 4 also illustrates the need to correct for the various detector responses. Even though all of the impurities are at the same concentration, the peak area of each impurity can differ dramatically. To correct for the different molar extinction characteristics, a normalization factor is determined for each impurity. The reference compound selected was 6,11-di-O-methylerythromycin A. This compound was selected for various reasons: it is the most likely produced impurity, it has very similar absorption properties to clarithromycin and it can easily be synthesized in good purity. The procedure for determination of the normalization factor is quite simple. First, the structure and purity of each compound was determined by using techniques such as HPLC, thin-layer chromatography, ^{1}H NMR, ^{13}C NMR and mass spectrometry. Then, binary mixtures of the impurity and the reference compound were made at six different concentrations ranging from approximately 0.6 to 20 μg/ml. The peak response calibration curve for each compound was obtained and the slope of the calibration curve was defined as the response factor of that compound. If the intercept of the calibration curve is essentially the origin, then the normalization factor can be obtained from the ratio of the response factor of the related substance to the response factor of 6,11-di-O-methylerythromycin A. This relationship is only true if the intercept of the calibration curve is zero. If the intercept is positive, then at low concentrations the concentration will be overestimated and at high concentrations, a low bias will result. If the intercept is negative then the reverse systematic error exists. The inability to accurately integrate the area of N-demethyl-6-O-methylerythromycin A, which is located on the front slope of the major peak, causes a positive intercept and thus peak heights were used to obtain a zero intercept that guarantees the accuracy of the quantitation. Table I summarizes the normalization factors and relative capacity factors of the most common impurities. The preliminary peak identifications are made

TABLE I

RELATIVE CAPACITY FACTOR AND NORMALIZATION FACTORS FOR CLARITHROMYCIN RELATED SUBSTANCES

Compound[a]	*Abbreviation*	*Rel. capacity factor*	*Normalization factor*
Decladinosyl-6-O-Me-ery A	DECLAD	0.21	1.12
Ery A-9-oxime (E)	EM=NOH (E)	0.30	1.39
6-O-Me-ery A 9-oxime (Z)	=NOH (Z)	0.37	2.00
N-De-Me-6-O-Me-ery A-9-oxime (E)	=NOH,NH (E)	0.44	2.48
6-O-Me-ery A-9-oxime (E)	=NOH (E)	0.50	2.41
N-De-Me-6-O-Me-ery A	-NH	0.56	1.72
10,11-Anhydro-6-O-Me-ery A	10,11-ANHYDRO	0.89	2.62
6,11-Di-O-Me-ery A	6,11-Di	1.00	1.00
Di-O-Me-ery A	Di-O-Me	1.10	1.00
6-O-Me-ery A-N-oxide	N-oxide	1.10	0.95
6,4″-Di-O-Me-ery A	6,4″-Di	1.15	0.88
6-O-Me-ery A-N-oxime (Z)	=NOMe (Z)	1.28	2.34
3-Decladinosyl-8,9;10,11-dianhydro--9,12-hemiketal	9,12-HEMIKETAL	1.70	4.50
6-O-Me-ery A-9-Me-oxime (E)	=NOMe (E)	2.02	2.74
N-De-Me-N-formyl-6-O-Me-ery A	N-formyl	2.15	5.47

[a] De-Me = Demethyl; Me = methyl; ery = erythromycin.

TABLE II

DIRECT *VERSUS* INDIRECT QUANTITATION FOR IDENTIFIED IMPURITIES IN A REPRESENTATIVE LOT

Compound	*Direct (%, w/w)*	*Indirect (%, w/w)*
=NOH,NH (E)	≤0.25	≤0.25
=NOH (E)	0.25	0.23
-NH	0.24	0.29
10,11-ANHYDRO	≤0.25	≤0.25
N-oxide	0.48	0.40

based on the relative capacity factor of each peak. The calculation of both the relative capacity factor and % (w/w) for each peak is performed by the integrator using in-house developed software which is based on the following equation:

$$\% \text{ (w/w)} = \frac{\text{(Response impurity) (concentration of 6,11-Di) (dilution factor)}}{\text{(Response 6,11-Di) (sample weight) (normalization factor)}}$$

All of the related substances were found to exhibit linearity from at least 1 to 10 μg/ml and had zero intercepts. The detection limit was obtained for 6,11-di-O-methyl-erythromycin A from a calibration curve of areas. The linear analysis of a range of 0.14 to 34 μg/ml gave an intercept of −967 counts, a slope of 3216 counts ml/μg, with a correlation coefficient of 0.9996. The detection limit for a peak with a signal-to-noise ratio of 3 was found to be 0.14 μg/ml which was approximately 0.04% (w/w). Due to the limited availability of all related substances and since almost all compounds have a normalization factor of greater than one, a minimal reportable value of 0.10% (w/w) was defined.

To prove that identification and quantitation *versus* a reference compound gives

TABLE III

REPRODUCIBILITY OF THE METHOD

A representative lot was assayed ten times (1–10), values are % (w/w).

Impurity	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Mean*	*S.D.*
DECLAD	0.04	0.01		0.04	0.04	0.02	0.05	0.04	0.04	0.02	0.04	±0.013
EM=NOH (E)	0.02	0.01	0.01	0.02	0.01			0.02	0.02		0.02	±0.005
=NOH (Z)	0.02	0.01		0.02	0.03	0.03	0.03	0.03	0.02	0.03	0.02	±0.007
=NOH, NH (E)			0.02								0.02	±0.000
=NOH (E)	0.12	0.17	0.08	0.12	0.12	0.12	0.10	0.27	0.12	0.12	0.13	±0.053
-NH	0.16	0.17	0.16	0.13	0.14	0.17	0.06	0.13	0.12	0.19	0.14	±0.037
6,11-Di	0.17	0.20	0.15	0.18	0.16	0.22	0.14	0.18	0.19	0.23	0.18	±0.029
Di-O-Me	0.21	0.23	0.19	0.20	0.33	0.22	0.20	0.21	0.30	0.24	0.23	±0.046
6,4″-Di	0.08	0.10	0.08	0.08	0.07	0.08	0.06	0.09	0.08	0.10	0.08	±0.012
=NOMe (Z)	0.04	0.05		0.03	0.03	0.04	0.03	0.04	0.04	0.04	0.04	±0.007
=NOMe (E)	0.37	0.39	0.29	0.32	0.37	0.38	0.34	0.32	0.36	0.36	0.35	±0.032
N-formyl	0.04			0.03	0.04	0.05	0.02	0.02	0.04	0.06	0.04	±0.014

compatible results with a direct quantitation using individual standards, a typical production intermediate sample was assayed against individual standards as well as indirectly *versus* 6,11-di-O-methylerythromycin A. For five selected impurities, individual calibration curves were obained and the representative lot was quantitated directly *versus* the individual standards curves. This direct value was compared with the concentrations obtained using the normalization factors and a single 6,11-di-O--methylerythromycin A reference standard. The results (Table II) indicate an excellent agreement between the two techniques.

Table III demonstrates the reproducibility of the method. A representative lot was assayed a total of ten times by two different analysts using entirely different HPLC systems. To better illustrate the reproducibility of the methodology, all values below 0.10% (w/w) were estimated to the nearest 0.01% and standard deviations (S.D.) to $\pm$0.001%. Considering the low levels of all of the related substances, the agreement between various analysts and chromatographic systems is very good.

CONCLUSIONS

Clarithromycin is a complex antibiotic and has several potential impurities. The related substances are structurally very similar to the parent compound and the separation is quite difficult. By compensating for differences in absorbance properties of different compounds, this method allows for an accurate, simple procedure using a single standard to identify and quantitate all related substances. Thus, this method provides identification and quantitation of most of the related substances at the 0.10% (w/w) level. When used with other techniques for the determination of moisture, residual solvents and inorganic material, a complete impurity profile of the bulk drug is possible. This type of normalization procedure can be adapted for any set of compounds and thus eliminates errors associated with techniques such as peak area percent.

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