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Study of stability of methotrexate in acidic solution Spectrofluorimetric determination of methotrexate in pharmaceutical preparations through acid-catalyzed degradation reaction

Suzy M. Sabry *, M. Abdel-Hady, M. Elsayed, Osama T. Fahmy, Hadir M. Maher

Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt

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Abstract

Study of the degradation reaction of methotrexate (MTX) in acidic solution was carried out. Optimization of the experimental parameters of MTX acid hydrolysis was investigated. Spectrofluorimetric method for determination of MTX through measurement of its acid-degradation product, 4-amino-4-deoxy-10-methylpteroic acid (AMP), was developed. Stability of the standard solution of MTX prepared in sulfuric acid was discussed in the view of accelerated stability analysis. Two other comparative spectroflourimetric methods based on measuring the fluorescence intensities from either a condensation reaction with acetylacetone–formaldehyde (Hantzsch reaction) or a reaction with fluorescamine were also described. Beer's law validation, accuracy, precision, limits of detection, limits of quantification, and other aspects of analytical merit are presented in the text. The proposed methods were successfully applied for the analysis of MTX in pure drug and tablets dosage form. The sensitivity of the developed methods was favorable, so it was possible to be adopted for determination of MTX in plasma samples for routine use in high-dose MTX therapy.

Keywords: Methotrexate; Spectrofluorimetric determination; Acidic solution

1. Introduction

Methotrexate (4-Amino-10-methylfolic acid or 4-Amino-4-deoxy-10-methylpteroyl-L-glutamic acid, MTX), is an antineoplastic which acts as an antimetabolite of folic acid. It is employed in maintenance programs and in the prophylaxis and treatment of meningeal leukaemia. Methotrexate is of value in the treatment of psoriasis. It is also used in rheumatoid arthritis and in bone marrow transplantation [1].

* Corresponding author. Tel.: +2-3-483-3810; fax: +2-3-487-3273.
E-mail address: suzymsabry@hotmail.com (S.M. Sabry).

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The USP XXIV [2] and BP 1998 [3] specified HPLC methods for the assay of MTX in bulk drug and pharmaceutical dosage forms.

A survey of the literature revealed that different analytical techniques for the assay of MTX have been reported. HPLC with fluorimetric [4–13] and UV [14–19] detection methods have been reported. Using the former methods, the derivatization reactions include; photo-oxidative irradiation at 254 nm in the presence of hydrogen peroxide to yield 2,4-diaminopteridine-6-carboxylic acid [6,11–13], oxidation with permanganate in presence of acetate buffer (pH 4) to give 2-amino-4-hydroxypteridine-6-carboxylic acid [10], and oxidative cleavage using either phosphate buffer containing 0.2 of 30% hydrogen peroxide [4,8,9] or pre-column of cerium (IV) trihydroxyhydroperoxide [5,7] to get 2,4-diaminopteridine-6-carboxylic acid. The latter reaction has been applied in flow injection technique [20].

Early, spectrofluorimetric methods based on oxidation of MTX to pteridine carboxylic acid, using permanganate, have been described [21,22]. The latter [22] has been applied to study plasma levels in cancer patients with a limit of determination of 100 ng ml^{-1}. Spectrophotometric methods including color reactions [23,24] and UV measurements [25] have been described. Polarographic and voltammetric methods [26–29] for the quantitation of MTX in pharmaceuticals and plasma samples have been published.

The main subject of this article is the spectrofluorimetric determination of MTX through acid-catalyzed degradation reaction. The latter is based on the hydrolysis across the amide linkage. The previously described fluorimetric derivatization [4–13,21,22] has concerned with the oxidative cleavage at the methylamino bridge. The present work was channeled through three approaches. First, to study the fluorescence and UV–Vis spectral characteristics of MTX and its acid-degradation product, AMP. Second, to investigate the kinetics of the degradation reaction of MTX in solutions of different acid-strengths. Third, to optimize the experimental conditions to establish a simple and sensitive spectroflourimetric method for the analysis of MTX based on measuring the fluorescence emission of its degradation product, AMP. As a continuation of this study, the stability of MTX in sulfuric acid solution (standard working solution) was discussed in the view of accelerated stability analysis, based on UV-derivative spectrophotometric measurement. The latter was evaluated as a stability-indicating assay. Hitherto, there has been no spectrofluorimetric or spectrophotometric study in the literature to evaluate the stability of MTX in acidic solution or to follow the kinetic of its hydrolytic degradation reaction.

Other comparative spectrofluorimetric methods have also been of interest. These include derivatization reactions (Hanztsch reaction and fluorescamine reaction) concerned with the primary amino group of the intact drug molecule.

Hantzsch reaction has many applications in the field of pharmaceutical analysis of some amine drugs. Of the latter, fluorimetric and/or spectrophotometric assays of beta-lactam antibiotics [30], gentamycin [31], mexiletine and heptaminol [32], lisinopril [33] and amikacin, kanamycin, neomycin and tobramycin [34] have been reported. Determination of aldehydes using Hantzsch reaction has also been of some interest [35–37].

Fluorescamine (fluram) is a fluorogenic agent which has been extensively used in the field of pharmaceutical analysis. It reacts instantaneously with primary amines in aqueous solutions to give highly fluorescent pyrrolinone derivatives [38]. Spectrofluorimetric assays of, amino acids [39,40], peptides and proteins [41], catecholamines [42] and folic acid [43] using fluorescamine derivatization reaction, have been reported. Also, fluorescamine has been used as derivatizing reagent in HPLC, for instances; analysis of sulfonamides in foods [44] and aniline derivatives in environmental waters [45], and in HPTLC [46].

In the above described reactions; degradation-, Hantzsch- and fluorescamine-reaction, the spectrophotometric determination of the respective reaction product was proposed as an alternative analytical technique.

The applicability of the developed methods was evaluated through the determination of MTX in pure form, tablets dosage form and after a spiking the plasma samples.

2. Experimental

2.1. Apparatus

Fluorescence spectra and measurements were taken on a Perkin–Elmer 650-10S spectrofluorimeter, equipped with 1-cm quartz cell, a 150 W Xenon arc lamp, excitation and emission grating monochromators and a Perkin–Elmer recorder model 56. Slit-widths for both monochromators were set at 10 nm.

For spectrophotometric measurements, a Perkin–Elmer Lambda EZ 201 UV–Vis spectrophotometer with 1-cm cuvettes was used.

2.2. Reagents and standard/assay solutions

2.2.1. Preparation of stock and standard methotrexate solutions

Stock solution of MTX containing 200.0 μg ml^{-1} in 0.1 M sulfuric acid was prepared and kept in refrigerator. This stock solution was diluted further with 0.1 M sulfuric acid to obtain working standard solution. The stock solution should be freshly prepared daily.

2.2.2. Preparation of methotrexate tablets assay solutions

A total of 20 tablets were weighed and powdered. A portion of the powder, equivalent to 10 mg of MTX, was weighed accurately and about 20 ml of 0.1 M sulfuric acid was added. Methotrexate was dissolved using ultrasonic bath and the mixture was filtered into a 50-ml volumetric flask. The residue was washed with two 10-ml portions of 0.1 M sulfuric acid and washings were added to the filtrate and diluted to volume. Further dilutions were made to appropriate concentrations (similar to standard solutions).

2.2.3. Preparation of acetylacetone–formaldehyde solution

Walpole's acetate buffer solution containing 0.2 M each of acetic acid and sodium acetate in water was prepared. The pH was adjusted to 5 with sodium hydroxide, measured by a pH-meter. To a 2.5-ml portion of the buffer, 2.5 ml of colorless redistilled acetylacetone (Tedia Co., USA) and 5 ml of formaldehyde solution (BDH, 40%) were added; the mixture was brought to 25 ml with water and thoroughly mixed. The reagent was prepared daily.

2.2.4. Preparation of fluorescamine solution

Solution of fluorescamine (Aldrich, 98%) containing 600.0 μg ml^{-1} in acetone was prepared and kept in fridge, to be used for 1 week.

2.3. Analytical procedures for calibration graphs

2.3.1. Assay procedure for acid degradation reaction

Into a set of glass tubes, aliquots of standard/assay solution (to give final concentration ranges specified for spectrophotometric and fluorimetric measurements, listed in Table 1) were mixed with 1-ml portions of 0.5 M sulfuric acid solution. The tubes were covered, heated in a boiling water bath for 75 min, cooled to room temperature, and the contents were made up to a final volume of 5 ml with acetonitrile (fluorimetric measurements) or water (spectrophotometric measurements). The fluorescence intensities and the derivative values (spectrophotometry) were measured at the specified wave-lengths (Table 1) using reagent blank.

2.3.2. Assay procedure for Hantzsch reaction

Into a set of glass tubes, aliquots of standard/assay solution (to give final concentration ranges specified for spectrophotometric and fluorimetric measurements, listed in Table 1) were mixed with 1-ml portions of the acetylacetone–formaldehyde solution. The tubes were covered, heated in a boiling water bath for 15 min, cooled to room temperature, and the contents were made up to a final volume of 5 ml with water. The fluorescence intensities and absorbances were measured at the specified wave-lengths (Table 1) using reagent blank.

2.3.3. Assay procedure for fluorescamine reaction

Into a set of 5-ml volumetric flasks, aliquots of standard/assay solution (to give final concentration ranges specified for spectrophotometric and fluorimetric measurements, listed in Table 1) were mixed with 2 ml acetate buffer of pH 4.8. Then, to

Table 1
Analytical features for the determination of methotrexate using different analytical methods

Method	Measurement wave-length (nm)	Linearity range ($\mu g\ ml^{-1}$)	Regression data			$S_{y/x}$	S_a	S_b	LOD ($\mu g\ ml^{-1}$)	LOQ ($\mu g\ ml^{-1}$)
			a	b	r					
Spectrofluorimetry[a]										
Acid degradation reaction	$\lambda_{em} = 485$ ($\lambda_{ex} = 405$)	0.1–0.4	0.500	141	0.9986	1.162	1.423	5.196	0.019	0.063
Hantzsch reaction	$\lambda_{em} = 485$ ($\lambda_{ex} = 425$)	0.04–0.2	−0.043	362.2	0.9982	1.375	1.375	10.762	0.006	0.028
Fluorescamine reaction	$\lambda_{em} = 495$ ($\lambda_{ex} = 405$)	0.2–0.6	1.14E−13	179	0.9998	0.489	0.655	1.545	0.034	0.114
Spectrophotometry										
Acid degradation reaction	$^1D_{416}$	1–40	0.0012	−0.0200	0.9999	0.002	0.001	5.64E−5	0.200	0.700
Hantzsch reaction	A_{338}	3–15	0.0133	0.0691	0.9990	0.017	0.016	0.002	0.599	1.999
Fluorescamine reaction	A_{409}	2–8	−0.0240	0.1073	0.9993	0.009	0.009	0.002	0.412	1.498

a, Intercept; b, slope; r, correlation coefficient; $S_{y/x}$, standard error of estimate; S_a, S.D. of intercept; S_b, S.D. of slope; LOD, detection limit; LOQ, quantification limit.
[a] The instrument sensitivity range was adjusted at 1, 0.1 and 0.3 for acid degradation reaction, Hantzsch reaction and fluorescamine reaction, respectively.

each, 0.5 ml of fluorescamine solution (600.0 μg ml^{-1}) was added and the contents were mixed. The reaction solutions were made up to 5 ml with acetonitrile. After standing for 5 min, the fluorescence intensities and absorbances were measured at the specified wave-lengths (Table 1) using reagent blank.

2.4. Analysis of plasma samples

Frozen plasma was thawed at room temperature. Into a set of centrifuge tubes, separate aliquots of 0.5 ml plasma were transferred and spiked with suitable amounts of standard MTX solution within the concentration ranges listed in Table 2. Protein deposition step was carried out with ~4 ml of a suitable organic solvent (Table 2), this was followed by centrifugation for 20 min at 2000 rpm. A flow of nitrogen was used to flush out the organic solvent (supernatant), of each of the prepared sample solution. Separate residues were reconstituted in 5-ml portion of 0.1 M sulfuric acid. These final assay solutions were processed as described in the analytical procedure (Section 2.3).

3. Results and discussion

3.1. Aspects of hydrolytic degradation of methotrexate and spectrofluorimetric analysis

3.1.1. Hydrolytic degradation reaction of methotrexate in acidic solution

Generally, some amide compounds are liable to hydrolysis in aqueous solutions. Such a degradation reaction is either acid- or base-catalyzed. In MTX molecule, the amide link associates pteroic acid and glutamic acid moieties. In the review, it has been referred that MTX can be hydrolyzed in strongly acidic aqueous solutions to give glutamic acid and 4-amino-4-deoxy-10-methylpteroic acid (AMP) [47]. The latter has also been prepared from MTX by alkaline hydrolytic reaction [48].

Methotrexate acts as a weak acid (COOH of glutamic acid moiety)-weak base (aminopteridine moiety). It is practically insoluble in water, alcohol, chloroform and ether. It dissolves in dilute solution of mineral acids and of alkali hydroxides

Table 2
Analytical results of methotrexate in plasma samples

Fluorimetric method	Solvent[a]	Spiking (μg ml^{-1})	Final measured (μg ml^{-1})	Found ± S.D.[b]	Recovery [c](%)
Acid degradation reaction	Acetone	20	0.1	0.099 ± 0.0007	99.0
		80	0.4	0.405 ± 0.004	101.25
Hantzsch reaction	Acetonitrile	4	0.04	0.0398 ± 0.0002	99.50
		20	0.2	0.195 ± 0.0009	97.50
Fluorescamine reaction	Acetonitrile	40	0.2	0.202 ± 0.002	101.0
		120	0.6	0.598 ± 0.009	99.67

[a] Organic solvent used for deproteinization process.
[b] Mean ± S.D. of four determinations.
[c] Mean of four determinations.

and carbonates [1]. In accordance with this fact, the need to prepare solution of MTX in acid or alkali solution is probable. In this concern information about the stability of MTX in sulfuric acid, which was used for preparation of standard solution, was of interest.

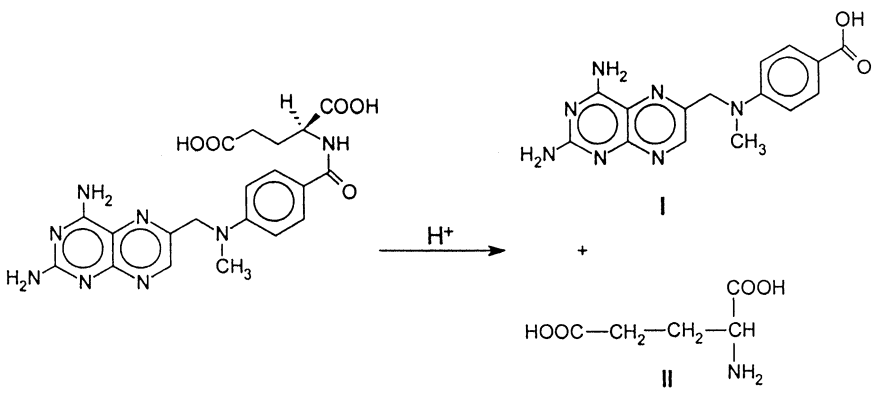
Scheme 1 presents the degradation reaction of MTX in sulfuric acid solution. AMP (I) and glutamic acid (II) are the degradation products.

3.1.2. Spectral characteristics of methotrexate and its acid degradation product

The yellow fluorescence of AMP exhibits excitation and emission wave-lengths of 405 and 485 nm, respectively. Fig. 1A shows the excitation and emission spectra of 360 ng ml^{-1} solution of MTX (after degradation reaction) in acetonitrile. The parent drug has no intrinsic fluorescence emission in acetonitrile. The UV–Vis absorption spectrum (Fig. 1B) of AMP (taken in aqueous solution) shows overlapping spectral features compared with the parent drug, MTX, in the vicinity of UV region. Meanwhile, AMP has its own characteristic absorption band with a maximum at 400 nm. The latter is missed in MTX spectrum. Fig. 1C presents the first derivative spectra of MTX and its degradation product, AMP. The ^{1}D values at 416 nm (zero-^{1}D value of MTX) allow selective measurement of AMP. By analogy, MTX can be determined without any interference from AMP through ^{1}D measurement at 354 nm (zero-crossing of AMP).

3.1.3. Study of the degradation reaction in solutions of different acid-strengths through spectrofluorimetric measurement

The MTX-degradation rate sequence showed the effect of sulfuric acid strength-reaction time. The fluorescence of solutions (each at 3 μg ml^{-1} MTX) was followed over the sulfuric acid strength



Scheme 1.

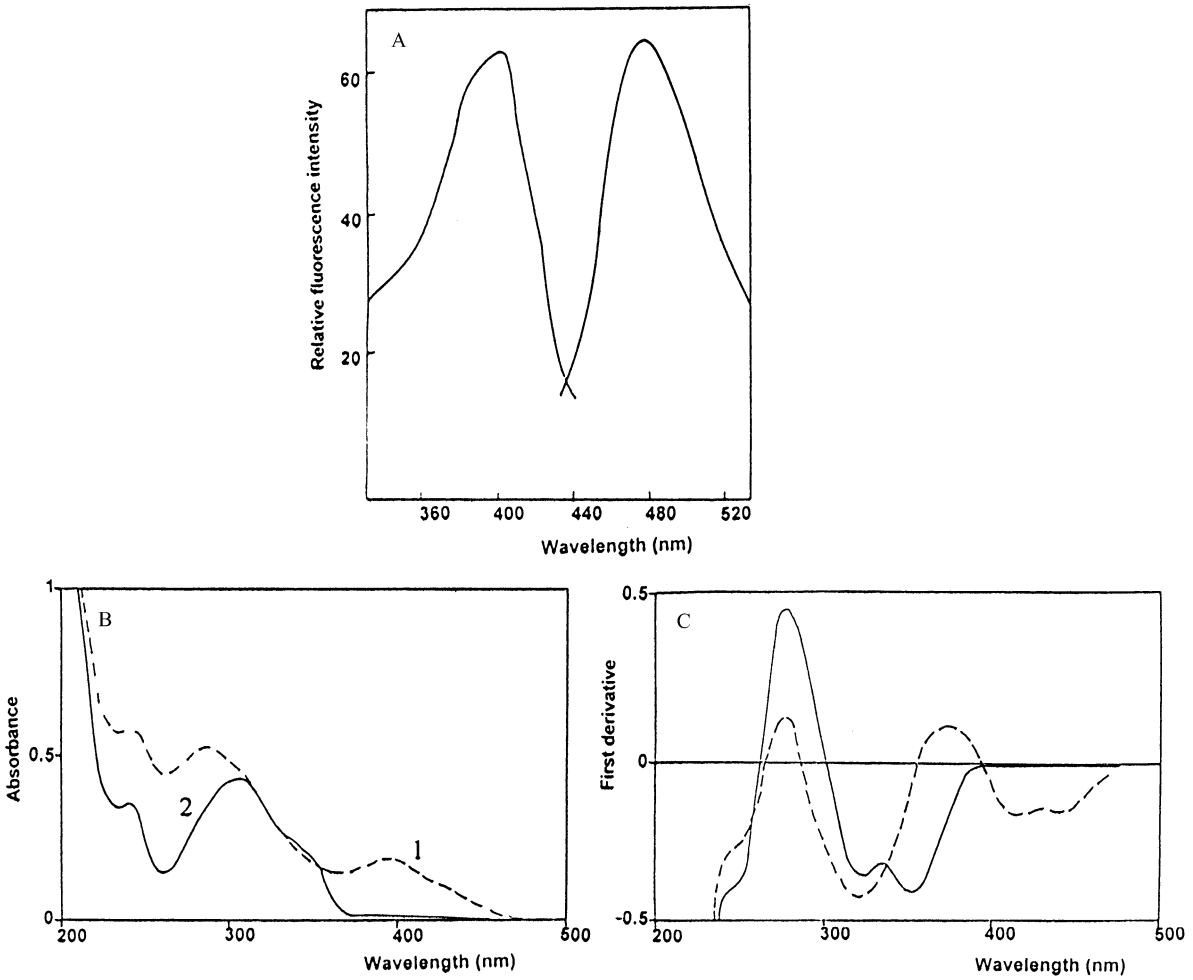


Fig. 1. (A) Excitation and emission spectra of the degradation reaction product of MTX (0.4 μg ml^{-1}) measured in acetonitrile (uncorrected spectra for blank). (B) Absorption spectra of (1) the degradation reaction product, AMP (8.0 μg ml^{-1}) in 0.05 M sulfuric acid and (2) MTX (10.0 μg ml^{-1}) in 0.1 M sulfuric acid. (C) The corresponding first derivative spectra.

range of 0.2–1 M, for a heating time up to 3 h at 100 °C. At adequate time intervals, certain volume of each reaction solution was withdrawn and diluted to volume with acetonitrile to get a final concentration of 0.3 μg ml^{-1}, and the fluorescence intensity was measured at 485 nm ($\lambda_{ex} = 405$ nm). The graphical presentation of the data in term of relative fluorescence intensity–time curve (Fig. 2A), for different strengths of sulfuric acid, indicated a first order reaction. It is clear from the curve that the formation of the fluorogen, AMP, is much faster with 1 M sulfuric acid than the others. On the contrary, the fluorescence intensity obtained after the completion of reaction was maximum for 0.2 M sulfuric acid reaction solution.

3.1.3.1. Kinetic considerations. Considering the acid-catalyzed hydrolysis of MTX in solution, the apparent rate law [49] of the product formation (AMP) is given by:

$$d\mathrm{AMP}/d\mathrm{t} = k_{\mathrm{obs}}[\mathrm{MTX}]$$

in which

$$k_{\mathrm{obs}} = k_1[\mathrm{H}^+]$$

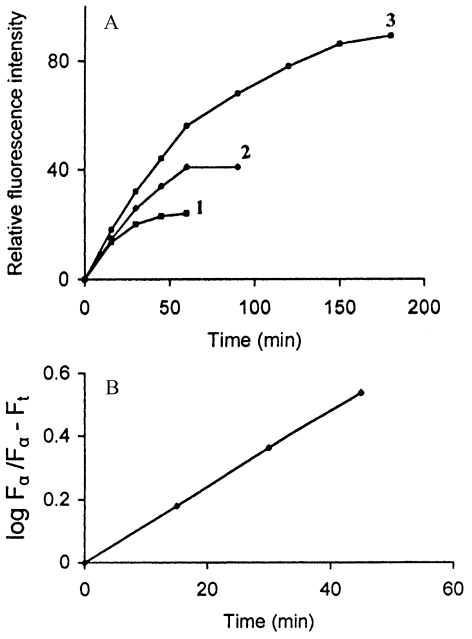


Fig. 2. (A) Time courses of the acid catalyzed hydrolysis of MTX (0.3 μg ml^{-1}) at different sulfuric acid strengths, 1.0 M (1), 0.5 M (2) and 0.2 M (3). (B) First order plot for the acid catalyzed hydrolysis of MTX (0.3 μg ml^{-1}) in 0.5 M sulfuric acid.

$$d\mathrm{AMP}/d\mathrm{t} = k_1[\mathrm{MTX}][\mathrm{H}^+]$$

where k_{obs} denotes the reaction rate constant. The hydrogen ion concentration term in the previous equation indicates that the process is a specific hydrogen-ion-catalyzed reaction. This was proved by the aforementioned study.

Applying Beer's law to fluorescence data, the AMP formation reaction (i.e. MTX degradation reaction) was found to be described as the first-order model:

$$\log \mathrm{F}_\alpha/(\mathrm{F}_\alpha - \mathrm{F}_\mathrm{t}) = k_{obs}/2.303\mathrm{t}$$

where F_t and F_α are the fluorescence of the degradation reaction system at a given time t and at the end of the experiment when a constant value is reached, respectively. The plot of this equation ($\log F_\alpha/(F_\alpha - F_t)$ vs. t) (Fig. 2B), for MTX degradation reaction in 0.5 M sulfuric acid solution heated at 100 °C, gives a straight line with a slope of 0.014, from which k_{obs} equals 0.032 min^{-1}. The corresponding k_{obs} values for 1 and 0.2 M sulfuric are 0.058 and 0.014 min^{-1}, respectively.

3.1.4. Derivative spectrophotometry as a stability-indicating assay

Referring to Fig. 1C, the first derivative peak of MTX, $^1D_{354}$, (zero-crossing of AMP) allows selective measurement of MTX. Accordingly the first derivative technique ($^1D_{354}$ measurement) was suggested for the assay of the intact drug, MTX, in presence of its degradation product, AMP. The linearity of calibration graph and conformity of the $^1D_{354}$ measurement to Beer's law were proved by the high value of the correlation coefficient, r, ($r = 0.9999$) of the regression equation; $^1D_{354} = 0.0077 + 0.0395C$. The calibration curve is linear over the concentration rang of 1.0–20.0 μg ml^{-1} of MTX.

In order to assess the precision, as percentage relative standard deviation (R.S.D.%) and the accuracy, as percentage relative error (Er%) of the proposed method, five replicate determinations were carried out on synthetic mixtures. The data shown in Table 3 indicated good accuracy and precision of the proposed derivative spectrophotometric method as a stability-indicating assay. So, it was important to use this method to evaluate the stability of MTX in 0.1 M sulfuric acid solution at room temperature.

3.1.4.1. Accelerated stability analysis. According to the method based on the principles of kinetics demonstrated by Garrett and Carper [50], the k value (reaction rate constant) for the decomposition of a drug in solution at various elevated temperatures are obtained from the linear expression in equation ($\log c = \log c_0 - kt/2.303$) where the slope of the line is $-k/2.303$. In view of the derivative spectrophotometric method, the latter equation can be rewritten as follows:

$$\mathrm{Log}\ ^1\mathrm{D}_\mathrm{t} = \mathrm{Log}\ ^1\mathrm{D}_0 - k/2.303\mathrm{t}$$

where ^1D_t and $^1D_0$ refer to $^1D_{354}$ values measured for MTX solution at a given time t and at zero time, respectively.

The log of the rates of decomposition are then plotted against the reciprocals of the absolute temperature (Arrhenius plot) [49]. The $K_{25\,°C}$ is

Table 3
Precision and accuracy for the derivative spectrophotometric determination of methotrexate in presence of its degradation product

Intact/degraded ratio	Added intact (nominal value) (μg ml^{-1})	Found ± S.D.[a]	R.S.D. (%)[b]	Er (%)[c]
0.5:1	2.5	2.52 ± 0.02	0.87	0.68
1:1	5	5.01 ± 0.04	0.72	0.22
2:1	10	9.99 ± 0.05	0.58	−0.04
3:1	15	14.88 ± 0.09	0.60	−0.80
4:1	20	20.15 ± 0.09	0.45	0.75

[a] Mean ± S.D. of five determinations.
[b] Percentage R.S.D.
[c] Percentage relative error.

used as a measure of the stability of the drug at room temperature.

A set of three solutions of MTX, prepared in 0.1 M sulfuric acid at concentration level 200 μg ml^{-1}, were heated at adjusted temperatures of 100, 80, and 60 °C, separately. The reaction sequence was followed. At a time, 0.5-ml volume was withdrawn from each solution, diluted to 5 ml with water and the $^{1}D_{354}$ values were measured for the solutions. The first-order plots were derived and the corresponding k values were calculated from the slopes. Arrhenius plot is presented in Fig. 3. By extrapolation on the plot, the $K_{25\,^{\circ}C}$ obtained has the value of 0.0001 min^{-1}.

The time required for the drug concentration to fall to 95, 90, 80% ... etc, of original value is calculated from the equation:

$$t = 2.303/k_{25\,^{\circ}C} \log c_0/c$$

According to this equation, it was found that 5% of MTX solution will degrade within 7.8 h or 1% within 1.6 h ($t_{1/2}$, half life-time, = 108 h). Finally it could be concluded that MTX solution prepared in 0.1 M sulfuric acid can be used for the analytical purpose within the few working hours. Longer life-time is expected if the solution is kept in refrigerator at 4 °C. So, it is advised that the stock solution to be kept in the refrigerator over the day and on time the working solutions, needed to be used, are prepared by suitable dilution.

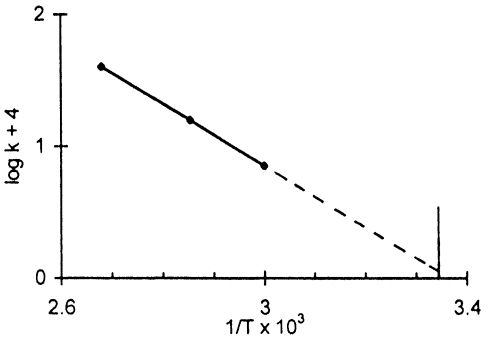


Fig. 3. Arrhenius plot for the degradation of MTX in 0.1 M sulfuric acid using first derivative measurement.

3.1.5. Spectrofluorimetric and spectrophotometric determination of MTX through acid-catalyzed degradation reaction

3.1.5.1. Optimization of the analytical procedure. The reaction conditions with respect to the sulfuric acid strength, the reaction time and temperature, and the diluting solvent were optimized to achieve maximum possible sensitivity. The first preliminary experiments indicated that 100 °C is just adequate to activate the reaction. Lower temperatures do not meet the rapidity requirement. So all the studies were carried out at 100 °C.

With reference to the previous study in Section 3.1.3, obviously, the sulfuric acid strength, needed to be selected, did not give by necessity the highest fluorescence intensity to avoid long reaction time. Accordingly, 0.5 M sulfuric acid was chosen as the most appropriate to compromise between short reaction time and reasonable fluorescence intensity.

To attain better fluorescence intensity enhancement, the fluorescence of the solutions as a function of the diluting solvents was compared. Water, ethanol, methanol, acetonitrile, dimethyl-

formamide and acetone were tested. Decrease in fluorescence intensity was in the order; acetonitrile, acetone, ethanol, methanol, dimethylformamide, water. Therefore, acetonitrile was chosen as the most appropriate solvent.

3.1.5.2. Measurements. The proposed spectrofluorimetric and spectrophotometric methods deal with the measurement of the fluorescence intensity at 485 nm ($\lambda_{ex} = 405$ nm) and the first derivative value at 416 nm (Fig. 1C), respectively, for the degradation reaction product solution.

3.2. Other comparative methods

3.2.1. Spectrofluorimetric and spectrophotometric methods using Hantzsch reaction

Based on Hantzsch reaction method, MTX was condensed with formaldehyde and acetylacetone to form a yellow fluorescent dihydrolutidine derivative. The latter shows characteristic excitation and emission spectra presented in Fig. 4A. The spectrofluorimetric method is based on fluorescence emission measurement at 485 nm (excitation at 425 nm). The absorption spectrum of the reaction product exhibits a maximum at 338 nm (Fig. 4B). The influence of some variables on the reaction was tested to establish the most favorable conditions to achieve maximum fluorescence sensitivity and obedience to Beer's law. In this concern, the buffer pH (Fig. 4C), the reagent concentration, the time–temperature and the diluting solvent were optimized in accordance with the experimental Section 2.3.2. A 15-min reaction time at 100 °C was necessary for the completion of the reaction. Of the diluting solvents examined are, water, methanol, ethanol, acetone, acetonitrile and dimethylformamide. No significant difference in fluorescence emission was observed. Water was chosen as an appropriate working diluting solvent.

3.2.2. Spectrofluorimetric and spectrophotometric methods using reaction with fluorescamine

The amine derivative MTX, reacts instantaneously with fluorescamine to yield a yellow fluorescent derivative. The developed method deals with the measurement of the fluorescence emission at 495 nm ($\lambda_{ex} = 405$ nm) and of the absorbance at 409 nm (Fig. 5A and B).

The early work with fluorescamine indicated that its reactions with primary amines occur in aqueous solutions of pH 8 to 9.5 [38]. Later, studies carried out by Silva et al. [51] showed that the primary aromatic amino compounds favored acidic pH (3–4) for optimal reactivity. Consequently the influence of pH of the buffer solution on MTX–fluorescamine reaction was tested. Of the buffers used, to cover the pH range from 2 to 10, are Sorensen's phosphate buffer (monopotassium phosphate/disodium phosphate), Walpole's acetate buffer (0.2 M acetic acid/0.2 M sodium acetate), borate buffer (0.1 M boric acid in 0.1 M potassium chloride/0.1 M sodium hydroxide) and Britton Robinson buffer (0.04 M in each of acetic, *o*-phosphoric and boric acids). The experiments clarified that maximum fluorescence intensity was obtained at pH 4.8 (Fig. 5C). Such a finding agreed with the study reported for the structurally related compound, folic acid [43].

To achieve better sensitivity, the fluorescence intensity for MTX–fluorescamine reaction using different diluting solvents, was followed. The solvent's efficiency was of the order; acetonitrile, dimethylformamide, methanol, ethanol, acetone, water. Accordingly, acetonitrile was used as a working solvent.

3.3. Stability concern

The stability of the reactions products (degradation reaction, Hantzsch reaction and fluorescamine reaction) was examined. The fluorescence measurements (15-min interval) show no variation over at least 2 h.

3.4. Statistical analysis of results

3.4.1. Concentration ranges and calibration graphs

Using the optimized reactions conditions, the spectrofluorimetric and the spectrophotometric measurements at the working wave-lengths were found to be linearly correlated to the MTX concentration. Data recorded in Table 1 summarizes the characteristics of the calibration plots. These include linear regression equations, concen-

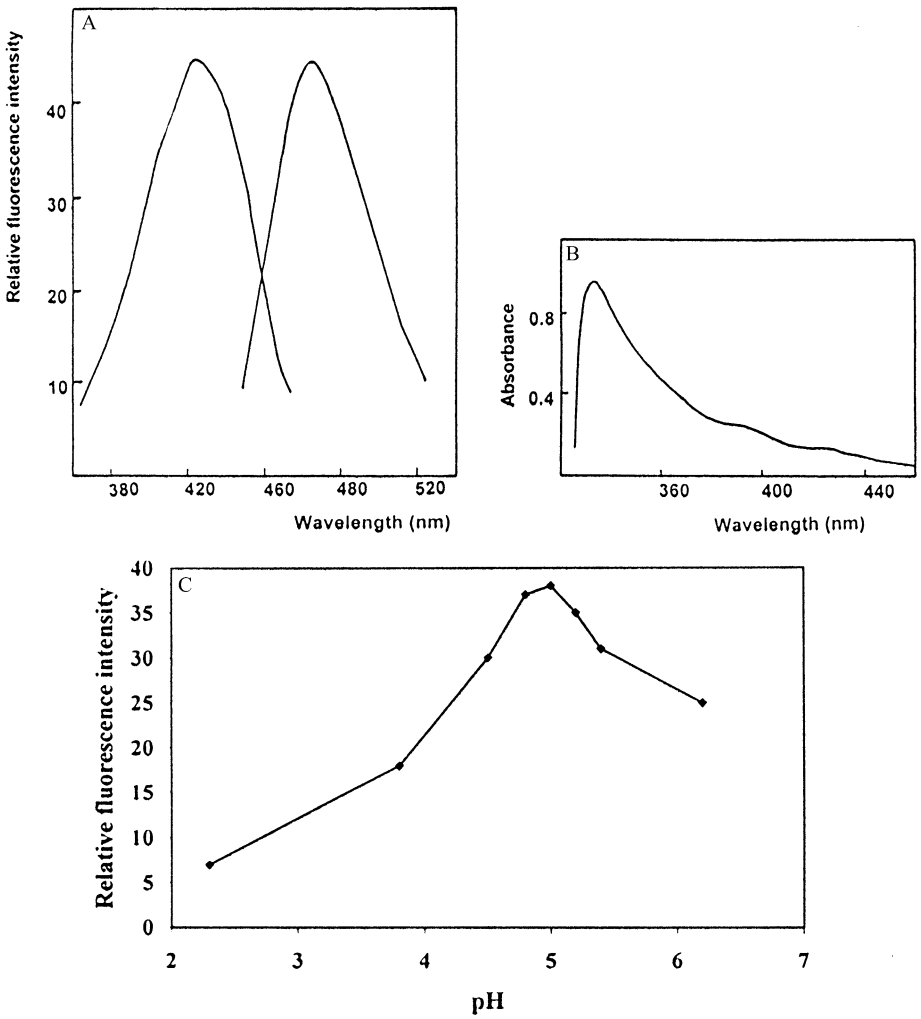


Fig. 4. (A) Excitation and emission spectra of the reaction product of MTX (0.1 μg ml^{-1}) with acetylacetone–formaldehyde in aqueous solution (uncorrected spectra for blank). (B) The corresponding absorption spectrum (13.0 μg ml^{-1} MTX). (C) Effect of pH on the reaction (0.1 μg ml^{-1} MTX), blank was subtracted.

tration ranges, correlation coefficients (r), and standard deviations (S.D.) of the intercept (S_a) and slope (S_b).

3.4.2. Detection and quantification limits

In accordance to the official compendial methods [2] and IUPAC [52], the limit of detection, LOD = $3s$ per slope, where s is the S.D. of replicate blank responses (under the same conditions as for sample analysis). Using this formula, the detection limits obtained for the investigated spectrofluorimetric and spectrophotometric methods are listed in Table 1. The limits of quantification, LOQ, defined as $10s$ per slope, were found to be 0.06, 0.03 and 0.11 μg ml^{-1} for the spectrofluorimetric measurements of the fluorogenic products following acid degradation reaction, Hantzsch condensation reaction and fluorescamine reaction, respectively. The corresponding values for the spectrophotometric measurements

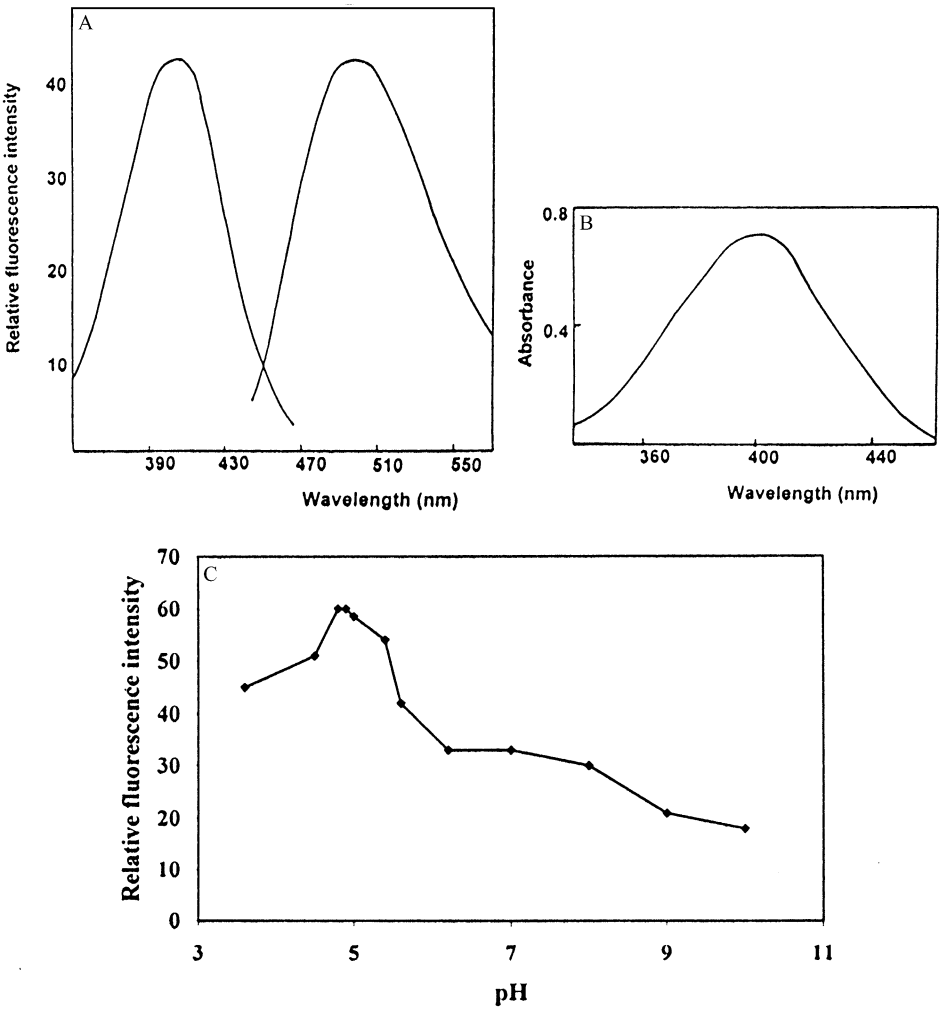


Fig. 5. (A) Excitation and emission spectra of the reaction product of MTX (0.2 μg ml^{-1}) with fluorescamine measured in acetonitrile (uncorrected spectra for blank). (B) The corresponding absorption spectrum (6.5 μg ml^{-1} MTX). (C) Effect of pH on the reaction (0.33 μg ml^{-1} MTX), blank was subtracted.

of the reactions products, as in the latter sequence, are 0.70, 2.0 and 1.5 μg ml^{-1}, respectively.

3.4.3. Precision and accuracy

In order to assess the precision, as percentage R.S.D.% and the accuracy, as Er% of the proposed methods, five replicate determinations were carried out on calibration samples. The data obtained from this investigation is summarized in Table 4. The results can be considered adequate for the quality control analysis of pharmaceutical preparations.

3.4.4. Specificity and interferences

It is well known that the reaction with fluorescamine and Hantzsch condensation reaction with acetylacetone–formaldehyde are specific for primary amino group-containing compounds. However, because of the dependence of (a) the previous reactions on the presence of primary amino groups attached to the pteridine nucleus in MTX molecule and (b) the acid degradation reaction on the break down of the amide link followed by determination of the pteroic acid derivative (AMP), the impurities of closely related

Table 4
Precision and accuracy for the determination of methotrexate by the proposed methods

Method	Nominal value (μg ml^{-1})	Found ± S.D.[a]	R.S.D. (%)[b]	Er (%)[c]
Spectrofluorimetry				
Acid degradation reaction	0.1	0.101 ± 0.0002	0.21	1.0
	0.4	0.402 ± 0.002	0.57	0.50
Hantzsch reaction	0.04	0.041 ± 0.0006	1.14	1.5
	0.2	0.203 ± 0.0003	0.14	1.50
Fluorescamine reaction	0.2	0.201 ± 0.001	0.55	0.50
	0.6	0.599 ± 0.003	0.55	− 0.02
Spectrophotometry				
Acid degradation reaction	1	1.003 ± 0.005	0.48	0.30
	20	20.10 ± 0.08	0.40	0.50
Hantzsch reaction	3	2.95 ± 0.01	0.41	− 1.67
	15	14.93 ± 0.08	0.50	− 0.47
Fluorescamine reaction	3	2.99 ± 0.02	0.70	− 0.23
	8	7.99 ± 0.03	0.39	− 0.13

[a] Mean ± S.D. of five determinations.
[b] Percentage R.S.D.
[c] Percentage relative error.

compounds [53] which commonly found in commercial MTX samples will interfere.

In USP [2], MTX is defined as a mixture of 4-amino-10-methylfolic acid and closely related compounds (specified to be not more than 2%). So, the possible interference from such impurities with the developed assays will not exceed 2%. In pharmaceutical industry, samples of bulk powder of MTX have to pass the pharmacopeial chromatographic purity test to check that these related compounds do not exceed 2% of the content of the examined sample. Accordingly, it could be concluded that the proposed methods are suitable for routine analysis in control laboratories for the purpose of content uniformity of MTX in tablets formulated from MTX powder agreed with the pharmacopeial purity requirements.

It was also shown that excipients and diluents such as starch, talc, magnesium stearate, and microcrystalline cellulose, which are commonly formulated in tablets, do not interfere with the proposed methods.

3.5. Analysis of pharmaceutical formulation

The proposed spectrofluorimetric and spectrophotometric methods were applied to the determination of MTX in tablets dosage form. From the results shown in Table 5, the methods gave satisfactory recovery data. Also the S.D. for the assays results show good precision.

To compare the average results of the spectrofluorimetric and spectrophotometric methods, the statistical procedure, one-way analysis of variance (ANOVA test) was used. The data from Table 5 was used for a comparison of the methods applying six replicates (three replicate determinations at each concentration level) per method. The observed ANOVA F ratio [54] was compared with the theoretical F value (table of F distribution) at 1% significant level. Since the calculated F (3.40) is smaller than the table value for F (3.69), therefore, there is no significance difference between the methods compared.

3.6. Analysis of plasma samples

Very high doses of MTX have been given by intravenous infusion as part of combined therapy in patients with osteosarcoma. Following intravenous infusion of 50–250 mg kg^{-1} over 6 h to 14 subjects, peak plasma concentration of 45–450 μg ml^{-1} were attained [55]. It is thought that the higher blood concentration achieved with high

Table 5
Assay results for the determination of methotrexate in emthexate tablets[a]

Method	Declared (μg ml^{-1})	Found ± S.D.[b]	Recovery[c]
Spectrofluorimetry			
Acid degradation reaction	0.1	0.099 ± 8.0E − 5	99.94
	0.4	0.398 ± 0.002	99.50
Hantzsch reaction	0.04	0.0395 ± 0.0004	98.75
	0.2	0.198 ± 0.0008	99.00
Fluorescamine reaction	0.2	0.200 ± 0.001	100.00
	0.6	0.590 ± 0.009	99.95
Spectrophotometry			
Acid degradation reaction	1	1.002 ± 0.003	100.20
	20	20.01 ± 0.07	100.05
Hantzsch reaction	3	2.95 ± 0.01	98.33
	15	14.93 ± 0.1	99.26
Fluorescamine reaction	3	2.96 ± 0.03	98.50
	8	7.95 ± 0.05	99.38
ANOVA test F = 3.40[d]			

[a] Labeled to contain methotrexate sodium equivalent to 2.5 mg MTX per tablet. It is manufactured by Pharmachemie, Netherlands.
[b] Mean ± S.D. of three determinations.
[c] Mean percentage recovery.
[d] The tabulated value at a significance level 1% is 3.69.

dose MTX will result in greater anti-tumor effect [56]. However, high blood levels of MTX if maintained for prolonged periods can result in the development of severe toxicity. So, there is a need to monitor blood MTX levels following this dosage regime to indicate whether a patient is able to rapidly eliminate the drug, or whether toxicity may arise as a result of maintained blood levels.

It can be seen that the sensitivity of the developed spectrofluorimetric methods is adequate and promising. So, it was advantageous to evaluate the applicability of these methods to analyze MTX in plasma samples. The analytical procedures described in the experimental Section 2.3, for the spectrofluorimetric methods, were applied to plasma samples, spiked with varying amounts of MTX (4-120 μg per 1 ml plasma), after a prior purification step of protein deposition with suitable organic solvent. Determination of MTX at the previously mentioned levels showed errors between ~1 and 3% (Table 2). It should be noted that the dilution (100-fold) of plasma samples following the purification step and before applying the considered reaction is necessary. The dilution cancels the high background fluorescence measured for blank samples. This interference results from extraneous substances in plasma samples even after the purification step.

To assess the reproducibility, four determinations for each concentration examined were conducted and the S.D. was calculated. The results obtained are listed in Table 2. The method is quite effective for determination of MTX in plasma and meeting the requirement of rapidity for routine use in high-dose MTX therapy. Also, the advantage of a low-cost and easily handling instrument should be taken into consideration.

4. Conclusion

The proposed methods introduced a promising approach to the fluorimetric analysis of MTX. A concentration down to 40 ng ml^{-1} could be measured. Therefore, analysis of plasma samples spiked with MTX at a level of 4 ug ml^{-1} was possible. The sensitivity of the fluorimetric method following Hantzsch reaction is somewhat better than that of acid-catalyzed degradation method.

However, the latter keeps the advantage of no need for special chemical reagent.

The methods are cheap to operate compared with alternative HPLC [4–13] methods currently available. They are, therefore, suitable for routine clinical analysis and in control laboratories, to be applied for the analysis of MTX in pure form and in tablets. However, the reported HPLC-fluorimetric detection methods offered a highly sensitive technique for quantification of low-therapy MTX plasma levels.

The spectrophotometric measurements were more sensitive than [24,25] or at least as sensitive as [23] the reported spectrophotometric methods. A potential advantage appears in the stability-indicating derivative spectrophotometric study, the matter, which has not been discussed in the previously reported spectrophotometric methods.

References

[1] K. Parfitt, S.C. Sweetman, P.S. Blake, A.V. Parsons, Martindale, the Extra Pharmacopoeia, 32th ed, Pharmaceutical Press, London, 1999, pp. 547–551.
[2] United States Pharmacopeia XXIV, The National Formulary 19, US Pharmacopeial convention, INC, 2000, pp. 984–985, 2151.
[3] British Pharmacopoeia, Stationery Office, London, 1998, pp. 872, 1805.
[4] E.A. McCrudden, S.E. Tett, J. Chromatogr. Biomed. Appl. 721 (1999) 87–92.
[5] S. Emara, H. Askal, T. Masujima, Biomed. Chromatogr. 12 (1998) 338–342.
[6] Z. Yu, D. Westerlund, K.S. Boos, J. Chromatogr. Biomed. Appl. 689 (1997) 379–386.
[7] S. Emara, S. Razee, A. Khedr, T. Masujima, Biomed. Chromatogr. 11 (1997) 42–46.
[8] F. Albertioni, C. Rask, S. Eksborg, J.H. Poulsen, B. Pettersson, O. Beck, H. Schroeder, C. Peterson, Clin. Chem. 42 (1996) 39–44.
[9] F. Albertioni, B. Pettersson, O. Beck, C. Rask, P. Seideman, C. Peterson, J. Chromatogr. Biomed. Appl. 665 (1995) 163–170.
[10] T. Suzuki, H. Hashimoto, N. Ichinose, Fresenius J. Anal. Chem. 351 (1995) 806–807.
[11] O. Beck, P. Seideman, M. Wennberg, C. Peterson, Ther. Drug Monit. 13 (1991) 528–532.
[12] J. Salamoun, M. Smrz, F. Kiss, A. Salamounova, J. Chromatogr. Biomed. Appl. 63 (1987) 213–223.
[13] J. Salamoun, J. Frantisek, J. Chromatogr. Biomed. Appl. 51 (1986) 173–181.
[14] H.N. Zhang, L.L. Wen, S.W. Zhang, P.Y. Ding, Yaowu. Fenxi. Zahi 20 (2000) 401–404.
[15] T. Hirai, S. Matsumoto, I. Kishi, J. Chromatogr. Biomed. Appl. 690 (1997) 267–273.
[16] H. Aboleneen, J. Simpson, D. Backes, J. Chromatogr. Biomed. Appl. 681 (1996) 317–322.
[17] M. Cociglio, D. Hillaire-Buys, C. Alric, J. Chromatogr. Biomed. Appl. 674 (1995) 101–110.
[18] T. Hirai, M. Kitamura, Y. Inoue, Yakugaku. Zasshi 114 (1994) 602–610.
[19] R.M. Mader, B. Rizovski, G.G. Steger, H. Rainer, R. Proprentner, R. Kotz, J. Chromatogr. Biomed. Appl. 124 (1993) 311–316.
[20] S. Emara, S. Razee, A.N. El-Shorbagi, T. Massujima, Analyst 121 (1996) 183–188.
[21] S.G. Chakrabarti, I.A. Bernstein, Clin. Chem. 15 (1969) 1157–1161.
[22] J.M.J. Kinkade, W.R. Vogler, P.G. Dayton, Biochem. Med. 10 (1974) 337–350.
[23] C.S.P. Sastry, J.S.V.M. Lingeswara-Rao, Anal. Lett. 29 (1996) 1763–1778.
[24] M.M. Ellaithy, M.F. El-Tarras, N.B. Tadros, M.M. Amer, Anal. Lett. 15 (1982) 981–988.
[25] R. Gandhi, A.J. Khopade, N.K. Jain, Indian Drugs 34 (1997) 455–458.
[26] H.Z. Fan, H.X. Hou, J.Q. Ren, J.H. Pan, Fenxi. Huaxue 24 (1996) 246.
[27] M.O. Kovbuz, N.M. Felitsin, Y.Z. Khim'yak, B.S. Zimenkovskii, Farm. Zh. 3 (1995) 80–83.
[28] M.A. Malone, A. Costa Garcia, P. Tunon Blanco, M.R. Smyth, Anal. Methods Instrum. 1 (1993) 164–171.
[29] A.J. Miranda Ordieres, A. Costa Garcia, J.M. Fernandez Alvarez, P. Tunon Blanco, Anal. Chim. Acta 233 (1990) 281–287.
[30] Q.L. Ma, J.H. Yang, X. Wu, F. Huang, L.M. Sun, Anal. Lett. 33 (2000) 2689–2699.
[31] H.Y. Wang, L.D. Liu, Y. Sun, L. Ma, J. Li, Talanta 52 (2000) 201–209.
[32] A.F.M. El Walily, F.A. El-Yazbi, S.F. Belal, O. Abdel-Razak, Anal. Lett. 30 (1997) 2029–2043.
[33] F.A. El-Yazbi, H.H. Abdine, R.A. Shaalan, J. Pharm. Biomed. Anal. 19 (1999) 819–827.
[34] V. Das-Gupta, K.R. Stewart, J.M. Gunter, J. Pharm. Sci. 72 (1983) 1470–1471.
[35] G. Zurek, U. Karst, J. Chromatogr. A 864 (1999) 191–197.
[36] A.E. Mansilla, A.M. de la Pena, F. Salinas, A. Alanon, J.A. Murillo, J. Agric. Food Chem. 45 (1997) 172–177.
[37] T.J. Kelly, C.R. Fortune, Int. J. Environ. Anal. Chem. 54 (1994) 249–263.
[38] K. Imai, T. Toyo'oka, H. Miyano, Analyst 109 (1984) 1365–1373.
[39] F.G. Sanchez, A.A. Gallardo, Anal. Chim. Acta 270 (1992) 45–53.
[40] S. Stein, P. Bohlen, J. Stone, W. Dairman, S. Udenfreind, Arch. Biochem. Biophys 155 (1973) 202–212.

[41] M. Weigle, J.F. Blount, J.P. Tengi, R.C. Czaikowski, W. Leimgruber, J. Am. Chem. Soc. 94 (1972) 4052–4054.
[42] D. Djozan, M.A. Faraj-zadeh, J. Pharm. Biomed. Anal. 10 (1992) 1063–1067.
[43] C.C. Blanco, A.S. Carretero, A.F. Gutiérrez, M.R. Ceba, Anal. Lett. 27 (1994) 1339–1353.
[44] T.A. Gehring, L.G. Rushing, H.C. Thompson, J. AOAC Int. 80 (1997) 751–755.
[45] D. Djozan, M.A. Faraj-zadeh, Chromatographia 41 (1995) 568–572.
[46] W. Funk, T. Kuepper, A. Wirtz, S. Netz, J. Plannar Chromatogr. Mod. TLC 7 (1994) 10–13.
[47] H.G. Brittain, in: Analytical Profiles of Drug Substances, vol. 5, Academic Press, California, 1986, pp. 284–306.
[48] D.A. Cairnes, W.E. Evans, Ther. Drug Monit. 5 (1983) 363–366.
[49] A. Martin, J. Swarbrick, A. Cammarata, in: Physical Pharmacy, third ed., Lea and Febiger, Washington Square, Philadelphia, USA, 1983, pp. 352–394.
[50] E.R. Garrett, R.F. Carper, J. Am. Pharm. Assoc. 44 (1955) 515.
[51] J.A.F. de Silva, N. Stonjny, Anal. Chem. 47 (1975) 714–718.
[52] J.N. Miller, Analyst 113 (1991) 3–14.
[53] J.F. Gallelli, G. Yokoyama, J. Pharm. Sci. 56 (1967) 387–389.
[54] S. Bolton, Pharmaceutical Statistics, Marcel Dekker, New York, 1984, pp. 218–225.
[55] A.C. Moffat, J.V. Jackson, M.S. Moss, B. Widdop, Clarke's Isolation and Identification of Drugs, second ed., London, 1986, p. 756.
[56] L. Djerassi, Cancer Chem. Rep. 6 (1975) 3–6.