Department of Pharmaceutical Chemistry[1], Faculty of Pharmacy, Health Science Center, Kuwait University, Kuwait, Department of Experimental Therapy[2], Cancer Research Institute, Slovak Academy of Sciences, Department of Organic Chemistry[3], Faculty of Chemical Technology, Slovak Technical University, and Institute of Experimental Endocrinology[4], Slovak Academy of Sciences, Bratislava, Slovak Republic

Stability of the new antileukemic 4-pyranone derivative, BTMP, using HPLC and LC-MS analyses

L. Novotny[1], M. Abdel-Hamid[1], H. Hamza[1] P. Rauko[2], M. Uher[3] and J. Brtko[4]

The stability of the new antileukemic kojic acid derivative, 5-benzyloxy-2-thiocyanatomethyl-4-pyranone (BTMP) was investigated. The degradation of BTMP was studied using specific and reproducible HPLC and LC-MS methods. Accelerated stability studies of BTMP were conducted in 0.1 M hydrochloric acid solution, physiological phosphate buffer solution (pH 7.5) and basic phosphate buffer solution (pH 9.0) at 30, 40 and 60 °C, respectively. The degradation of BTMP was found to follow pseudo-first order kinetics. In basic solution (pH 9.0), BTMP underwent rapid hydrolysis at a degradation rate constant (0.183–0.638 h^{-1}) and degradation half-life (3.67–1.06 h) depending on the temperature setting. On the other hand, BTMP was significantly stable in 0.1 M hydrochloric acid solution (k_{deg}: 0.0017–0.0052 h^{-1}; degradation half-life $t_{1/2}$: 408.6–135.7 h), whereas in physiological phosphate buffer solution (pH 7.5), BTMP was only moderately stable (k_{deg}: 0.006–0.231 h^{-1}; degradation half-life: 117.7–3.0 h). Arrhenius plots were constructed to predict the degradation kinetic parameters of BTMP at 25 °C and 4 °C. LC-MS analyses confirmed the degradation of BTMP in basic solutions and indicated at least two degradation products; namely 5-benzyloxypyran-2-ol-4-one (m/z 217.8) and 2-thiocyanatomethylpyran-5-ol-4-one (m/z 181.6).

1. Introduction

Cytotoxic activity the has been confirmed for many groups of natural compounds. The 4-pyranone skeleton, which is also a part of flavonoids [1, 2], is known to be associated with antineoplastic activity. This is reflected in the focusing of scientific attention at the cytotoxic effects of a variety of new chemically synthetic 4-pyranone derivatives. Consequently, synthetic derivatives of kojic acid and their biological activity were investigated in cell cultures. It was found that their activity depends on both their lipophilic and hydrophilic properties, as well as on their ability to form chelates [3]. One of the most interesting compounds of this class is 5-benzyloxy-2-thiocyanatomethyl-4-pyranone (BTMP) [4]. This compound was investigated *in vitro* and its effects on cell division of neoplastic cell lines, DNA, RNA, protein synthesis and cytoplasmatic protein phosphorylation were reported. It was shown that BTMP inhibits the growth of L1210 at a relatively low concentration [$IC_{50} = 2.60$ μM].

In addition to the previous biological studies, assessment of the stability of BTMP under controlled experimental conditions, is important for biopharmaceutical and drug formulation studies. There was been little reported in the literature on the stability profiles of BTMP. This paper discusses the effects of pH and temperature changes on the stability of BTMP and suggests a model for the determination of various degradation kinetics. The degradation pathways of BTMP in basic solutions are postulated.

2. Investigations, results and discussion

The decomposition of BTMP was followed by a HPLC procedure developed in our laboratory. Chromatographic analysis was based on the use of a Shim Pack CLC-SIL column and a mobile phase consisting of methanol and 5% acetic acid solution (1:1 v/v) at a flow rate of 1.0 ml min^{-1}. The effluent was monitored at 254 nm. Quantitation was based on PA measurements of BTMP. Linear relationship of the peak area (PA) and concentration of BTMP over the concentration range 2.5–10 μg ml^{-1} was obtained. The linear regression equation was: $PA \times 10^{-5} = 0.33 + 1.49$ C (r: 0.9881). The developed HPLC method was accurate and reproducible. The % deviation from the mean (DEV%) was 2.7–3.3%, whereas the RSD % was in the range of 0.11–0.41% using standard solutions of BTMP at concentrations of 5.0 and 10 μg ml^{-1}, respectively. Fig. 1 shows the HPLC

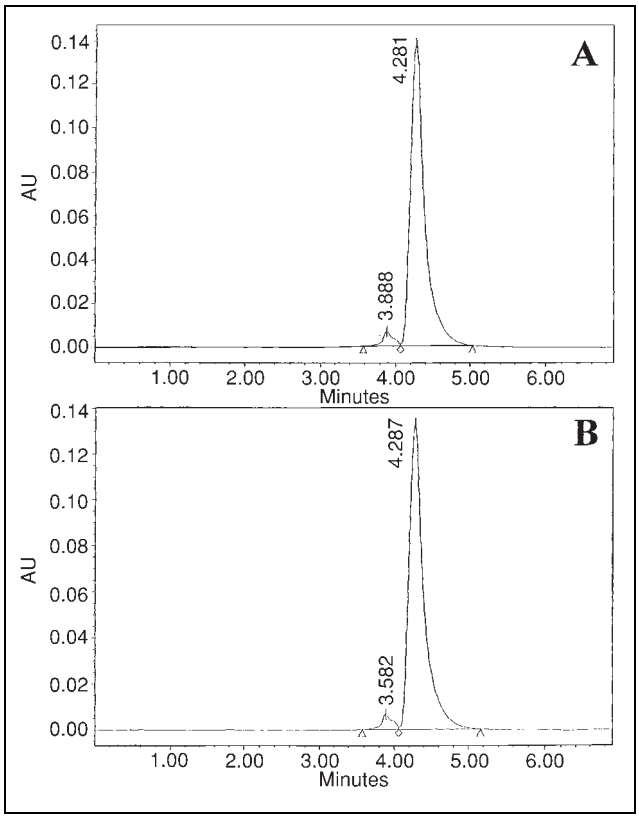


Fig. 1: HPLC chromatograms of BTMP in 0.1 M HCl solution at 60 °C at zero time (PA: 858503) (A) and after 3 hours (PA: 843032) (B)

Table 1: Degradation kinetic parameters of BTMP as derived from pseudo-first order plots

	0.1 M HCl			Phosphate buffer (pH 7.5)			Phosphate buffer (pH 9.0)		
	30 °C	40 °C	60 °C	30 °C	40 °C	60 °C	30 °C	40 °C	60 °C
K_{deg} (h^{-1})	0.0017	0.0027	0.0052	0.006	0.058	0.231	0.183	0.191	0.638
	± 0.0004	0.0002	0.0009	0.001	0.004	0.008	0.003	0.001	0.017
$t_{1/2}$ (h)	408.6	260.5	135.7	117.7	12.0	3.00	3.67	3.63	1.06
	± 24.1	15.2	22.0	19.9	0.74	0.10	0.06	0.03	0.03

chromatograms of BTMP in 0.1 M HCl solution at zero-time and after 3-h incubation at 60 °C. The chromatograms showed the base peak of BTMP at retention time ~4.26 min. The small peak at 3.89 min may be attributed to impurities present in the investigated compound. In physiological phosphate buffer (pH 7.5) and basic phosphate buffer (pH 9.0) solutions, the major peaks originating from BTMP appeared at shorter retention times (~3.2 min.). This may be attributed to the ionic character of BTMP molecule in basic solutions. The peak area of BTMP progressively decreased with increasing time of incubation. An approximately 45.2% reduction of the peak area was calculated following incubation of BTMP in basic solution for 3 hours at 30 °C. These data can be compared with those of samples of BTMP in 0.1 M HCl solution, where ~1% of compound was degraded. In physiological phosphate buffer solution (pH 7.5), BTMP displayed a slower rate of degradation compared to the basic solution at pH 9.0.

Determination of the kinetic parameters of degradation of BTMP was based on the decrease of the PA of BTMP under controlled experimental conditions. Plotting of the logarithmic values of the remaining concentrations, expressed as percentages, in various solutions versus time indicated that the degradation of BTMP followed pseudo-first order kinetics as the reaction was catalyzed by the presence of base. Furthermore, degradation was significantly enhanced by elevation of temperature. A summary of the various kinetic parameters for the degradation of BTMP is given in Table 1. To determine the degradation kinetics of BTMP at room temperature (25 °C) and at storage temperature (4 °C), Arrhenius plots were constructed [5]. Using least squares regression, linear correlations of the observed k_{deg}, computed from the degradation plots at different temperatures, versus 1/T were obtained (Table 2). As derived from Arrhenius plots, BTMP would appear to be highly stable in 0.1 M HCl, less stable in phosphate buffer solution (pH 7.5) and highly unstable in phosphate buffer solution (pH 9.0). The predicted kinetic parameters for the degradation of BTMP at 25 °C and 4 °C are summarized in Table 3. As indicated, the stability of BTMP solutions is optimized by adjusting the acidity to pH 1 and controlling the temperature at 4 °C. The data also suggest that BTMP would have better stability in simulated gastric fluid compared to intestinal fluid.

Although the degradation of BTMP in basic solution was proven using kinetic studies, it was not, however, possible to identify the chemical structure of the degradation products. In this context, LC/MS was selected because of its high specificity and sensitivity. LC/MS spectra of BTMP and its base-induced degraded solution at pH 9.0 were measured under the LC/MS conditions selected. The TIC chromatogram (Fig. 2) displayed a peak at 3.76 min which corresponds to a single molecular mass ion (m/z: 274.4) of BTMP. The TIC of the degraded solution of BTMP showed a peak at 3.84 min (Fig. 3), showed a peak at 3.84 min which corresponds to the fragment ions m/z 247.8, 217.8, 181.6, 167.3 and 149.7, respectively. This indicates that the LC peak refers to multiple components, which were not separated in the chromatogram. However, they were identified by their mass ratio values in the MS. A postulated Scheme for the elucidation of these fragments is reported. As indicated, there is good agreement between the measured and predicted values. According to these Schemes, two degradation pathways of BTMP (I and II) were proposed. The first pathway was through splitting of the thiocyanate moiety from to yield 5-benzyloxypyran-2-ol-4-one (m/z 217.8), whereas the other pathway was through splitting of the benzyl moiety from the molecule of BTMP to produce 2-thiocyanatomethyl-pyran-5-ol-4-one (m/z 181.6).

In conclusion, the new synthetic 4-pyranone (BTMP) was significantly stable in acidic solutions, but was highly unstable under basic conditions. Its stability to a large extent affected by storage temperature.

Table 2: Regression equations of Arrhenius plots of BTMP

Solvent	Regression equation
0.1 M HCl solution	$\log K_{deg} = 2.63 - 1637.9/T$ (r: 0.9912)
Phosphate buffer solution (pH 7.5)	$\log K_{deg} = 14.26 - 4939.9/T$ (r: 0.9617)
Phosphate buffer solution (pH 9.0)	$\log K_{deg} = 5.54 - 1922.1/T$ (r: 0.9570)

T: °C + 273

Table 3: Predicated kinetic parameters of BTMP as derived from Arrhenius plots at 25 °C and 4 °C

Solvent	Temperature 25 °C			Temperature 4 °C		
	K_{deg} (h^{-1})	$t_{1/2}$ (h)	t_{90} (h)	K_{deg} (h^{-1})	$t_{1/2}$ (h)	t_{90} (h)
0.1M HCl	0.0014	499.0	75.0	0.0006	1155.0	175.0
Phosphate buffer (pH 7.5)	0.0045	154.0	23.3	0.0016	433.1	65.5
Phosphate buffer (pH 9.0)	0.125	5.5	0.8	0.04	17.3	2.6

$t_{1/2}$: $0.693/k_{deg}$; t_{90}: $0.105/k_{deg}$

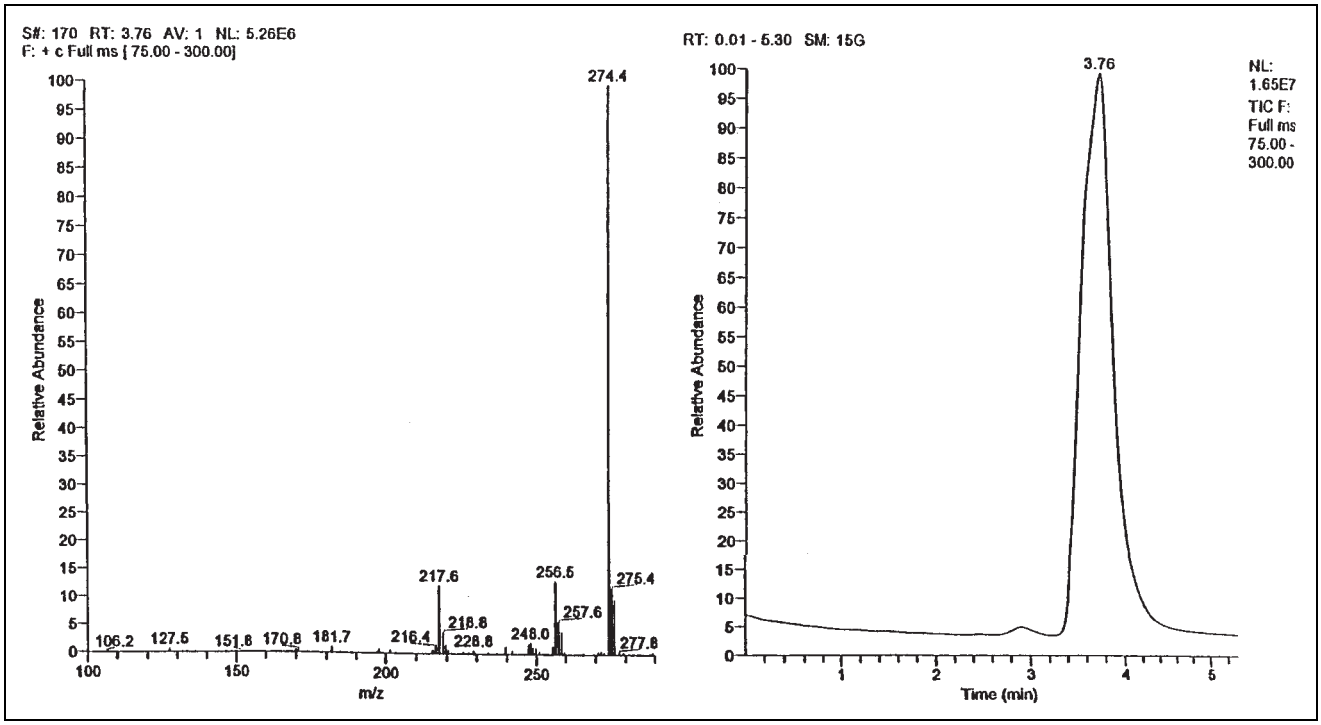


Fig. 2: Full-scan LC/MS spectra of BTMP

3. Experimental

3.1. Materials

BTMP (formula shown in the LC-MS degradation Scheme) was prepared according to the reported procedure [4]. Characterization and structure elucidation of BTMP were confirmed by elemental and spectral analyses. Water was purified by Milli-Q-System from Millipore Corporation (Milford, MA, USA). All chemicals and reagents were of analytical grade and the solvents were of HPLC grade.

3.2. Instruments

Accelerated stability studies were conducted using HPLC analysis. An isocratic high-performance liquid chromatograph (Waters 2690 Separation Module, USA) connected to an auto-sampler (Waters, USA) and photodiode array detector (Waters 996, USA) was used. Chromatographic separations were run at ambient temperature using a Shim Pack CLC-SIL (C18, 5 μ, 150 × 6 mm) column. The mobile phase consisted of methanol and 5% acetic acid solution (1 : 1 v/v). The flow rate was 1.0 ml min^{-1} and the effluent was monitored at 254 nm. Quantitative analyses were based on peak area measurements processed by the instrument's built-in Millennium software. Identification of the degradation products was performed using LC/MS. Mass spectrometric analyses were performed using a quadruple ion-trap Finnigan mass spectrometer. APCI was used as the ionization process. The APCI conditions were: vaporization temperature 430 °C, sheath gas flow 60 ml · min^{-1}, discharge current 5 μA, corona discharge 4.38 kV and capillary temperature 150 °C. The mass spectrometer was programmed

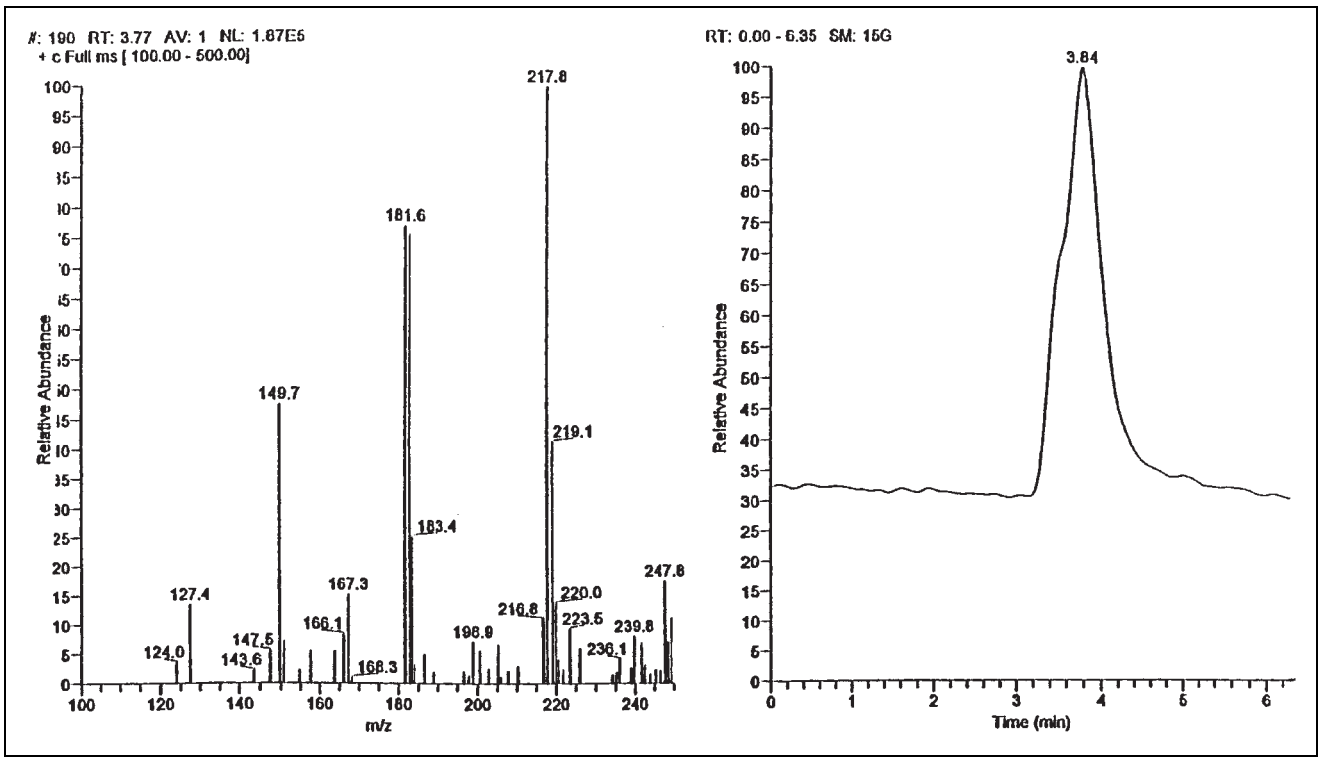
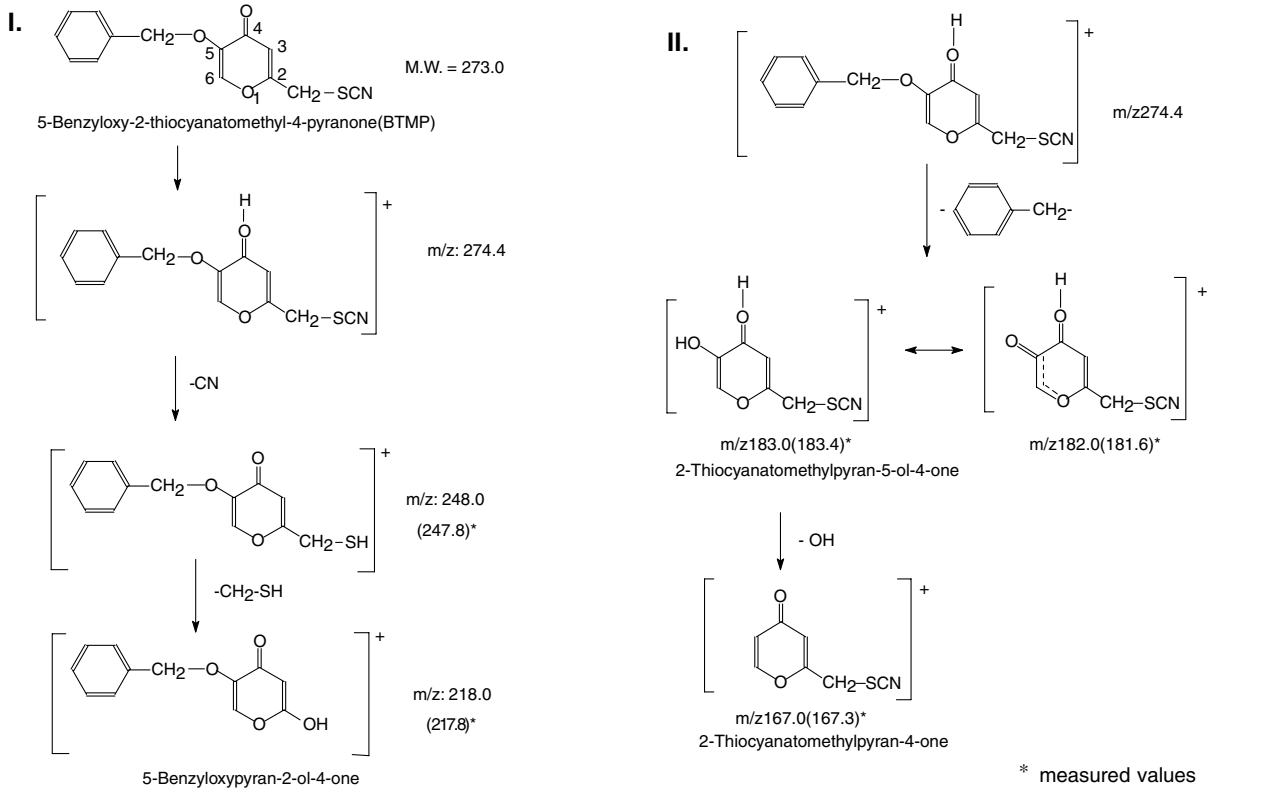


Fig. 3: Full-scan LC/MS spectra of base-induced degradation solution of BTMP

Scheme



to detect positive ions in the range m/z 100–500. Analytical data were processed by the instrument's built-in LCQ software. LC analyses were performed using an HPLC pump (Spectra System P 2000, TSP) linked to a Shim Pack CLC-CN, C18 (150 × 6 mm, 5 µ) column. The mobile phase was prepared from methanol and 1% acetic acid solution (4 : 1) and was pumped at a flow rate of 1 ml min^{-1}. Injection of the samples was done manually using a 10-µl syringe.

3.3. Methods

3.3.1. Calibration curve for HPLC

Approx ∼10 mg of BTMP powder was weighed accurately, transferred into a 10-ml volumetric flask and diluted to volume with methanol (1 mg · ml^{-1}). The solution was stable for ∼one week, when stored at 4 °C. A working standard solution of BTMP in methanol at a concentration of 100 ng · μl^{-1} was prepared. Aliquots were diluted to 1 ml with methanol to obtain a calibration curve in the concentration range 2.5–10 µg · ml^{-1}. A 10 µl-aliquot was injected into HPLC and the corresponding peak area (PA) was computed automatically by the instrument software.

3.3.2. Kinetic procedure of BTMP

Aliquots of the methanolic solution of BTMP (100 ng · μl^{-1}) were transferred into 1-ml vials and were diluted to 1 ml with 0.1 M HCl solution or phosphate buffer solutions at pH 7.5 or 9.0. The vials were vortexed and placed in a water bath at 30, 40 and 60 °C for the appropriate periods of time. An aliquot of 10 µl was automatically injected into the HPLC and analyzed. The concentrations of BTMP at zero- and at various time-intervals were determined from the regression equation representing the calibration curve using the HPLC procedure. The degradation kinetic parameters, k_{deg} and $t_{1/2,}$ in the designated solutions, were calculated from the degradation plots of the logarithmic values of the percentages remaining and time. The predicted kinetic parameters for the degradation of BTMP at 25 °C and 4 °C were extrapolated from Arrhenius plots [5].

3.3.3. Preparation of base-induced degradation products

In a 10-ml tube, an aliquot of 1 ml solution of BTMP in methanol (∼10 µg · ml^{-1}) was mixed with ∼1 ml of phosphate buffer solution (pH 9.0). The mixture was heated at 60 °C for 1. Aliquots were neutralized, injected into LC/MS and analyzed under the above instrumental conditions.

Acknowledgement: The authors are grateful to Kuwait University for supporting this work through Grant No. PC 03/99.

References

1 Ching, L.-M.; Baguley, B. C.:. Eur. J. Cancer Clin. Oncol. **23**, 1047 (1987)
2 Havsteen, B.: Biochem. Pharmacol. **32**, 1141 (1983)
3 Balaz, S.; Sturdik, E.; Ujhelyova, R.; Valigura, D.; Uher, M.; Veverka, M.; Konecny, V.; Adamcova, J.; Michalik, P.; Brtko, J.: Collect. Czech. Chem. Commun. **58**, 693 (1993)
4 Bransova, J.; Uher, M.; Novotny, L.; Brtko, J.: Anticancer Res. **17**, 1175 (1997)
5 Carstensen, J. T.: Drug Stability – Principles and Practices, 2nd Ed., Marcel Dekker, Inc., New York – Basel – Hong Kong 1995.

Received September 11, 2001
Accepted September 17, 2001

Prof. Ladislav Novotny
Department of Pharmaceutical Chemistry
Faculty of Pharmacy
Kuwait University
P.O. Box 24923 Safat
13110 Kuwait
Novotny@hsc.kuniv.edu.kw