BioImpacts, **2011**, 1(1), 53-62

http://bi.tbzmed.ac.ir/

Simultaneous Determination of 6-Mercaptopurine and its Oxidative Metabolites in Synthetic Solutions and Human Plasma using Spectrophotometric Multivariate Calibration Methods

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ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 11 February 2011
Revised: 22 February 2011
Accepted: 16 March 2011
ePublished: 9 June 2011

Keywords:
6-Mercaptopurine
Oxidative Metabolites
Multivariate
Spectrophotometric

ABSTRACT

Introduction: 6-Mercaptopurine (6MP) is an important chemotherapeutic drug in the conventional treatment of childhood acute lymphoblastic leukemia (ALL). It is catabolized to 6-thiouric acid (6TUA) through 8-hydroxo-6-mercaptopurine (8OH6MP) or 6-thioxanthine (6TX) intermediates. ***Methods:*** High-performance liquid chromatography (HPLC) is usually used to determine the contents of therapeutic drugs, metabolites and other important biomedical analytes in biological samples. In the present study, the multivariate calibration methods, partial least squares (PLS-1) and principle component regression (PCR) have been developed and validated for the simultaneous determination of 6MP and its oxidative metabolites (6TUA, 8OH6MP and 6TX) without analyte separation in spiked human plasma. Mixtures of 6MP, 8-8OH6MP, 6TX and 6TUA have been resolved by PLS-1 and PCR to their UV spectra. ***Results:*** Recoveries (%) obtained for 6MP, 8-8OH6MP, 6TX and 6TUA were 94.5-97.5, 96.6-103.3, 95.1-96.9 and 93.4-95.8, respectively, using PLS-1 and 96.7-101.3, 96.2-98.8, 95.8-103.3 and 94.3-106.1, respectively, using PCR. The NAS (Net analyte signal) concept was used to calculate multivariate analytical figures of merit such as limit of detection (LOD), selectivity and sensitivity. The limit of detections for 6MP, 8-8OH6MP, 6TX and 6TUA were calculated to be 0.734, 0.439, 0.797 and 0.482 µmol L-1, respectively, using PLS and 0.724, 0.418, 0783 and 0.535 µmol L-1, respectively, using PCR. HPLC was also applied as a validation method for simultaneous determination of these thiopurines in the synthetic solutions and human plasma. ***Conclusion:*** Combination of spectroscopic techniques and chemometric methods (PLS and PCR) has provided a simple but powerful method for simultaneous analysis of multicomponent mixtures

Introduction

6-Mercaptopurine (6MP) is an antimetabolite with antineoplastic and immunosuppressive activities used, usually in combination with other drugs, for treatment of leukemia (Schmiegelow *et al.*, 1994). The 6MP is also active metabolite of the immunosuppressive drug, azathioprine (Dubinsky, 2004). In fact, 6MP is a prodrug, which is activated to 6-thioguanosine-5'-phosphate and 6-thioinosine by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) inside the cell. These metabolites inhibit *de novo* synthesis of purine, thereby blocking the formation of purine nucleotide and inhibiting DNA synthesis. In addition, 6MP exerts its effect through incorporation into DNA in the form of deoxythioguanosine which results in the disruption of DNA replication (Lennard *et al.*, 1989, Zimm *et al.*, 1984). Apart from the above metabolic pathway, 6MP could also enter the catabolic pathway in which the drug is degraded to its inactive forms. In this pathway, 6MP is oxidized to 6-thiouric acid (6TUA) by xanthine oxidase as the major enzyme and aldehyde oxidase through either 8-hydroxo-6-mercaptopurine (8OH6MP) intermediate or 6-thioxanthine (6TX) intermediate (Fig.

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1) (Elion, 1967, Rashidi *et al.*, 2007, Van Scoik *et al.*, 1985).

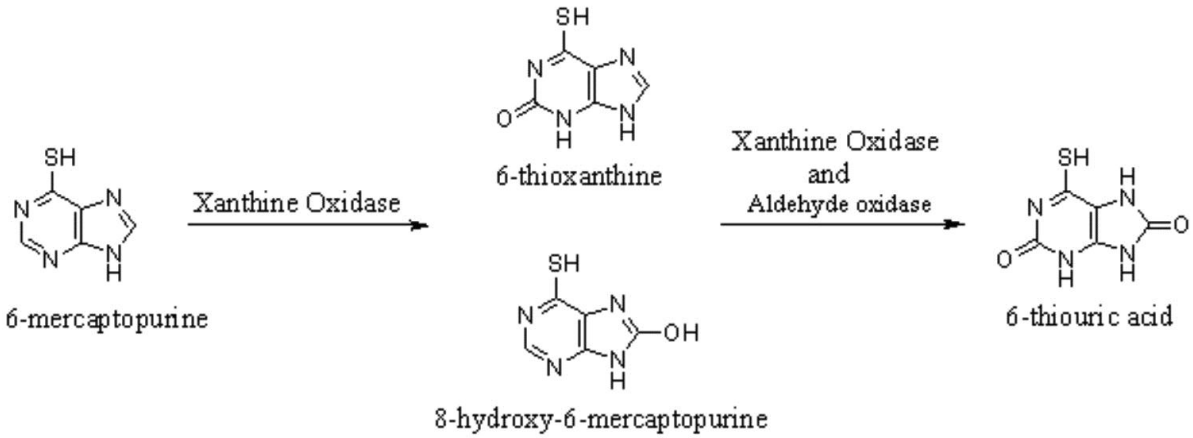

Fig. 1. Proposed catabolic pathways for 6MP

To study a metabolic pathway of a drug such as the oxidative metabolic pathway of 6MP, all components of the sample must be analyzed. The simultaneous determination of several components in the biological matrices, especially when their analytical characteristics are similar to each other, can be a difficult and challenging task. To tackle these challenges, chromatographic techniques are used frequently in analyzing of multicomponents sample. Accordingly, different liquid chromatographic methods have been developed and reported for simultaneous determination of 6MP and its metabolites in biological fluids (Breter and Zahn, 1977, Ding and Benet, 1979, Erb *et al.*, 2003, Hawwa *et al.*, 2009, Kato *et al.*, 1991, Oliveira *et al.*, 2004, Su *et al.*, 1999, Warren and Slordal, 1993, Weller *et al.*, 1995). However, chromatographic methods are usually expensive, tedious and time consuming processes. Other methods such as spectrophotometric techniques are among simple analytical methods for quantitative analysis. However, these techniques usually require that the components under study in a given sample to have different spectrum without overlapping. Recently, multicomponent systems based on chemometric methods have become an important tool in resolution of mixtures into their components in different fields including biomedical, clinical, environmental and drug analysis (Sorouraddin *et al.*, 2008, Fang and Liu, 2001).Taking into account the simplicity of spectrophotometry and higher efficiency of chemometric methods in resolution of mixtures into their components, a combination of these two techniques may provide simple and meanwhile powerful methods for simultaneous analysis of multicomponent mixtures, particularly in biological samples. Therefore, the use of chemometric-spectrophotometric methods could enhance the signal-to-noise ratio, improve selectivity of determination, optimize experimental conditions, raise analytical operation efficiency and provide muchscientific information. Among the various chemometric approaches applied to multicomponent analysis, classical least squares (CLS), principal component regression (PCR) and partial least-squares regression (PLS-1) have been successfully adopted in many quantitative assays of pharmaceutical formulations (Ferraro *et al.*, 2004, Haaland and Thomas, 1988, Frenich *et al.*, 1997, Ragno *et al.*, 2004).

In the present study, we have developed a simple spectrophotometric method for simultaneous quatification of 6MP and its three oxidative metabolites, 6TUA, 8OH6MP and 6TX in synthetic solutions and plasma using PLS-1 and PCR technique. According to the best of our knowledge, the simultaneous determination of 6MP and its metabolites by spectroscopic methods has not been reported. Resolution of binary, ternary and quaternary mixtures of analytes with minimum sample pre-treatment and without analyte separation has been successfully achieved by analyzing the UV spectral data. In order to evaluate and validate the results obtained by PLS-1 and PCR methods, the HPLC method was also employed and the results of both techniques were compared.

Materials and methods

Materials

All experiments were performed with analytical-reagent grade chemicals. 6MP and 6TX were purchased from Sigma Aldrich. Trichloroacetic acid (TCA) was obtained from Merck (Darmstatd, Germany). 6TUA and 8OH6MP were prepared as described previously (Li Loo *et al.*, 1959). Acetonitrile (HPLC grade) was prepared from Fluka. The stock solution of 5×10^{-3}mol L^{-1} was prepared by dissolving the four purine compounds (6MP, 6TX, 8OH6MP and 6TUA) in NaOH solution (0.1 mol L^{-1}). Then, the solution was diluted to a final volume in a 10-ml flask with doubly distilled water and the working solution was prepared by appropriate dilution with Sorenson's phosphate buffer (pH 7.0) containing 0.001 mol L^{-1} EDTA. Human plasma was obtained from the Iranian Blood Transfusion Organization.

Apparatus and software

Spectrophotometric measurements were performed by a Shimadzu 2550 UV–Vis spectrophotometer controlled by the Shimadzu UV Probe personal software package. A cuvette with a path length of 1.0 cm was used. The instrument was connected to a Shimadzu cell temperature control unit. The chromatographic analysis was carried out using a system supplied by Waters Associates, Northwich and Cheshire consisting of a Waters 515 pump, Waters 717 plus Autosampler, and Waters 2487 Dual λ Absorbance Detector.

The data were handled using MATLAB software (7.0) and PLS-1, and PCR were applied with MULTIVAR program, written in Visual Basic 5.0.

Procedures

Individual calibration

In order to determine the linear dynamic concentration range of each thiopurine, individual calibration in the range of 0.4-100.0 µmol L^{-1} was performed. To find the calibration curves of the compounds, the absorbance of 6MP, 6TX, 8OH6MP and 6TUA in Sorenson's phosphate buffer (pH 7.0) containing 0.001 mol L^{-1} EDTA was recorded at 325, 342, 255 and 349 nm, respectively. The blank solution was Sorenson's phosphate buffer (pH 7.0) containing 0.001 mol L^{-1} EDTA. Analytical figures of merit for individual calibration of 6MP, 8OH6MP, 6TX, and 6TUA are shown in Table 1. The spectra of analytes are significantly overlapped. Therefore, this calibration procedure failed to determine the concentration of the thiopurines in the multicomponent sample.

Standard solutions and calibration graphs For HPLC method

A C18 column and a mobile phase comprising acetonitrile (2%)/ water/ ammonium acetate (pH= 4.65) at a flow rate of 1.0 ml/min were operated. The column pressure, using this condition, was set to be at 1200 PSI. The absorbance measurement was performed at 255 nm for 8OH6MP and 340 nm for 6MP, 6TX and 6TUA. The standard solutions were prepared by further dilutions of the stock standard solutions with the mobile phase to reach the concentration range of 0.4-100 µmol L^{-1} for 6MP, 6TX and 8OH6MP and 0.2-100 µmol L^{-1} for 6TUA.

Triplicate 50 µl injections were made for each concentration and chromatographed under the specified chromatographic conditions described before. The peak height values were plotted against corresponding concentrations.

PLS-1 and PCR determination of thiopurines in pure solvent

Two sets of standard solutions were prepared. A orthogonal array designed calibration mixture of 6MP, 8OH6MP, 6TX and 6TUA, using a 25 sample set, were prepared as follows: appropriate volumes of the standard solutions were prepared in Sorenson's phosphate buffer (pH 7.0) containing 0.1 mmol L^{-1} EDTA. The concentrations of each compound in the resulting solutions were in the dynamic linear range of 0.1-100.0 µmol L^{-1}. The composition of calibration set was chosen by orthogonal array design, is shown in Table 3, and the composition of validation set was randomly chosen. The absorption spectra of the mixtures were recorded in the wavelength range 230- 400 nm with 1nm intervals.

PLS-1 and PCR determination of thiopurines in plasma

Plasma spiked 6MP, 6TX, 8OH6MP and 6TUA were obtained by diluting aliquots of the stock standard solutions of these compounds with the human plasma in a 1:1 ratio. To analyze plasma samples and determine the concentration of6MP, 6TX, 8OH6MP and 6TUA in the plasma, proteins were precipitated from plasma, before recording the absorbance spectra. For this purpose, 0.5 mL of TCA (1.5 mol L^{-1}) was added to 1.0 mL of the spiked plasma, and the precipitated proteins were separated by centrifugation for 15 min at 7500 rpm. The pH of the clear supernatant layer was adjusted to 7 with NaOH solution (1.5 mol L^{-1}). The same procedure was applied for the blank sample. The absorbance spectrum of each resulted solutions was recorded against the blank sample.

Analysis of 6MP, 6TX, 8OH6MP and 6TUA

A HPLC method was also employed for analysis of the mixtures of 6MP, 6TX, 8OH6MP and 6TUA in the synthetic samples and human plasma. The plasma proteins were precipitated according to the procedures discussed in the previous section. After the preparation of the solutions, a 50 µl aliquot of the samples was

injected into HPLC system. The mobile phase consisted of a mixture of 0.05 mol L^{-1} ammonium acetate (pH= 4.65)-acetonitrile (98:2, v/v). Separations were performed on a C-18 column (Perfectsil Target ODS-3 (5 µM), 250×4.6 mm) with a C-18 guard column (Perfectsil Target ODS-3 (5 µM), 10×4 mm). The effluent was monitored by UV detection at 255 and 340 nm at a flow rate of 1.0 ml/min.

Results and Discussion

Spectral features

The characteristics of the calibration graph and the statistical parameters for determination of analytes under optimum conditions are given in Table 1.

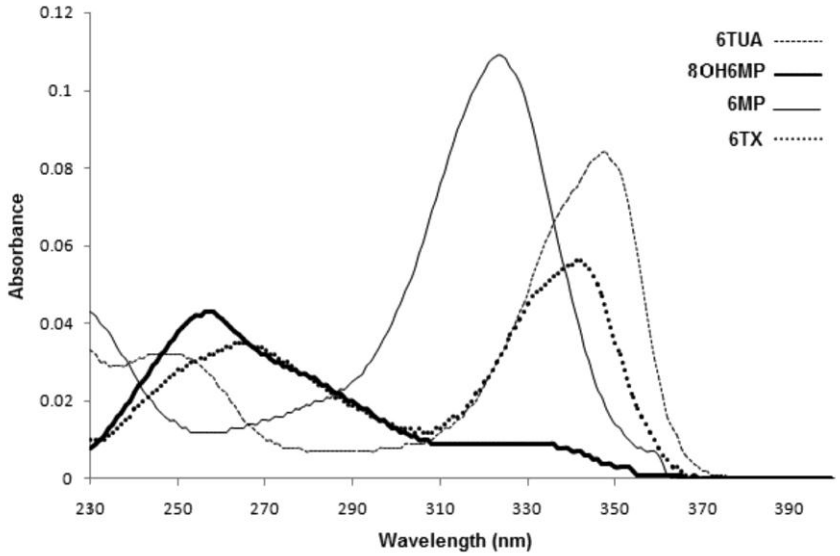

Fig. 2. Pure spectra obtained for 5×10−6 mol L−1 6TX, 8OH6MP, 6MP and 6TUA in 67 mmol L-1 Sorenson's phosphate buffer, pH 7 containing 0.1 mmol L-1 EDTA.

Analytical figures of merit of 6MP, 8OH6MP, 6TX, and 6TUA obtained for HPLC analysis are shown in Table 2. Fig. 2 shows the UV absorption spectra of 6MP, 6TX, 8OH6MP and 6TUA at their nominal concentrations. As can be seen, the UV absorption spectra of 6MP, 6TX, 8OH6MP and 6TUA are overlapped; and the UV absorption spectra of 6MP is also highly overlapped with 6TUA and 6TX spectra. The simultaneous determination of such drugs in their mixtures by conventional spectrophotometric methods is not possible due to strong spectral overlap throughout the wavelength range. Thus, PLS-1 as well as PCR calibration methods were used to overcome spectral overlapping and measure the concentration of each compound in the multicomponent sample.

The effects of pH changes on the spectrum of each thiopurine at a constant concentration, 5µmol L^{-1}, were investigated separately over the pH range of 2–12. No significant change was observed in spectrum of thiopurines over the pH range. Therefore, pH 7, as the physiological pH, was selected for simultaneous determination of these compounds.

Multivariate methods

The PLS and PCR techniques are typical full-spectrum methods, thereby making this method more powerful than the direct spectrophotometry (one-wavelength method). In the PLS and PCR techniques, the simultaneous inclusion of multiple spectral intensities can greatly improve the precision and applicability of quantitative spectral analysis of mixtures.

Before using the PLS-1 and PCR in human plasma for simultaneous determination of concentrations of 6MP and its metabolites, these methods were applied for measuring of concentration of these compounds in their synthetic mixtures.

The optimum number of factors to be used within the multivariate algorithms is an important parameter to achieve better performance in prediction. This allows modeling of the system with the optimum amount of information, avoiding over fitting. We have applied the cross-validation procedure in all cases, which consists of systematically removing one of the training samples, in turn, and using only the remaining ones for construction of the latent factors and/or regression coefficients. The predicted concentrations were then compared with the actual ones for each of the calibration samples, and the predicted error sum of squares [$PRESS=\sum(C_{act}-C_{pred})^2$] was calculated. The PRESS was computed in the same manner, each time a new factor was added to the PLS-1 model. The optimum number of factors or latent variables for each analytes is shown in Table 3. The PLS-1 and PCR methods were evaluated, and a comparative study of the prediction capabilities of the two chemometric approaches in this work was undertaken. To compare the effect of the type of regression method on the analysis of 6MP, 6TX, 8OH6MP and 6TUA by spectrophotometric technique, in addition to PLS-1, PCR method was employed. In the case of both techniques, the spectral range was set at 230-400 nm. Table 4 shows the obtained values for the PRESS, relative error of prediction (REP%) and optimum numbers of factors for the studied analytes given by PLS-1 and PCR of the spectral data.

The results obtained by PLS and PCR methods were in good agreement and both methods were suitable for analysis of these compounds. PCR is principal component analysis which predicts response variables from factors underlying the predictor variables. Whereas the PLS algorithm chooses X-scores of the latent independents to be paired as strongly as possible with Y-scores of the latent response variable(s), PCR selects X-scores to explain the maximum proportion of factor variation (Malthouse *et al.*, 1997, Ergon, 2003).To compare the PLS-1 and PCR methods, estimation of analytical figures of merit by multivariate analysis is possible.

Table 1. Analytical figures of merit for 6MP, 8OH6MP, 6TX, and 6TUA

Parameters	6TUA	8OH6MP	6TX	6MP
Λ	349	255	342	325
Dynamic linear range (µmol L^{-1})	0.4-100	0.4-100	0.4-100	0.4-100
Correlation coefficient	0.9986	0.9996	0.9999	0.9999
Limit of detection (µmol L^{-1}) (n=5)	0.055	0.064	0.061	0.053
Limit of quantification(µmol L^{-1}) (n=5)	0.183	0.213	0.203	0.177
RSD (%)[a]	0.81	0.57	0.68	0.52
Equation of calibration curve (n=5) (absorbance versus mol L^{-1} of analyte)	A=18088C -0.005	A=11505C -0.008	A=12657C -0.001	A=21710C+0.003

[a]The concentration of each analyte used for RSD determination was 5 µmol L^{-1}.

Table 2. HPLC Analytical figures of merit for 6MP, 8OH6MP, 6TX and 6TUA

Parameters	6TUA	8OH6MP	6TX	6MP
λ	340	255	340	340
Dynamic linear range (µmol L^{-1})	0. 2-100	0.4-100	0.4-100	0.4-100
Correlation coefficient	0.9989	0.9999	0.9999	0.9998
Limit of detection (µmol L^{-1}) (n=5)	0.034	0.060	0.064	0.060
Limit of quantification (µmol L^{-1}) (n=5)	0.113	0.200	0.213	0.199
RSD (%)[a]	2.66	0.83	1.17	1.27
Equation of calibration curve(n=5) (absorbance versus mol L^{-1} of analyte)	$A=4.0\times10^9C-6392$	$A=2.0\times10^9C-4.2$	$A=1.0\times10^9C-248$	$A=1.0\times10^9+1047$

[a] The concentration of each analyte used for RSD determination was 5 µmol L-1.

Different figures of merit such as selectivity (SEL), sensitivity (SEN) and limit of detection (LOD) have been reported in the literature to quantify the quality of a given multivariate method (Martínez Galera *et al.*, 2003).

Table 3. Composition of calibration set in coded values

Experiment	6MP	8H6MP	6TUA	6TX
1	0	0	0	0
2	0	-2	+2	-2
3	-2	-2	-1	+2
4	-2	+2	+2	-1
5	+2	-1	0	+2
6	-1	+2	-1	0
7	+2	0	-1	-1
8	0	-1	+1	-1
9	-1	-1	+2	+1
10	-1	+1	+1	+2
11	+1	+2	0	+1
12	+2	+1	+2	0
13	+1	0	+2	+2
14	0	+2	-2	+2
15	+2	+2	+1	-2
16	+2	-2	-2	+1
17	-2	+1	0	-2
18	+1	-2	+1	0
19	-2	0	+1	+1
20	0	+1	-1	+1
21	+1	+1	-2	-1
22	+1	-1	-1	-2
23	-1	-2	0	-1
24	-2	-1	-2	0
25	-1	0	-2	-2

[a] +2, +1, 0, -1, and -2 codes are 25, 15, 10, 2.5 and 0.5 µmol L^{-1}, respectively.

When expressing figures of merit for multivariate calibration methods, the part of the signal that relates to the analyte is more important than the total signal (Goicoechea and Olivieri, 2000). This unique signal is termed net analyte signal (NAS) and has an important role in the calculation of figures of merit for characterizing a calibration model. It could be defined as the part of the spectral signal that is orthogonal to the signal of the interferences present in the sample (Goicoechea and Olivieri, 2000).

The SEL which range from 0 to 1 is used to measure how unique the signal of the analyte is compared with the other species. It can be calculated as:

$$SEL = \frac{\left\| s_k^* \right\|}{\left\| s_k \right\|} \quad (1)$$

where || || means the Euclidian norm of vector and s_k is a signal containing analyte *k* at unit concentration, and s_k^* is its corresponding NAS. The changes in response as a function of the concentration of a particular analyte can be measured by SEN using the following equation:

$$SEN = \left\| s_k^* \right\| \quad (2)$$

The calculated values for analytical figures of merit of 6MP, 6TX, 8OH6MP and 6TUA have been summarized in Table 5.

Comparison of the results of PLS-1 and PCR with HPLC

In order to evaluate the results of the PCR and PLS-1 regression, an HPLC procedure was also employed to analyze these compounds in synthetic mixtures and in the human plasma samples. Table 2 shows the HPLC Analytical figures of merit for 6MP, 8OH6MP, 6TX and 6TUA.

Fig. 3 illustrates the chromatogramsof human plasmasample spiked with standard substances (in concentrations of 50×10^{-6}mol L^{-1}).

A satisfactory separation was achieved using a method developed in our laboratory (Fig. 3). The retention time for 6MP, 6TX, 8OH6MP and 6TUA were found to be 14.4, 18.5, 8.2 and 7.1 min, respectively. Table 6 indicates the recoveries and relative standard deviations for determination of these thiopurines in synthetic mixtures and human plasma samples using the HPLC method. Recovery (%) values and the relative standard deviations for determination of four components in synthetic mixtures and human plasma samples using the PCR and PLS-1 methods are represented in Tables 7 and 8, respectively.

As seen in Tables 6-8, the results obtained by HPLC were in good agreement with the results obtained by the PCR and PLS-1 methods. In addition, the simplicity of the multivariate calibration methods capable of analyzing a large number of samples in a shorter time period along with acceptable accuracy and precision are the great advantages of these methods in comparison with the chromatographic method.

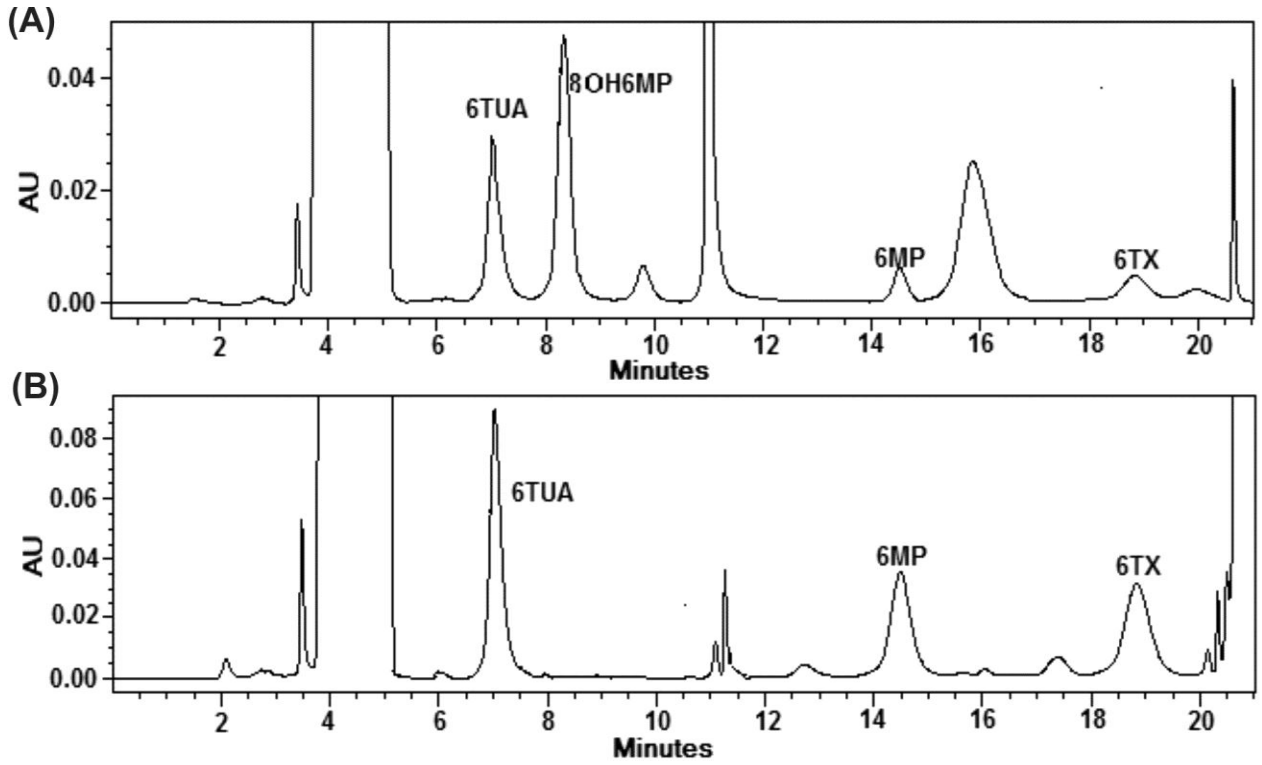

Fig. 3. HPLC chromatograms of spiked human plasma sample with 50×10−6 mol L−1 6TU, 8OH6MP, 6MP and 6TX monitored at 255 nm (a) and 340 nm (b) using a C18 column and a mobile phase comprising acetonitrile (2%)/ water / ammonium acetate (pH= 4.65) at a flow rate of 1.0 ml/min.

Table 4. Statistical parameters for 6MP, 6TX, 8OH6MP and 6TUA in validation sets of PLS-1 and PCR methods

Statistical parameters		Component			
		6MP	**8OH6MP**	**6TX**	**6TUA**
PLS-1	Spectral rage (nm)	230-400	230-400	230-400	230-400
	Factors	5	5	5	5
	PRESS	1.3	1.0	0.6	0.7
	REP (%)	6.75	8.80	8.38	9.82
PCR	Spectral rage (nm)	230-400	230-400	230-400	230-400
	Factors	5	5	5	5
	PRESS	1.6	1.2	0.7	0.8
	REP (%)	6.46	8.89	8.23	9.77

Table 5. Multivariate analytical figures of merit for 6MP, 6TX, 8OH6MP and 6TUA

Figures of merit	6MP		8OH6MP		6TX		6TUA	
	PLS-1	PCR	PLS-1	PCR	PLS-1	PCR	PLS-1	PCR
Limit of detection (μmol L^{-1})	0.730	0.724	0.439	0.418	0.767	0.783	0.482	0.535
Selectivity	0.51	0.49	0.32	0.36	0.57	0.62	0.38	0.40
Sensitivity (L μmol^{-1})	0.614	0.654	0.340	0.355	0.557	0.624	0.356	0.451

Table 6. Recoveries (%) for the target compound in synthetic mixtures and human plasma samples using HPLC method

Compound	Target concentration	In Synthetic mixtures		in human plasma	
		RSD%	Recovery	RSD%	Recovery
6MP	1	3.43	97.45	3.06	96.74
	5	2.11	101.74	1.86	98.40
	25	2.02	101.60	3.34	96.64
6TX	1	2.86	97.05	2.11	95.97
	5	1.08	101.34	1.95	99.42
	25	2.69	102.59	2.75	98.87
8OH6MP	1	3.47	98.12	1.86	94.57
	5	1.80	100.49	2.67	96.13
	25	2.12	101.48	1.60	98.08
6TUA	1	3.19	98.17	4.12	97.30
	5	0.86	101.04	2.59	98.49
	25	1.78	102.37	2.76	97.15

Table 7. Recoveries (%) andrelative standard deviations (RSD) of 6MP, 6TX,8OH6MP and 6TUA in the resolution of synthetic mixtures of these compounds by using PLS-1 and PCR

Concentration (μmol L^{-1})	6MP		8OH6MP		6TX		6TUA	
	PLS-1	PCR	PLS-1	PCR	PLS-1	PCR	PLS-1	PCR
1	97.7 (0.53)	97.3 (1.24)	97.5 (0.34)	99.4 (0.41)	100.7 (1.12)	101.1 (0.23)	95.9 (0.47)	102.4 (0.61)
5	105.2 (2.32)	103.6 (1.04)	97.5 (2.63)	98.9 (0.86)	100.9 (1.75)	102.9 (3.35)	94.9 (2.76)	98.1 (2.02)
25	103.5 (0.89)	102.9 (3.12)	102.1 (2.34)	104.8 (0.45)	99.8 (0.70)	102.4 (2.67)	98.4 (2.21)	96.2 (2.73)

Table 8. Recoveries (%) and relative standard deviations (RSD) for the target compound in human plasma samples using PLS-1 and PCR models

Concentration (μmol L^{-1})	6MP		8OH6MP		6TX		6TUA	
	PLS-1	PCR	PLS-1	PCR	PLS-1	PCR	PLS-1	PCR
1	94.5 (0.7)	96.7 (0.62)	98.9 (0.54)	97.9 (0.45)	96.8 (0.89)	103.3 (1.12)	93.4 (0.34)	94.3 (0.31)
5	95.7 (2.86)	101.3 (2.56)	96.6 (3.23)	98.8 (2.87)	96.9 (0.89)	97.4 (1.21)	95.8 (3.12)	106.1 (3.31)
25	97.5 (1.97)	96.7 (2.19)	103.3 (2.60)	96.2 (1.62)	95.1 (2.02)	95.8 (2.48)	93.8 (2.65)	96.5 (2.29)

Conclusion

Combination of spectroscopic techniques and chemometric methods (PLS and PCR) has provided a simple but powerful method for simultaneous analysis of multicomponent mixtures. PLS-1 and PCR were employed for simultaneous spectrophotometric determination of 6MP and its oxidative metabolites, and a comparative method using HPLC was employed to confirm the results obtained by the last two methods. The accuracy was tested with spiked samples, and the obtained recoveries were satisfactory. Although the HPLC method is mostly used to analyze the multicomponent samples, PLS and PCR methods are simpler, faster and less expensive. Thus, the proposed multivariate spectrophotometric calibration methods, as a simple, accurate and more precise method as compared to HPLC, can be used for simultaneous analysis of 6MP and its oxidative metabolites in plasma for biological studies such as pharmacokinetic analysis.

Ethical Issue

None to be declared.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgment

Authors are thankful to Tabriz University of Medical Sciences for the financial support.

References

Breter Hj and Zahn Rk. **1977**. The quantitative determination of metabolites of 6-mercaptopurine in biological materials. II. Advantages of a variable-wavelength HPLC spectrophotometric detector for the determination of 6-thiopurines. *J Chromatogr,* 137(1)**,** 61-8.

Ding Tl and Benet Lz. **1979**. Comparative bioavailability and pharmacokinetic studies of azathioprine and 6-mercaptopurine in the rhesus monkey. *Drug Metab Dispos,* 7(6)**,** 373-7.

Dubinsky Mc. **2004**. Azathioprine, 6-mercaptopurine in inflammatory bowel disease: pharmacology, efficacy, and safety. *Clin Gastroenterol Hepatol,* 2(9)**,** 731-43.

Elion Gb. **1967**. Symposium on immunosuppressive drugs. Biochemistry and pharmacology of purine analogues. *Fed Proc,* 26(3)**,** 898-904.

Erb N, Haverland U, Harms Do, Escherich G and Janka-Schaub G. **2003**. High-performance liquid chromatographic assay of metabolites of thioguanine and mercaptopurine in capillary blood. *J Chromatogr B Analyt Technol Biomed Life Sci,* 796(1)**,** 87-94.

Ergon R. **2003**. Constrained numerical optimization of PCR/PLSR predictors. *Chemometrics and Intelligent Laboratory Systems,* 65(2)**,** 293-303.

Fang G and Liu N. **2001**. Determination of eight essential amino acids in mixtures by chemometrics-spectrophotometry without separation. *Analytica Chimica Acta,* 445(2)**,** 245-253.

Ferraro Mc, Castellano Pm and Kaufman Ts. **2004**. Chemometric determination of amiloride hydrochloride, atenolol, hydrochlorothiazide and timolol maleate in synthetic mixtures and pharmaceutical formulations. *J Pharm Biomed Anal,* 34(2)**,** 305-14.

Frenich Ag, Galera Mm, Vidal Jm, Vázquez Pp and García Mg. **1997**. Comparative Study for Determining Diuron and Chlorpyrifos at PPB Levels by First Derivative Spectra and Multivariate Calibration Methods. *Analytical Letters,* 30(2)**,** 341 - 358.

Goicoechea Hc and Olivieri Ac. **2000**. MULTIVAR. A program for multivariate calibration incorporating net analyte signal calculations. *TrAC Trends in Analytical Chemistry,* 19(10)**,** 599-605.

Haaland Dm and Thomas Ev. **1988**. Partial least-squares methods for spectral analyses. 1. Relation to other quantitative calibration methods and the extraction of qualitative information. *Analytical Chemistry,* 60(11)**,** 1193-1202.

Hawwa Af, Millership Js, Collier Ps and Mcelnay Jc. **2009**. Development and validation of an HPLC method for the rapid and simultaneous determination of 6-mercaptopurine and four of its metabolites in plasma and red blood cells. *J Pharm Biomed Anal,* 49(2)**,** 401-9.

Kato Y, Matsushita T, Yokoyama T and Mohri K. **1991**. Determination of 6-mercaptopurine in acute lymphoblastic leukemia patients' plasma by high-performance liquid chromatography. *Ther Drug Monit,* 13(3)**,** 220-5.

Lennard L, Van Loon Ja and Weinshilboum Rm. **1989**. Pharmacogenetics of acute azathioprine toxicity: relationship to thiopurine methyltransferase genetic polymorphism. *Clin Pharmacol Ther,* 46(2)**,** 149-54.

Li Loo T, Michael Me, Garceau Aj and Reid Jc. **1959**. 6-Thiouric Acid—a Metabolite of 6-Mercaptopurine. *Journal of the American Chemical Society,* 81(12)**,** 3039-3041.

Malthouse Ec, Tamhane Ac and Mah Rsh. **1997**. Nonlinear partial least squares. *Computers & Chemical Engineering,* 21(8)**,** 875-890.

Martínez Galera M, Picón Zamora D, Martínez Vidal Jl, Garrido Frenich A, Espinosa-Mansilla A, Muñoz De La Peña A*, et al.* **2003**. Determination of carbendazim, thiabendazole and fuberidazole using a net analyte signal-based method. *Talanta,* 59(6)**,** 1107-1116.

Oliveira Bm, Romanha Aj, Alves Tm, Viana Mb and Zani Cl. **2004**. An improved HPLC method for the quantitation of 6-mercaptopurine and its metabolites in red blood cells. *Braz J Med Biol Res,* 37(5)**,** 649-58.

Ragno G, Ioele G and Risoli A. **2004**. Multivariate calibration techniques applied to the spectrophotometric analysis of one-to-four component systems. *Analytica Chimica Acta,* 512(1)**,** 173-180.

Rashidi Mr, Beedham C, Smith Js and Davaran S. **2007**. In vitro study of 6-mercaptopurine oxidation catalysed by aldehyde oxidase and xanthine oxidase. *Drug Metab Pharmacokinet,* 22(4)**,** 299-306.

Schmiegelow K, Schroder H and Schmiegelow M. **1994**. Methotrexate and 6-mercaptopurine maintenance therapy for childhood acute lymphoblastic leukemia: dose adjustments by white cell counts or by pharmacokinetic parameters? *Cancer Chemother Pharmacol,* 34(3)**,** 209-15.

Sorouraddin Mh, Fooladi E, Naseri A and Rashidi Mr. **2008**. A novel spectrophotometric method for determination of kinetic constants of aldehyde oxidase using multivariate calibration method. *J Biochem Biophys Methods,* 70(6)**,** 999-1005.

Su Y, Hon Yy, Chu Y, Van De Poll Me and Relling Mv. **1999**. Assay of 6-mercaptopurine and its metabolites in patient plasma by high-performance liquid chromatography with diode-array detection. *J Chromatogr B Biomed Sci Appl,* 732(2)**,** 459-68.

Van Scoik Kg, Johnson Ca and Porter Wr. **1985**. The pharmacology and metabolism of the thiopurine drugs 6-mercaptopurine and azathioprine. *Drug Metab Rev,* 16(1-2)**,** 157-74.

Warren Dj and Slordal L. **1993**. A sensitive high-performance liquid chromatographic method for the determination of 6-mercaptopurine in plasma using precolumn derivatization and fluorescence detection. *Ther Drug Monit,* 15(1)**,** 25-30.

Weller S, Thurmann P, Rietbrock N, Gossmann J and Scheuermann Eh. **1995**. HPLC analysis of azathioprine metabolites in red blood cells, plasma and urine in renal transplant recipients. *Int J Clin Pharmacol Ther,* 33(12)**,** 639-45.

Zimm S, Grygiel Jj, Strong Jm, Monks Tj and Poplack Dg. **1984**. Identification of 6-mercaptopurine riboside in patients receiving 6-mercaptopurine as a prolonged intravenous infusion. *Biochem Pharmacol,* 33(24)**,** 4089-92.