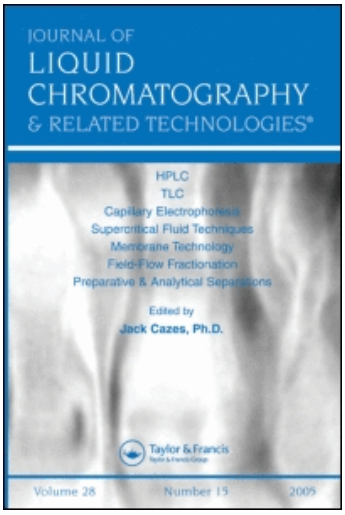

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Validation of a HPLC Method for the Determination of Propylthiouracil in Plasma

L. Kabanda[a]; C. de Muynck[a]; R. A. Lefebvre[b]; J. P. Remon[a]
[a] Laboratory of Pharmaceutical Technology, University of Gent, Gent, Belgium [b] Heymans Institute of Pharmacology, University of Gent Medical School, Gent, Belgium

To cite this Article Kabanda, L. , de Muynck, C. , Lefebvre, R. A. and Remon, J. P.(1994) 'Validation of a HPLC Method for the Determination of Propylthiouracil in Plasma', Journal of Liquid Chromatography & Related Technologies, 17: 9, 2069 — 2083
To link to this Article: DOI: 10.1080/10826079408013480
URL: http://dx.doi.org/10.1080/10826079408013480

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(9), 2069-2083 (1994)

VALIDATION OF A HPLC METHOD FOR THE DETERMINATION OF PROPYLTHIOURACIL IN PLASMA

L. KABANDA[1], C. DE MUYNCK[1], R. A. LEFEBVRE[2], AND J. P. REMON[1]

[1]*Laboratory of Pharmaceutical Technology*
University of Gent
Harelbekestraat 72
B-9000 Gent, Belgium

[2]*Heymans Institute of Pharmacology*
University of Gent Medical School
De Pintelaan 185
B-9000 Gent, Belgium

ABSTRACT

A high-performance liquid chromatography method for the analysis of propylthiouracil in plasma was validated. Methylthiouracil was used as the internal standard. A one step extraction procedure and an isocratic HPLC method with UV detection were used. No plasma components were found to interfere in the assay. Linear calibration ($r^2 > 0.99$) curves using water and plasma as matrices in the range of 0.05 to 15 $\mu g.mL^{-1}$ were obtained. Good recoveries for propylthiouracil (> 85%) and methylthiouracil (> 65%) were seen both in water and in plasma. The coefficient of variation for repeatability was < 7%, for reproducibility < 8% and for accuracy <6%. The limit of detection was 2 $ng.mL^{-1}$ in water and 5 $ng.mL^{-1}$ in plasma. A poor stability of propylthiouracil was observed even at -20°C and it is recommended to perform the analyze shortly after sampling.

INTRODUCTION.

Colorimetry, the first method reported for propylthiouracil (PTU) determination in serum or plasma [1], was lacking both specificity and sensitivity. Gas chromatographic, high-performance liquid chromatographic (HPLC) and radioimmunoassay methods were developed [2-11] to overcome this problem. Because gas chromatography can only be carried out after conversion of PTU into a salt, this method is difficult and time consuming [2]. Several radioimmunoassay methods have been published [10,11] but they all require extensive sample pretreatment and overnight incubation.

Several HPLC methods were developed, using ion exchange [3] or reversed-phase systems [4-9] with UV detection. Some of these methods do not use an internal standard [4] or add the internal standard only after extraction [5]. Other methods require a deproteinisation step before PTU quantization [7]. Extraction recoveries for the internal standard [8] or for both the internal standard and PTU [9] are often low.

The chromatographic method and the extraction procedure in this paper were adapted from Rosseel M.T. and Lefebvre R.A. [8] in order to obtain a better specificity and sensitivity with low intra and inter day variability. This optimized method is used to analyze plasma samples from dogs and humans in the development of a sustained release formulation (data not included).

MATERIALS AND METHODS.

Chemicals.

The products used were: PTU (USP reference standard, Rockville, MD, USA), PTU and methylthiouracil (MTU) (Sigma, St. Louis, MO, USA), methyl

alcohol (HPLC grade, Janssen Chimica, Geel, Belgium), dichloromethane (HPLC grade, Fluka AG, Buchs, Switzerland), acetone (pro analyze, UCB, Leuven, Belgium) tetrahydrofuran (HPLC grade, LAB-SCAN, Brussels, Belgium), sodium hydroxide (pro analyze, Novolab, Gent, Belgium), potassium dihydrogen orthophosphate (highest purity, UCB, Leuven, Belgium), hydrochloric acid (pro analyze, Novolab, Gent, Belgium), N,N-dimethylacetamide (E.Merck, Darmstadt, F.R.Germany) and distilled water. Stock solutions of PTU (1 $mg.mL^{-1}$) and MTU (0.5 $mg.mL^{-1}$) were prepared in methanol. These solutions were stored at 8°C and were stable for at least 20 days. Working solutions were made by diluting the stock solutions in distilled water.

Instrumentation.

The HPLC system consisted of an Iso-Chrom LC pump (Spectra Physics, San Jose, California, USA) and a Spectra SYSTEM UV 2000 variable wavelength detector (Spectra Physics, San Jose, California, USA) set at 275 nm (λ max of PTU and MTU). Compound separation was performed at ambient temperature on a reversed-phase column (5 μm particles Lichrospher RP-18; 125 mm x 4 mm, E.Merck, Darmstadt, F.R.Germany) equipped with a precolumn (5 μm particles Lichrospher RP-18; E.Merck, Darmstadt, F.R.Germany). A 0.9% tetrahydrofuran solution in phosphate buffer pH 6.0 $\pm$ 0.1 was used as the mobile phase. The composition of the buffer was 34 g of potassium dihydrogen orthophosphate, 170 mL of NaOH 0.2 N and enough distilled water to make 5 L [17]. The mobile phase was degassed before use. The flow rate was 1.5 $mL.min^{-1}$. The samples were injected via a septumless syringe-loaded injector loop of 20 μL (Valco

Instruments Corporation, Houston, USA). Peak area response was calculated using the Spectra Station software (Spectra Physics, San Jose, California, USA).

Sample preparation.

The stock solutions of PTU (1 $mg.mL^{-1}$) and MTU (0.5 $mg.mL^{-1}$) were prepared in methanol. Dilutions in distilled water were prepared in borosilicate volumetric flask and extractions were performed in 15-mL borosilicate glass tubes (16 x 100 mm, Corning, NY, USA). Next, 450 μL of water or plasma (from dogs) were pipetted into a 15-mL borosilicate glass followed by the addition of 60 μL of hydrochloric acid 0.1 N and 50 μL of the internal standard solution (25 $\mu g.mL^{-1}$ in water). The mixture was vortexed for 10 s. followed by the addition of 6 mL of organic phase (dichloromethane-acetone: 75-25, v/v). After vortexing during 2 min. and centrifugation at 1500 g for 5 min., the organic phase was transferred to another tube and evaporated to dryness at 60°C under nitrogen. The samples were redissolved in 250 μL of water and vortexed for 20 s. Twenty μL were injected and analyzed using the chromatographic conditions described above.

Calibration Curves (in water and in plasma).

The solutions used to prepare the calibration curves contained PTU in the following concentrations: 0.5, 5, 10, 30, 50, 100 and 150 $\mu g.mL^{-1}$. MTU was used in a concentration of 25 $\mu g.mL^{-1}$.

The calibration graphs were prepared by adding 50 μL of PTU and internal standard (MTU) to blank samples (450 μL) in order to provide concentrations in

the range of 0.05 to 15 $\mu g.mL^{-1}$ (0.05, 0.5, 1, 3, 5, 10 and 15 $\mu g.mL^{-1}$) for PTU and 2.5 $\mu g.mL^{-1}$ for MTU.

Stability of PTU in plasma.

Known amounts of PTU were added to blank plasma in order to obtain concentrations of 1 $\mu g.mL^{-1}$ and 10 $\mu g.mL^{-1}$. These samples were stored at -20°C and analyzed after various storage times (0, 15, 90, 150 and 180 days). The stability of the samples was expressed as the amount recovered with the coefficient of variation (n=4) and related to the freshly prepared samples.

Calculations.

The calibration graphs were obtained by plotting the peak-area ratio of PTU to the internal standard versus the PTU concentration calculating the regression line parameters. Unknown concentrations were determined from the regression equation.

RESULTS AND DISCUSSION.

Optimisation of mobile phase.

PTU is a very weak acid (pKa of 7.5) [16]. An extraction in the unionised form was performed after acidification of the medium. During the optimisation of the extraction procedure it was observed that PTU showed, even in the unionised form, a better solubility in polar solvents (acetone, methyl alcohol, N,N-dimethylacetamide, etc.). Optimal extraction yield was obtained with a dichloromethane-acetone mixture (75:25; v/v). The volume of the extraction

liquid was increased up to 6 mL. Using MTU as the internal standard, a better separation was obtained from PTU than when 5-PTU was used. Two absorption maxima were found for PTU and MTU at 214 and 275 nm. At 214 nm interferences of endogenous compounds with PTU and the internal standard were observed while at 275 nm no such interferences occurred. A detection at 300 nm as proposed by Rosseel and Lefebvre [8] caused a sensitivity loss of about 50%.

Selectivity.

Figure 1 shows chromatograms from extracted plasma (A), extracted plasma spiked with PTU and MTU (B), and extracted plasma after I.V. administration of 300 mg of PTU (C). No interference of endogenous compounds or anticoagulant (Heparin Novo 5000 U.I.mL^{-1}, Novo Nordisk A/S, Bagsvaerd, Denmark) used during sample collection were detected. Under the chromatographic conditions used, there was a very good separation between PTU and the internal standard with retention times of 15.20 ($\pm$ 10%) and
2.60 ($\pm$ 10%) min., respectively. It can be concluded that the proposed method is selective for PTU [12].

Stability of PTU in plasma.

In plasma, drugs may be degraded due to storage conditions such as temperature, light, air and enzymes [13]. Plasma samples were stored over a six month period at -20°C. Two different concentrations were used: 1 $\mu g.mL^{-1}$ and 10 $\mu g.mL^{-1}$. For 1 $\mu g.mL^{-1}$ the measured concentrations were 0.87 $\mu g.mL^{-1}$

(CV: 5.4), 0.77 $\mu g.mL^{-1}$ (CV: 2.6), 0.46 $\mu g.mL^{-1}$ (CV: 5.5), 0.39 $\mu g.mL^{-1}$

(CV: 1.1), 0.25 $\mu g.mL^{-1}$ (CV: 2.2) respectively for 0, 15, 90, 150 and 180 days of storage and 9.47 $\mu g.mL^{-1}$ (CV: 3.13), 8.44 $\mu g.mL^{-1}$ (CV: 1.6), 6.58 $\mu g.mL^{-1}$ (CV: 0.7), 5.98 $\mu g.mL^{-1}$ (CV: 3.9) and 4.5 $\mu g.mL^{-1}$ (CV: 1.7) respectively for 10 $\mu g.mL^{-1}$. These results showed the poor stability of PTU in plasma when stored at -20°C indicating that analysis should be performed shortly after sampling.

Reference standard.

The standard curves were made using PTU obtained from Sigma (Sigma, St. Louis, MO, USA). A comparison of the purity of this PTU source to the USP reference standard was performed. From both PTU sources, a solution of 2 $\mu g.mL^{-1}$ in distilled water was prepared. After analysis (n=6) peak areas were compared. The mean area of PTU obtained from Sigma was 100.22% (CV: 1.31) in comparison to the USP reference standard.

Calibration and linearity.

Linear calibration curves were obtained in water and in plasma and were forced through the origin. In Table 1, the x coefficient and correlation coefficient are shown for the plasma calibration curves. The response was linear over the entire concentration range (0.05 to 15 $\mu g.mL^{-1}$).

Run-by-run variability in plasma was determined from calibration curves by calculating the coefficient of variation on the x coefficient of different calibration curves obtained on ten different days over a 3 month period. An average value of 1.11 (CV: 3.21%) was calculated.

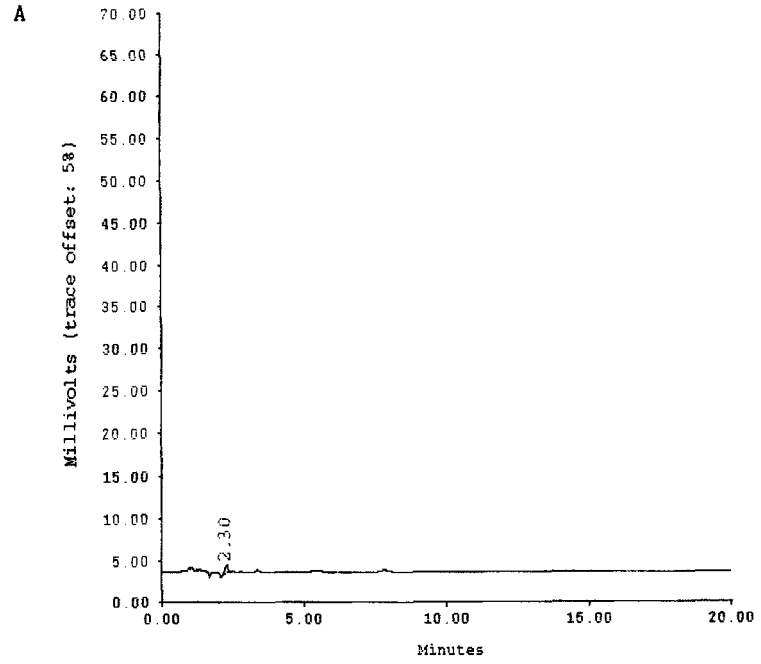

FIGURE 1.

A-Representative Chromatogram for Extracted Blank Plasma.

B-Representative Chromatogram for Extracted Plasma spiked with MTU (2.5 $\mu g.mL^{-1}$) and PTU (15 $\mu g.mL^{-1}$).

C-Representative Chromatogram for Extracted Plasma after I.V. Administration of 300 mg of PTU.

For within-run variability an average value of 1.09 (CV: 0.67%) was calculated (n=5).

Precision, repeatability and reproducibility.

Precision of a test procedure is defined as the closeness of agreement between a series of measurements obtained from multiple sampling of the same

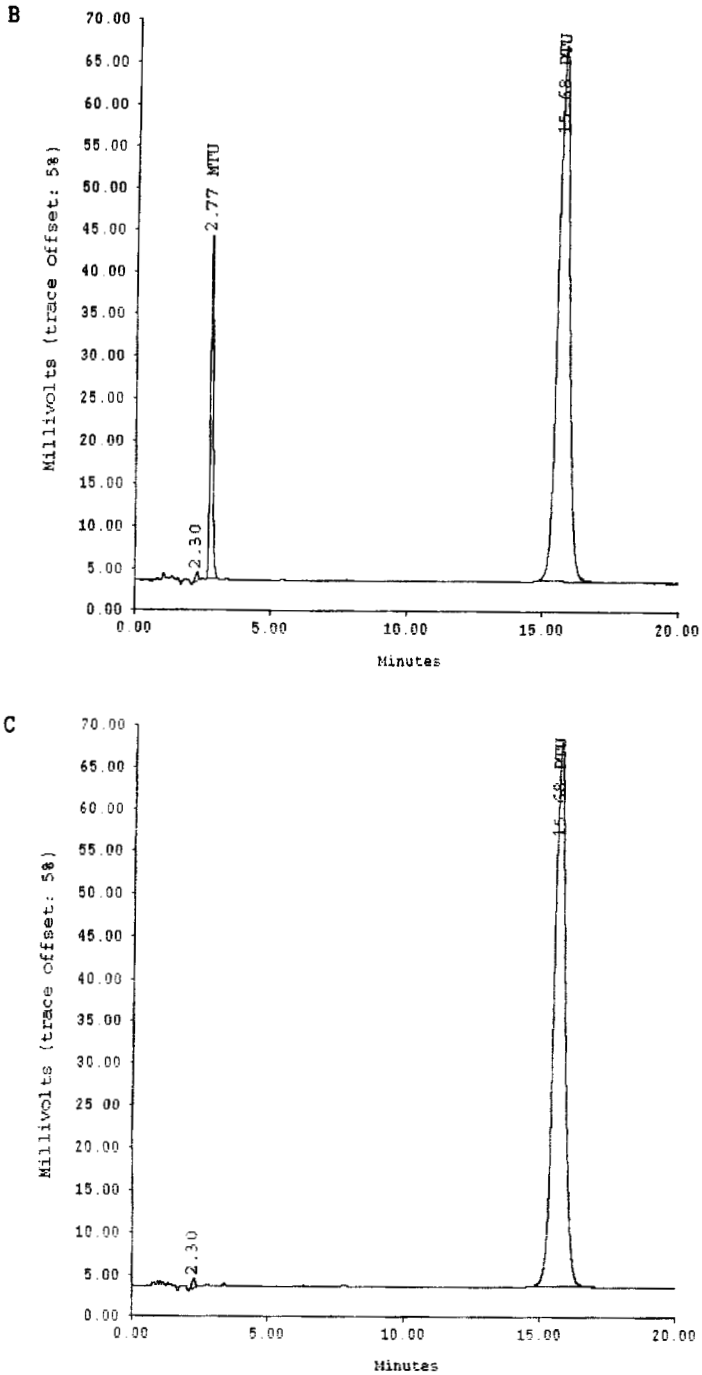

FIGURE 1 (continued)

TABLE 1.

X Coefficients and Correlation Coefficients for Calibration Curves (Mean ± C.V.) in Plasma for both within-Run (n=5) and Run-by-Run (n=10) Experiments. Calibration Curves were made for PTU Concentrations between 0.05 and 15 $\mu g.mL^{-1}$. The Concentration of the Internal Standard was 2.5 $\mu g.mL^{-1}$.

Within-run variability	
X coefficient	1.09 (± 0.67)
R squared	0.999 (± 0.03)
Run-by-run variability	
X coefficient	1.11 (± 3.21)
R squared	0.998 (± 0.29)

homogeneous sample under prescribed conditions (within-run variability: repeatability) or under different conditions (run-by-run variability: reproducibility) [12].

Repeatability and reproducibility were calculated for the different PTU concentrations in plasma (n=6). Table 2 shows the results obtained for different concentrations. Coefficients of variation for the repeatability test was within a 2.86-6.84% range of the expected concentration. For the reproducibility test it was within a 2.08-7.17% range. As can be seen in Table 2, higher variations were found only at the lowest concentration (0.5 $\mu g.mL^{-1}$). The acceptance criteria for precision is that the CV should not be greater than 15% [15].

Accuracy.

Accuracy is the closeness of agreement between the value accepted as the conventional true value (USP reference standard) and the values obtained by

TABLE 2.

Accuracy and Precision for Plasma containing PTU (n=6; Mean ± C.V.)

Concentration $\mu g.mL^{-1}$	Accuracy	Precision	
		Reproducibility (C.V)	Repeatability (C.V)
0.50	0.63 (±5.45)	0.52 (±7.17)	0.51 (±6.84)
1.00	1.07 (±3.51)	1.05 (±3.72)	1.07 (±2.86)
7.50	7.52 (±1.20)	7.62 (±2.08)	7.68 (±3.57)
9.00	8.66 (±4.74)	9.48 (±4.24)	9.29 (±3.88)
15.00	14.97 (±3.75)	15.12 (±2.67)	15.06 (±4.72)

applying the test procedure a number of times [12].

Different concentrations were tested and the results are shown in Table 2. The accuracy is within 6% (n=6) of the theoretical value at the concentrations tested. The acceptance criteria for accuracy is that the CV should not be greater than 15% [15].

Analytical recovery.

Sample preparation procedures are often causing loss of drug substance. Recovery is the percentage of reference material measured to the amount which has been added to water without performing an extraction [14].

The experiments were performed at different concentrations of PTU and at one concentration for the internal standard. At the different concentrations tested (n=10) the extraction was above 85% for PTU and around 66% for MTU in both media (Table 3). Extraction recovery reported in others HPLC methods were 59.4% [9], 71.1% to 81.6% [8], 74% [7] for PTU and 52.1% [8] for MTU. The

TABLE 3.

Recovery of PTU and MTU from Water and Plasma (n=10; Mean ± C.V.)

		Concentration ($\mu g.mL^{-1}$)	Recovery expressed as the percentage of the area of PTU or MTU obtained after extraction in water or in plasma to the area which has been measured in water without performing an extraction.
PTU	Plasma	0.5	94 (± 10.64)
		1	89 (± 4.83)
		7.5	90 (± 9.89)
		9	92 (± 3.69)
		15	95 (± 5.16)
	Water	1	85 (± 4.59)
		7.5	90 (± 4.89)
		15	92 (± 2.61)
MTU	Plasma	2.5	66 (± 4.09)
	Water	2.5	77 (± 3.24)

extraction recovery was improved for both PTU and MTU by the proposed procedure.

Limit of detection and limit of quantification.

The limit of detection is the lowest concentration of an analyte that the analytical process can reliably differentiate from background levels [15].

Using a bunching factor (BF) of 7 and a peak threshold (PT) of 291, the limit of detection was 2 and 5 ng.mL^{-1} in water and plasma, respectively. In other HPLC methods, limits of detection in plasma were 40 ng.mL^{-1} [9] and 50 ng.mL^{-1} [8]. The limit of quantification is the lowest concentration of an analyte that can be measured with a stated level of confidence [15]. With the same conditions (BF: 7 and PT: 291) and taking 15% CV as the level of acceptance, the limit of quantification was 20 and 40 ng.mL^{-1} in water and in plasma respectively.

Quality control.

Over a twelve month period, more than 500 plasma samples containing PTU were analyzed. During these experiments a quality control of the method was performed. Using two concentrations (2 and 10 μg.mL^{-1}) in plasma, one spiked sample was analyzed every 25 samples. The acceptability was evaluated using the bracket approach [14] not allowing a larger coefficient of variation than 15% for more than 10% of the spiked samples. This requirement was fulfilled over the period the analyses were carried out.

REFERENCES

1.C.R.Ratliff, P.F.Gilliland and F.F.Hall, Serum propylthiouracil: determination by a direct colorimetric procedure. Clin. Chem., 18 1373-1375 (1972).

2.D.Schuppan, S.Riegelman, B.V.Lehmann, A.Pilbrant and C.Becker, Preliminary pharmacokinetic studies of propylthiouracil in humans. J.Pharmacokinet.Biopharm., 1 307-318 (1973).

3.D.S.Sitar and D.B.Hunninghake, Pharmacokinetics of propylthiouracil in man after a single oral dose. J.Clin.Endocrinol.Metab., 40 26-29 (1975).

4.H.P.Ringhand and W.A.Ritschel, High-pressure liquid chromatographic determination of propylthiouracil in human plasma. J.Pharm.Sci., 68 1461-1462 (1979).

5. H.G.Giles, R.Miller and E.M.Sellers, High-performance liquid chromatographic determination of plasma propylthiouracil. J.Pharm.Sci., 68 1459-1460 (1979).

6.B.MacArthur and J.N.Miceli, Micro high-performance liquid chromatographic procedure for the quantization of serum propylthiouracil. J.Chromatogr., 278 464-468 (1983).

7.C.Kim, Simple and sensitive method for the determination of propylthiouracil in blood by high-performance liquid chromatography. J.Chromatogr. 272 376-379 (1983).

8.M.T.Rosseel and R.A.Lefebvre, High-performance liquid chromatographic determination of propylthiouracil in plasma. J.Chromatogr., 507 247-251 (1990).

9.G.R.Cannell and J.P.Williams, Selective liquid chromatographic assay for propylthiouracil in plasma. J.Chromatogr. 564 310-314 (1991).

10.D.S.Cooper, V.C.Saxe, F.Maloof and E.C.Ridgway, Studies of propylthiouracil using a newly developed radioimmunoassay. J.Clin.Endocrinol.Metab., 52 204-213 (1981).

11.R.Halperen, D.S.Cooper, J.D.Kieffer, V.Saxe, H.Mover, F.Maloof and E.C.Ridgway, Propylthiouracil (PTU) pharmacology in the rat. I.Serum and thyroid PTU measurements by radioimmunoassay. Endocrinology, 113 915-920 (1983).

12.Commission of the European Communities. GMP working party on quality of medicinal products. Note for guidance for analytical validation (August, 1989)

13.W.G.Peng and W.L.Chiou, Analysis of drugs and other toxic substances in biological samples for pharmacokinetic studies. J.Chromatogr., 531 3-50 (1990).

14.H.T.Karnes, G.Shiu and V.P.Shah, Validation of bioanalytical methods. Pharm.Res., 8 421-426 (1991).

15.V.P.Shah, K.K.Midha, S.Dighe, I.J.McGilveray, J.P.Skelly, A.Yacobi, T.Layloff, C.T.Viswanathan, C.E.Cook, R.D.McDowall, K.A.Pittman and S.Spector, Analytical methods validation: Bioavailability, bioequivalence and pharmacokinetic studies. Int.J.Pharm., 82 1-7 (1992).

16.J.P.Kampmann and J.M.Hansen, Clinical pharmacokinetics of antithyroid drugs. Clin.Pharmacokinet., 6 401-428 (1981).

17.U.S.Pharmacopoeial Convention, Inc., Rockville, USA., XXI (1985) p.1420.

Received: December 3, 1993
Accepted: December 11, 1993