Research Article

Biomedical Chromatography

Received: 24 January 2009, Revised: 8 February 2009, Accepted: 9 February 2009 Published online in Wiley Interscience: 29 April 2009

(www.interscience.wiley.com) DOI 10.1002/bmc.1229

Assay of free captopril in human plasma as monobromobimane derivative, using RPLC/(+)ESI/MS/MS: validation aspects and bioequivalence evaluation

Andrei Medvedovici,[a,b] Florin Albu,[a] Iuliana Daniela Sora,[a] Stefan Udrescu,[a] Toma Galaon[a] and Victor David[b]*

ABSTRACT: A sensitive method for determination of free captopril as monobromobimane derivative in plasma samples is discussed. The internal standard (IS) was 5-methoxy-1*H*-benzimidazole-2-thiol. Derivatization with monobromobimane immediately after blood collection and plasma preparation prevents oxidation of captopril to the corresponding disulfide compound and enhances the ionization yield. Consequently, derivatization enhances sample stability and detection sensitivity. Addition of the internal standard was made immediately after plasma preparation. The internal standard was also derivatized by monobromobimane, as it contains a thiol functional group. Preparation of plasma samples containing captopril and IS derivatives was based upon protein precipitation through addition of acetonitrile, in a volumetric ratio 1:2. The reversed-phase liquid chromatographic separation was achieved on a rapid resolution cartridge Zorbax SB-C_{18}, monitored through positive electrospray ionization and tandem MS detection using the multiple-reaction monitoring mode. Transitions were 408–362 amu for the captopril derivative and 371–260 amu for the internal standard derivative. The kinetics of captopril oxidation to the corresponding disulfide compound in plasma matrix was also studied using the proposed method. A linear log–log calibration was obtained over the concentration interval 2.5–750 ng/mL. A low limit of quantitation in the 2.5 ng/mL range was obtained. The analytical method was fully validated and successfully applied in a three-way, three-period, single-dose (50 mg), block-randomized bioequivalence study for two pharmaceutical formulations (captopril LPH 25 and 50 mg) against the comparator Capoten 50 mg. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: captopril; monobromobimane; derivatization; RPLC separation, positive electrospray ionization; oxidation kinetics; nonlinear calibration; validation; bioequivalence

Introduction

Captopril, (2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline (CAS number 62571-86-2), is known to be an inhibitor of angiotensin-converting enzyme (ACE), acting directly on the adrenal gland to stimulate the release of aldosterone. It prevents high blood pressure by inhibiting the enzymatic conversion of angiotensin I to angiotensin II (i.e. Khalil *et al*., 2001). During the metabolic pathway it is converted to a disulfide compound, which is eliminated together with unchanged captopril and metabolites in urine. Recently, it has been proved that this antihypertensive drug can exert an anticoagulant effect upon clotting time as followed by prothrombin time and activated partial thromboplastin time (Brecher *et al*., 2008).

Thiol containing compounds impose serious challenges in terms of the extreme reactivity of the sulhydryl group (Srinivas and Mamidi, 2003). Determination of captopril in biological fluids has been extensively studied in the last 10 years. Major problems related to the determination of this compound in human plasma have been solved in various ways and by means of different approaches. Improvement of the chemical stability and enhancement of detectability has been solved by derivatization with monobromobimane (Kok *et al*., 1997; Tache *et al*., 2002), ThioGlo™ 3{3H-naphtho[2,1-b]pyran-9-acetoxy-2-[4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)phenyl-3-oxo-]} (Aykin *et al*., 2001), *p*-bromophenacyl bromide (Li *et al*., 1996; Bahmaei *et al*., 1997), 1-benzyl-2-chloropyridinium bromide (Sypniewski and Bald, 1996), 1-benzyl-2-chloropyridinium bromide (Bald *et al*., 1996), *N*-(1-pyrenyl)maleimide (Arroyo *et al*., 1997), 2-bromo-2′-acetonaphthone (Amini *et al.,* 1999) and 3-bromomethyl-propyphenazone (Khedr and el-Sherief, 1998).

Various sample preparation procedures have been proposed in order to improve selectivity and detection sensitivity in liquid chromatography (LC): liquid–liquid extraction using ethyl acetate (Arroyo *et al*., 1997), dichloromethane (Huang *et al*., 2006), diethylether:dichloromethane (Salem *et al*., 2005) and solid-phase extraction (Bahmaei *et al*., 1997; Bald *et al*., 1996; Sypniewski and Bald, 1996; Rezende *et al*., 2007).

* Correspondence to: V. David, University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, Sos. Panduri, no. 90, Bucharest, 050663, Romania. E-mail: Vict_David@yahoo.com

[a] Labormed Pharma S.A., Splaiul Independentei no. 319 E, Bucharest 060044, Romania

[b] University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, Sos. Panduri, no. 90, Bucharest, 050663, Romania

Contract/grant sponsor: CNCSIS; Contract/grant number: PN2-Idei, no. 55/2007.

Abbreviations used: ACE, angiotensin-converting enzyme.

Liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) has been extensively used during the last decade for drug assay in biological fluids, owing to its performance in detecting low concentrations of analyte(s) and to improve the selectivity of the determination. So far, only two papers have reported the application of the tandem MS technique for determination of captopril in plasma, by monitoring transition from the precursor captopril protonated molecular ion ($m/z = 218$ amu) to its product $m/z = 172$ amu (Salem *et al*., 2005; Rezende *et al*., 2007).

This paper reports a new high-throughput analytical method for the assay of free captopril in human plasma based on derivatization with monobromobimane, protein precipitation through organic solvent addition, LC separation, positive electrospray ionization and MS/MS detection. Derivatization with monobromobimane has a double role: it preserves analyte oxidation to the corresponding disulfide (increase of stability) and enhances the detection sensitivity (Medvedovici *et al*., 2008). Some insights about the kinetics of the oxidation process of captopril are also discussed. CID ionization patterns for the derivatized compound are proposed. The method was fully validated according to the regulations in place. Once the method quality attributes had been assessed, it was successfully applied in a three-way, three-period, single-dose (50 mg), block-randomized bioequivalence study for two pharmaceutical formulations (captopril LPH 25 mg and 50 mg) against the reference product Capoten 50 mg (E.R. Squibb and Sons Ltd) in male and female healthy volunteers.

Experimental

Reagents

Acetonitrile and methanol were HPLC-grade from Merck (Darmstadt, Germany). Captopril was a USP-certified reference substance (USP-09120-0, batch H). The internal standard (IS) 5-methoxy-1*H*-benzimidazole-2-thiol (batch S02632-296, assay min. 99%) and the derivatization reagent monobromobimane (batch 016K1026, assay >97.0%) were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). For derivatization of samples, a solution in acetonitrile of the reagent, having a concentration of 4 mg/mL, was used. Formic acid, ammonium acetate and ammonia solution 25%, all of extra pure grade, were from Merck.

Instrumentation

Experiments were performed with an Agilent 1200 SL series LC/MSD (Agilent Technologies) system consisting of the following modules: degasser (G1379A), binary pump (G1312A), autosampler (G1313A), column thermostat (G1316A), AP-ESI standard interface (G1948B) and triple quadrupole mass spectrometric detector (G2571A). System control, data acquisition and interpretation were made with the Agilent MassHunter software version B 01.00. The system was operationally qualified before and after the bioequivalence study completion. The vortex system was a model Multi Reax from Heidolph (Schwabach, Germany) and the thermostated centrifuge was a model Universal 320R from Hettich (Tuttlingen, Germany).

Mass Spectrometric Conditions

The parameters controlling the AP-ESI interface were as following: drying gas, N_2; drying gas temperature, 350°C; flow rate of the drying gas, 10 L/min; nebulizer gas pressure, 60 psi; capillary voltage, 4000 V. MRM transitions were: for the captopril derivative from $m/z = 408$ amu to $m/z = 362$ amu; for IS derivative from $m/z = 371$ amu to $m/z = 260$ amu

Sample Preparation

Two phases are distinguishable within the sample preparation procedure: (a) derivatization; (b) protein precipitation. During phase 1, aliquots of 2.5 mL whole blood collected onto the anticoagulant are centrifuged. Once plasma had been obtained, a 1 mL aliquot was mixed with the IS standard solution (0.1 mL, 500 ng/mL in aqueous 500 mM ammonium acetate at pH = 7.5), followed by the addition of the derivatization reagent solution (0.05 mL, 4 mg/mL in acetonitrile). After vortexing (15 s at 2000 rpm), 30 min were needed to wait for a complete derivatization. Samples were frozen at −40°C and stored until analysis. Phase 2 consisted of the unassisted thawing at room temperature and addition to an aliquot of 0.2 mL plasma in a 0.4 mL volume of acetonitrile (protein precipitation agent). After vortexing for 5 min at 2000 rpm, precipitated protein separation was done under centrifugation (25°C, 5 min, 9000***g***) and the supernatant was transferred to the injection vial after a further dilution with 0.4 mL of water. Five microliters of sample were thus injected onto the chromatographic column.

Derivatization was made immediately after blood sample collection and plasma preparation, and improved stability over an increased storage period under freezing conditions (as further described under sample stability). Without derivatization, free captopril was quantitatively oxidized to disulfide over 3 months of storage at −80°C. Internal standard was added prior to reagent addition, as it was derivatized as well as the target compound due to its thiol functional group. Obviously, IS behaved similarly to the target compound all over the steps of the analytical method.

The second sample preparation stage was applied before separation and analysis. Protein precipitation through acetonitrile addition was used. Water addition to the supernatant before injection dealt with elimination of focusing effects, as the mobile phase at the beginning of the chromatographic run contained 85% aqueous component.

Chromatographic Method

LC separations were achieved on a Zorbax SB-C_{18} rapid resolution cartridge (30 mm length × 2.1 mm internal diameter × 3.5 µm particle size), fitted with a Phenomenex C_{18} (4 × 2 mm) guard column. The column temperature was kept at 60°C. The mobile phase contained acetonitrile and aqueous component (0.2% formic acid) in volumetric proportions 15:85. Separation is practically achieved isocratically in 2.3 min. The step gradient to 80% ACN in 0.01 min, followed by another isocratic plateau of 1.2 min, was used for a run to run column washout (for elimination of residual endogenous matrix compounds having an increased apolar character). The initial mobile phase composition was obtained after another stepwise modification of the mobile phase composition in 0.01 min and column equilibration took 0.5 min. A whole separation cycle took 4 min. The flow rate was set to 0.8 mL/min, and an injection volume of 5 µL was used. A typical separation is shown in Fig. 1.

Methodology for Validation

Validation was carried out according to available guidelines (US Department of Health and Human Sercices, FDA, CDER, CVM,

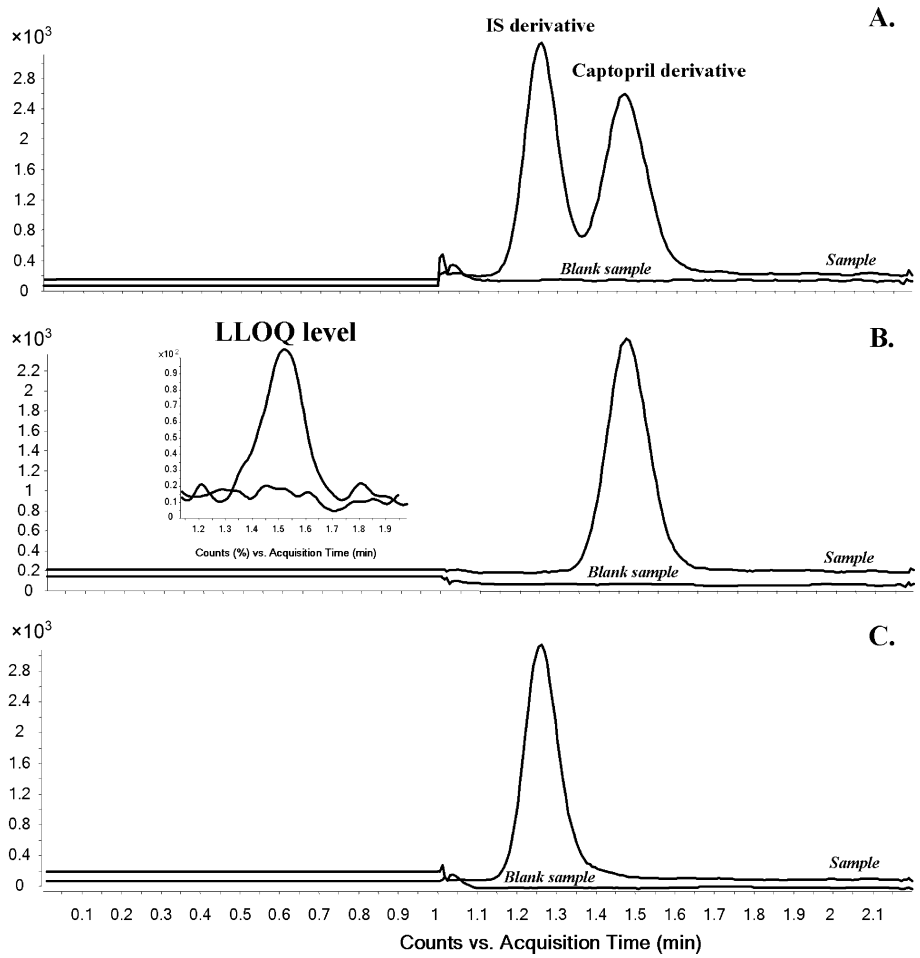

Figure 1. Separation of captopril and IS derivatives with monobromobimane from spiked plasma samples. (A) MRM of the two specific mass transitions; (B) EIC characteristic to the specific product ion of captopril derivative; (C) EIC characteristic to the specific product ion of IS derivative.

2001; European Medicines Agency, 2008) and refers to the following aspects: selectivity, linearity domain, determination of the low limit of quantitation (LLOQ), intra- and inter-day precision, recovery, sample stability (long-term, short-term, freeze–thaw, post-preparative stability, stock solution stability) and dilution integrity. Allthough not included in the guidelines, the robustness of the method was also investigated. Statistical interpretation of data collected through study completion was used to validate the choice of the analytical sequence length (number of samples from volunteers associated to one calibration and one quality control set). Trends in the variation of the experimental data were also controlled.

Methodology and Pharmacokinetic Parameters

In this three-way, three-period, crossover, block-randomized study, 25 healthy volunteers (male/female) aged between 18 and 45 years, having a body mass index between 19 and 27.5, received under fasting conditions one dose of 50 mg of captopril (two tablets × 25 mg or one tablet × 50 mg) from the tested products (T1 and T2) and one dose (one tablet × 50 mg) of the reference product (R), in the sequence determined by randomization, with a 7 day wash-out period between consecutive administrations. The reference product was Capoten® 50 mg from E.R. Squibb & Sons Ltd. The volunteers were non-smokers or ex-smokers (given up smoking at least 12 years prior to the study). All volunteers signed an informed written consent before initiation of the screening procedure. The protocol of the study was formally accepted by the evaluation department of the Romanian National Drug Agency and received the approval of the Institutional Ethics Committee. Subjects were hospitalized 12 h before and after T1, T2 or R drug administration. Venous blood samples were collected pre-dose (0 h) and at the following post-dose intervals of time: 0.33, 0.67, 1.0, 1.33, 1.67, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10 and 12 h. Medical examinations were performed in the screening, at the beginning of the in-house day and at the end of each study period. No clinical significant changes in laboratory values were observed after the study.

Evaluation of bioequivalence was made based on primary pharmacokinetic parameters AUC_{total} (area under plasma concentration–time plot extrapolated to infinity), AUC_{last} (area under plasma concentration–time plot until the last quantifiable value) and C_{max}, observed maximum plasma concentration for captopril. Evaluation of secondary (T_{max}, sampling time of the maximum plasma concentration) and auxiliary (T_{half}, terminal elimination half-life; %AUC extra, percentage of AUC_{extra} with respect to AUC_{tot}; MRT, mean residence time) parameters was also achieved.

The pharmacokinetic parameters were statistically treated by means of ANOVA (effects, period, subject nested in sequence, treatment and sequence) after log transformation of data for primary pharmacokinetic parameters, with determination of 90% confidence intervals for the intra-individual ratios test (1 or 2)/reference products. The non-parametric test (Friedman) was applied for T_{max}. Acceptance range for concluding bioequivalence was the conventional 90% confidence interval of 80–125% around the geometric mean ratios test/reference of log-transformed data for AUC_{last}, AUC_{tot} and C_{max}. Pharmacokinetic parameters were determined by means of the Kinetica™ software (version 4.4.1.) from Thermo Electron Corporation, USA.

Results and Discussion

Conditions used for Derivatization

Thiol group derivatization with monobromobimane is controlled through appropriate pH of the medium and time need for process completion. A pH value of 7.5 was found optimal. Below this value, the derivatization yield is poor. Over this value, the competitive hydrolysis of the derivatization reagent also affects transformation yield. In the 500 mM acetate buffer, at pH = 7.5 a decrease in monobromobimane concentration of about 4.5% was observed after 2 h. A period of 30 min for completion of derivatization may be considered as satisfactory. We also observed that peak area ratios between captopril and IS derivatives stabilizes 20 min after initiation of the derivatization reaction.

The concern related to the possibility that endogenous compounds from the plasma matrix may extensively consume the derivatization reagent was considered. Consequently, seven different plasma blanks were spiked at 750 ng/mL level with captopril. The sample preparation procedure was then applied to the resulting samples. The values corresponding to peak area ratios (captopril to IS derivatives) were characterized by a relative standard deviation (RSD%) of 10.4%.

Kinetics of Captopril Oxidation in Plasma Matrices

In order to evaluate the rate of captopril oxidation to the parent disulfide compound in plasma samples and at room temperature, bulk blank plasma was spiked with captopril at 2 µg/mL concentration level. Aliquots were prepared with the protein precipitation procedure at different moments, over an interval of 7 h and analyzed through the LC/MS/MS method. As illustrated in Fig. 2, the concentration of the free captopril was halved after 2 h and was practically consumed over an 8 h period. It became evident that derivatization of free captopril should be produced during the first hour after plasma preparation to prevent noticeable modifications in its concentration.

Ionization/fragmentation Patterns

Surprisingly, the S–C bond formed through derivatization resists to electrospray ion source conditions. Both IS and captopril derivatives produced protonated molecular ions. Adducts with sodium and potassium ions were also formed, but were less intense. If protonated molecular ions were selected as precursors, collision-induced dissociation with nitrogen molecules (collision cell potential was set at 10 V) generated the following fragments: for IS $m/z = 260$ (major product ion), 193 and 180 amu (intense signals); for captopril $m/z = 362$ (major product ion), 293 and 184 (intense signals), 265 and 216 amu (weak signals). Tentative fragmentation patterns are given in Fig. 3.

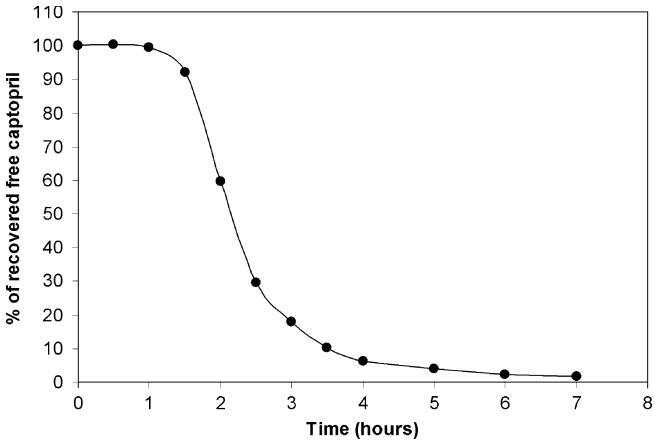

Figure 2. Kinetics of captopril oxidation to the corresponding disulfide compound at 25°C, determined by means of the assay of the free captopril remaining in plasma samples through derivatization with monobromobimane, chromatographic separation and MS/MS detection.

It can be observed that protonation may be located on N, O and S atoms. However, the bond S–C formed through derivatization is still stable, cleavage arising usually only in α position. This may reflect the chemical stability of monobromobimane derivatives and strongly recommends its use for long-term stability enhancement. Considering the intensity of the MS/MS signals obtained from the free captopril and from captopril–monobromobimane derivative in protein-precipitated plasma (monitored in the MRM mode, transitions $m/z = 218–172$ amu for free captopril and $m/z = 408–362$ amu for captopril derivative), an increase of the ionization yield by a factor of 6.6 was observed. Enhancement in the method sensitivity was thus produced.

The influence of the anticoagulants used for blood collection on the ionization yields was also considered. Plasma obtained from blood samples collected on lithium–heparin, ammonium–heparin, potassium edetate and sodium citrate were spiked with free captopril at the 300 ng/mL level and were processed according to the procedure. Six replicates were made for each case. Relative biases from the theoretical concentration were in the –5 to 12.8% range, while relative standard deviations within data sets were excellent (from 1 to 2.5%). One can conclude that blood sample collection can be made on all commonly used anticoagulants without affecting derivatization and ionization yields. This information together with observations made under recovery (as further discussed) suggests that no major ionization effect (suppression or enhancement) was produced by blood processing reagents or plasma matrix effects.

Method Validation

Detector response function was evaluated over the concentration interval 2.5–750 ng/mL, for plasma samples spiked with free captopril and prepared at eight concentration levels (2.5, 5, 25, 50, 100, 250, 500 and 750 ng/mL). Six replicates were prepared for each concentration level. Relative standard deviations calculated at each of the concentration levels ranged from 0.7 to 7.1%. The response function was found to be nonlinear over the studied interval, as is often cited in the literature for MS/MS detection (Georgita *et al*., 2008; Srinivas, 2008). To obtain linearization,

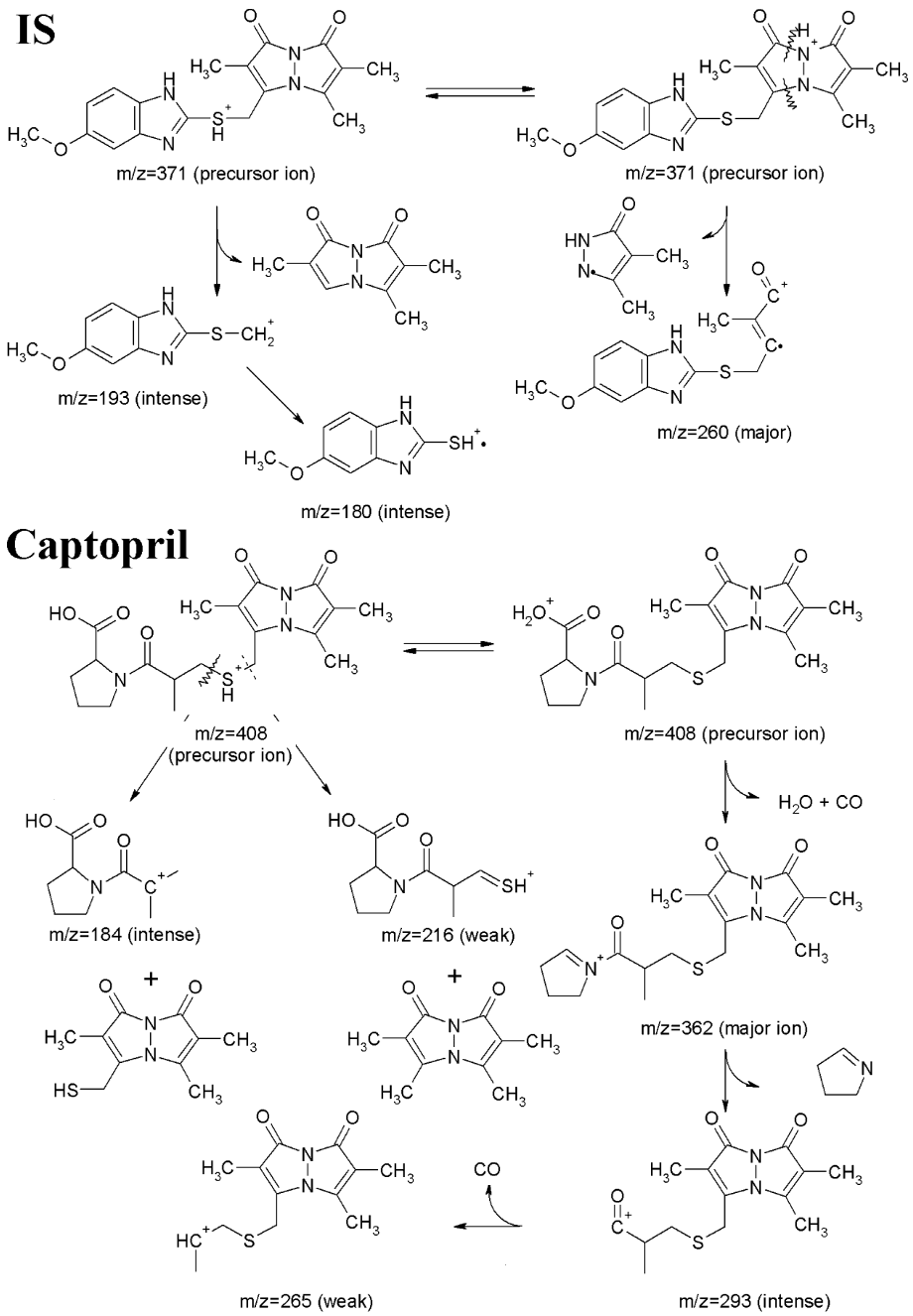

Figure 3. Tentative CID fragmentation pathways for IS and captopril derivatives with monobromobimane.

the logarithm (10 base) of the peak areas ratio of captopril and IS derivatives was represented against the logarithm of concentration. The log–log function was linear, being characterized by a correlation coefficient of 0.9999. The parameters of the log–log linear regression were: B (slope) = 1.119 ± 0.007 (standard deviation) and A (intercept) = –2.09 ± 0.01. Concentrations obtained through back-interpolation of the experimental data in the linear regression equation revealed relative biases ranging from –4.42 to 3.37%. Over the linearity study, the peak areas of the IS derivative were characterized by an RSD% of 3.7%, no trend being observed. For peaks corresponding to a free captopril concentration in plasma samples of 2.5 ng/mL, a mean signal-to-noise ratio of 5.3 was found. Consequently, the concentration of 2.5 ng/mL was considered to be the lower limit of quantitation (LLOQ). As the concentration of IS in samples was set at 50 ng/mL, the detector response function was also checked for the IS over the interval 10–100 ng/mL. The IS derivative response function (peak area vs concentration) was found to be linear over the studied interval (B = 1150 ± 27; A = –1454 ± 1453). The LLOQ for the IS derivative calculated from the relationship $(5 \times s_A - A)/B$ was found to be 7 ng/mL. The IS concentration added to samples was about 7 times greater than its LLOQ in the given conditions and was placed in the middle of the studied response linear interval.

The selectivity of the method was considered for seven different blank plasma samples being processed as such and after spiking with 2.5 ng/mL free captopril (LLOQ). Residual peak areas having the absolute retention of the captopril derivative peak in

the blank samples represented between 5.3 and 14.5% of the corresponding captopril peak in samples spiked at LLOQ. None of the samples obtained from volunteers at pre-dose collection moments in phases 1–3 of administration exhibited residual peak areas at captopril retention times higher than 20% of the mean response at LLOQ of samples analyzed over study completion. This confirms the method selectivity and the correct choice of the wash-out periods.

Intra-day precision was checked for three sets of plasma samples spiked with captopril at 10, 300 and 600 ng/mL concentration levels (IS was spiked at 50 ng/mL), prepared and analyzed as 10 replicates within a single experimental session. RSDs obtained within data sets ranged from 0.9 to 3.1%. Recovered mean concentrations compared against the theoretical values were between 95.4 and 103.5%. Inter-day precision was verified at the same concentration levels. Spiked samples were prepared and analyzed in experimental sessions separated by a period of at least 24 h. Six replicates were considered. The relative standard deviations found at each concentration level were within the 0.8–2.7% interval while recoveries range from 96.3 to 104%.

Recovery of free captopril was evaluated by comparing spikes made in water and in blank plasma samples at three concentration levels (10, 300 and 600 ng/mL). Six independent replicates were made at each of the concentration levels. Recovery was calculated as the percentage of the peak area in the chromatogram of the spiked blank plasma sample from the peak area in the chromatogram of the corresponding spiked water sample. IS was also considered for evaluation of the recovery at a single concentration level (50 ng/mL). Mean recovery for captopril was 96% (considering all concentration levels) with a normal variation interval (mean $\pm 2 \times$ standard deviation) ranging between 94.1 and 98.7%. For the IS, the mean recovery ($n = 18$) was 97.2%, with a normal variation interval placed within 96 and 98.2%. These results support the conclusion that the plasma matrix does not adversely affect derivatization, isolation of the derivatives, their separation and ionization within the source.

Method accuracy was verified over the bioanalytical study completion. Each analytical sequence contains one calibration set (eight concentration levels: 2.5, 5, 25, 50, 100, 250, 500 and 750 ng/mL of free captopril and 50 ng/mL IS), one quality control set (10, 300 and 600 ng/mL free captopril and 50 ng/mL IS spiked to blank plasma samples, two replicates per concentration level) and 48 samples obtained from one volunteer over the three phases of the study. Injection of samples from volunteers was done in order to analyze consecutively the same blood sampling time over the three phases of the study. None of the samples from calibrations failed back-interpolation within the issuing response function (accuracy threshold of ±15% was considered). For quality control sets, none of the experimental concentration values failed to comply with the imposed precision (RSD% = 15%) and accuracy (relative bias = ±15%). To illustrate results in terms of precision and accuracy, the values found for QC samples at the first concentration level (10 ng/mL, which is considered the most critical one due to its closeness to LLOQ) are shown in Fig. 4.

A simple statistical interpretation of the data obtained for IS peak area values over study completion (samples from volunteers) was made. It shows that the RSD% characterizing this statistical significant population of data ($n = 1125$) was 28.7%. Such a result almost doubles the commonly accepted interval for variation in bioanalytical studies. It became evident that long-term variability of the MS/MS detector induces significant changes in terms of response. An ion source cleaning operation was done once for each 24 h interval, which contributed to enhanced variability. Over bioanalytical study completion (6 days), no evident variation trend was obtained for IS peak areas. However, data collected between two consecutive ion source cleaning operations (one day) exhibited a negative trend (correlation coefficient of 0.96). Excessive ion source loading with residual matrix from plasma samples produced signal suppression through reduction of the ionization yield. In such conditions, the length of the analytical sequence became extremely important.

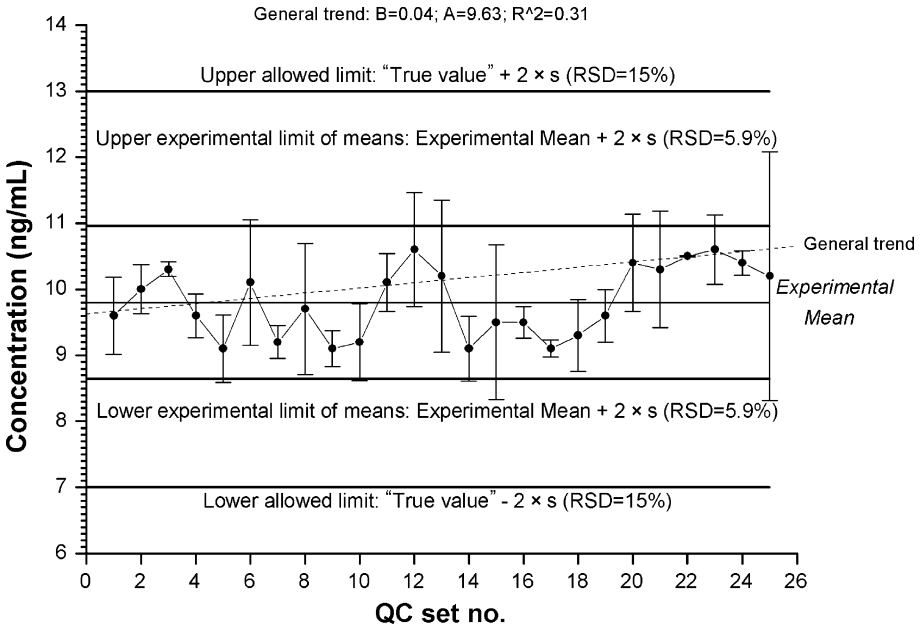

Figure 4. Variation and trend of experimental values obtained for the first concentration level (10 ng/mL) of the QC sets run over bioequivalence study completion.

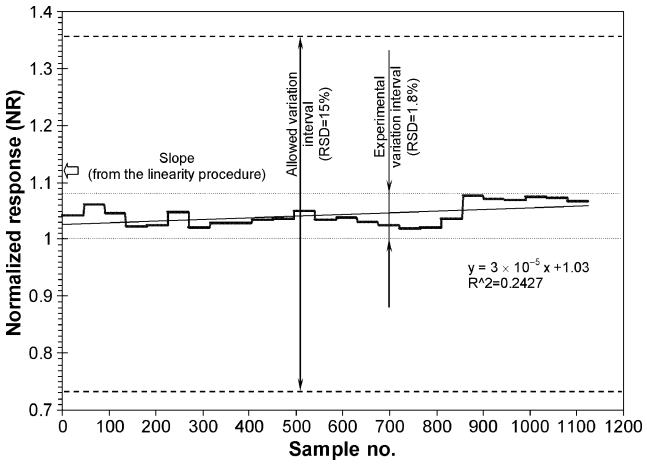

Figure 5. Trend of the normalized responses calculated from 1125 samples obtained from volunteers, over study completion.

To demonstrate that the proper choice had been made in relation to the length of an analytical sequence, the calculation of normalized responses (NR) obtained for samples belonging to volunteers and monitoring their trend throughout the study was considered. The normalized response was obtained with the formula $NR = [\log (PAR) - A]/\log (c)$, where PAR is the peak area ratio between captopril and IS derivatives in each chromatogram, A is the intercept given by the calibration associated to the respective sample and c is the computed concentration of the target compound in the sample. In fact, the normalized response represents the response of the detection system corresponding to one unit of concentration, and should be close to the slope of the calibrations being made during method validation and study completion. As shown in Fig. 5, variation of the normalized response for 1125 plasma samples corresponds to an RSD% of 1.8%. No specific trend can be observed (the slope of the linear regression is practically null, the correlation coefficient very is low and the intercept is close to the experimental mean value). A good fit to the value determined during method validation was obtained. These results demonstrate that the length of the analytical sequences was appropriate and sufficiently counterbalanced the variability of the detector.

The study of the method's robustness was oriented toward the operational parameters having potential influence over detector response. The ion source parameters, namely drying gas temperature (varied from 330 to 350°C), drying gas flow (varied from 8 to 12 L/min), nebulizer gas pressure (varied from 50 to 60 psi) and capillary voltage (varied from 3500 to 4500 V) were first considered. The concentration of formic acid in the aqueous phase component (varied from 0.15 to 0.25%) was evaluated, as ionization yield may be influenced by the pH of the effluent in ESI. Trials were made on spiked plasma samples with captopril at 300 ng/mL and IS (50 ng/mL). For each specific value of an operational parameter, five replicates were prepared, analyzed and detected. The relative standard deviations of peak areas corresponding to captopril and IS derivatives were 1.8–8.8% interval. The increase in formic acid concentration in the mobile phase was the single modification to most affect the ionization yield of the captopril derivative. One can conclude that formic acid content in the mobile phase should be adequately controlled to avoid increased variability for the captopril derivative response.

Stability of Derivatives

The stability study of the target compounds was deliberately treated with enhanced attention, as the major role of the derivatization process focuses on this feature. Stability data are presented in Table 1. Conditions, concentration levels, replicates and sampling frequency were also included in the table. One can conclude that the monobromobimane derivatives of captopril and IS are stable for short-term and long-term storage as well as during freeze and thaw cycles. The stock solution of the IS is stable at room temperature for at least 1 week.

Table 1. Stability data for captopril derivatized with monobromobimane and for the internal standard stock solution

Stability Conditions	Analyte	Spiked concentration level (ng/mL)	Mean found concentration level (ng/mL)	*n*	% RSD	Mean recovery (%)	Max. recovery (%)	Min. recovery (%)
Freeze and thaw (six consecutive cycles)	Captopril	10	10.3	6	6.5	103.2	94.4	112.4
		300	283.4		3.4	94.5	90.3	98.3
		600	598.5		4.3	99.8	92.7	102.4
Long-term stability: – 40°C/60 days		10	10.0	6	5.9	100	90.4	110.2
Samplingfrequency: 0, 1, 5, 7, 9, 10,		300	286.4		5.8	95.5	90.8	104.1
20, 30, 60 days		600	591.8		6.5	98.6	87.6	107.5
Short-term stability 25°C/24 h		10	10.2	6	4.0	102.4	99.0	107.8
Sampling		300	289.4		4.2	96.5	92.5	102.1
frequency: 0, 4, 8, 24 h		600	603.8		3.1	100.6	97.7	104.5
Sample post-preparative stability		10	10.2	6	1.3	102.2	100.6	106.6
(in the autosampler) 25°C/48 h		300	281.7		1.3	93.9	92.9	95.9
Sampling frequency: 0, 4, 8, 15,		600	598.4		1.2	99.7	98.7	101.8
24, 36 and 48 h								
Stock solution stability 25°C/7 days	IS	50	Peak area	5	8.6	—	Maximum	Minimum
Sampling frequency: 0, 1, 3, 5, 7 days			62566				peak	peak
							area 56740	area 69752

Table 2. Statistic interpretation of pharmacokinetic parameters corresponding to captopril in tested pharmaceutical formulations against the reference product

Pharmacokinetic parameter	Tested		Reference		Geometric mean ratio T/R	90% Confidence interval (%)
	Mean	SD	Mean	SD		
T1 formulation (2 × 25 mg)						
AUC_{tot} (ng/mL h)	1082.1	672.4	1074.5	737.0	1.0535	0.9872–1.1700
AUC_{last}(ng/mL h)	1057.6	658.1	1050.7	728.2	1.0566	0.9883–1.1747
C_{max} (ng/mL)	698.3	442.5	728.4	545.1	1.0165	0.9157–1.1591
T_{max} (h)	0.87	0.25	0.75	0.17	No significant difference between means has been determined through the non-parametric Friedman test	
$\%AUC_{extra}$	2.31	0.89	2.59	1.47	—	
T_{half} (h)	3.45	1.02	3.35	1.02	—	
MRT	2.24	0.29	2.20	0.34	—	
T2 formulation (1 × 50 mg)						
AUC_{tot} (ng/mL h)	1099.2	659.9	1074.5	737.0	1.0745	10.9872–1.1695
AUC_{last}(ng/mL h)	1076.8	651.4	1050.7	728.2	1.0775	0.9883–1.1747
C_{max} (ng/mL)	692.5	368.7	728.4	545.1	1.0302	0.9157–1.1591
T_{max} (h)	0.82	0.24	0.75	0.17	No significant difference between means has been determined through the non-parametric Friedman test	
$\%AUC_{extra}$	2.32	1.12	2.59	1.47	—	
T_{half} (h)	3.31	0.93	3.35	1.02	—	
MRT	2.17	0.34	2.20	0.34	—	

Pharmacokinetic Data and Bioequivalence Assessment

Pharmacokinetic parameters computed for free captopril are given in Table 2. From the experimental data, the bioequivalence between the two tested pharmaceutical formulations (T1 and T2) and the reference product (R) was determined with respect to the rate and extent of absorption of captopril.

No major adverse effects were observed or reported during the study. No clinical significant changes in clinical investigation laboratory values were observed after study completion.

Conclusions

Derivatization with the thiol-specific reagent monobromobimane was successfully used to stabilize free captopril in human plasma. The derivatization procedure should be carried out immediately after blood sample collection and plasma preparation. An internal standard containing a thiol group (5-methoxy-1*H*-benzimidazole-2-thiol) was added to sample prior to derivatization and behaved similarly to the target compound. The chemical stability of the derivatives was sustained by the ESI/MS ionization and by the CID fragmentation patterns. Derivatization is necessary, as free captopril readily oxidizes in plasma samples at room temperature, being quantitatively transformed to its parent disulfide compound within 8 h. It has been proved that derivatization also enhances the detector response, by favorable ionization yield in the MS interface. Once derivatives have formed, samples are treated for protein precipitation through addition of a miscible organic solvent (ACN, for instance). The procedure is fast and allows almost quantitative recoveries of the compounds from plasma. The high-throughput chromatographic separation of monobromobimane derivatives of captopril and internal standard contains a gradient step for run-to-run column wash-out. Separation is achieved within 4 min, including column equilibration. Specific MRM transitions were found for both derivatives. The LLOQ for captopril was 2.5 ng/mL. Linearization of the detector response was needed over the studied concentration interval (2.5–750 ng/mL), this being achieved through log–log representations. The method was found to be selective, sensitive, precise, accurate and robust. The appropriate choice of the analytical sequences length was supported through statistical interpretations of data relating to peak areas of IS and the normalized response for captopril. A three-way bioequivalence study between two formulations of captopril (25 and 50 mg, respectively) and the reference product was thus supported by the proposed method. Pharmacokinetic parameters were determined and their statistic interpretation led to assessment of the bioequivalence of the investigated formulations.

Acknowledgements

One of the authors (V.D.) takes this opportunity to acknowledge the financial support from Romanian Agency, CNCSIS, through the research grant, PN2-Idei, no. 55/2007.

References

Amini M, Zarghi A and Vatanpour H. Sensitive high-performance liquid chromatographic method for determination of captopril in plasma. *Pharmaceutica Acta Helvetiae* 1999; **73**: 303–306.

Arroyo C, Lopez-Calull C, Garcia-Capdevila L, Gich I, Barbanoj M and Bonal J. Determination of captopril in plasma by high-performance liquid chromatography for pharmacokinetic studies. *Journal of Chromatography B, Biomedical Sciences and Applications* 1997; **688**: 339–344.

Aykin N, Neal R, Yusof M and Ercal N. Determination of captopril in biological samples by high-performance liquid chromatography with ThioGlo 3 derivatization. *Biomedical Chromatography* 2001; **15**: 427–432.

Bahmaei M, Khosravi A, Zamiri C, Massoumi A and Mahmoudian M. Determination of captopril in human serum by high performance liquid chromatography using solid-phase extraction. *Journal of Pharmaceutical and Biomedical Analysis* 1997; **15**: 1181–1186.

Bald E, Sypniewski S, Drzewoski J and Stepien M. Application of 2-halopyridinium salts as ultraviolet derivatization reagents and solid-phase extraction for determination of captopril in human plasma by high-performance liquid chromatography. *Journal of Chromatography B, Biomedical Sciences and Applications* 1996; **681**: 283–289.

Brecher AS, Murrey SJ, Gray KD and Poulimenos JN. Anticoagulant activity of captopril. *Journal of Cardiovascular Pharmacology* 2008; **51**: 99–105.

European Medicines Agency. *Guideline of the Investigation of Bioequivalence* (draft). CPMP/EWP/QWP/1401/98 rev. 1, 2008.

Georgita C, Albu F, David V and Medvedovici A. Non-linear calibrations on the assay of diltiazem and two of its metabolites from plasma samples by means of liquid chromatography and ESI/MS^2 detection: application to a bioequivalence study. *Biomedical Chromatography* 2008; **22**: 289–297.

Huang T, Yang B, Shao L, Zheng X and Duan G. Simultaneous determination of captopril and hydrochlorothiazide in human plasma by RP-HPLC from linear gradient elution. *Journal of Pharmaceutical and Biomedical Analysis* 2006; **41**: 644–648.

Khlalil ME, Basher AW, Brown EJ and Alhaddad IA. A remarkable medical story: benefits of angiotensin-converting enzyme inhibitors in cardiac patients. *Journal of the American College of Cardiology* 2001; **37**: 1757–1764.

Khedr A and el-Sherief H. 3-Bromomethyl-propyphenazone as a new derivatization reagent for high performance liquid chromatography of captopril and hydrochlorothiazide with UV-detection. *Biomedical Chromatography* 1998; **12**: 57–60.

Kok RJ, Visser J, Moolenaar F, De Zeeuw D and Meijer DKF. Bioanalysis of captopril: two sensitive high-performance liquid chromatographic methods with pre- or postcolumn fluorescent labeling. *Journal of Chromatography B, Biomedical Sciences and Applications* 1997; **693**: 181–189.

Li K, Tan L and Zhou JA. HPLC determination of captopril in human plasma and its pharmacokinetic study. *Biomedical Chromatography* 1996; **10**: 237–239.

Medvedovici A, Farca A and David V. Derivatization reactions in liquid chromatography for drug assaying in biological fluids. *Advances in Chromatography*, 2008, **47**: 283–322.

Rezende KR, Mundim IM, Teixeira LS, Souza WC, Ramos DR, Cardoso CR, Souza IC, Gratao MZ and Bellorio KB. Determination of captopril in human plasma using solid phase extraction and high-performance liquid chromatography, coupled to mass spectrometry: application to bioequivalence study. *Journal of Chromatography B—Analytical Technologies in Biomedical and Life Sciences* 2007; **850**: 59–67.

Salem II, Saif WA, Jmeian Y and Al Tamimi JI, A selective and rapid method for the quantification of captopril in human plasma using liquid chromatography/selected reaction monitoring mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* 2005; **37**: 1073–1080.

Srinivas NR. Applicability of nonlinear regression for quantitative determination of parent and metabolite(s) in bioequivalence. *Biomedical Chromatography* 2008; **22**: 1315–1317.

Srinivas NR and Mamidi RNVS. Bioanalytical considerations for compounds containing free sulfhydryl group. *Biomedical Chromatography* 2003; **17**: 285–291.

Sypniewski S and Bald E. Determination of captopril and its disulphides in whole human blood and urine by high-performance liquid chromatography with ultraviolet detection and precolumn derivatization. *Journal of Chromatography A* 1996; **729**: 335–340.

Tache F, Farca A, Medvedovici A and David V. Validation of an HPLC-FLD method for determination of free captopril in human plasma, using a pre-column derivatization reaction with monobromobimane. *Journal of Pharmaceutical and Biomedical Analysis*, 2002; **28**: 549–557.

US Department of Health and Human Sercices, FDA, CDER, CVM. *Guidance for Industry: Bioanalytical Method Validation*, 2001.