

# Development of an Analytical Methodology for Simultaneous Determination of Vincristine and Doxorubicin in Pharmaceutical Preparations for Oncology by HPLC–UV

Ana Sofia Rodrigues<sup>1</sup>, Ana Rita Lopes<sup>1</sup>, Ana Leão<sup>1</sup>, Alda Couceiro<sup>1</sup>, Ana Bela Sarmiento Ribeiro<sup>2,3</sup>, Fernando Ramos<sup>1,\*</sup>, Maria Irene Noronha da Silveira<sup>1</sup>, and Catarina Resende de Oliveira<sup>2</sup>

<sup>1</sup>Grupo de Bromatologia—Centro de Estudos Farmacêuticos, Faculdade de Farmácia da Universidade de Coimbra, 3000-295 Coimbra, Portugal and <sup>2</sup>Instituto de Bioquímica e Biologia Molecular, Faculdade de Medicina da Universidade de Coimbra, 3000-295 Coimbra, Portugal, and <sup>3</sup>CIMAGO—Centre for Research of Environment, Genetics, and Oncobiology, 3001-301 Coimbra, Portugal

## Abstract

A high-performance liquid chromatography–UV methodology ( $\lambda = 230$  nm) was developed and validated for the simultaneous determination of vincristine and doxorubicin in pharmaceutical preparations used in oncology. The chromatography was carried out on a C<sub>18</sub> column using acetonitrile 90% in water–potassium hydrogenphosphate buffer 50mM, pH 3.2  $\pm$  0.1 (32:68, v/v) as mobile phase at a flow rate of 1.5 mL/min. The method proved to be specific, exact, and accurate, in accordance with the ICH standards, presenting linearity in the 1–5  $\mu$ g/mL and 5–100  $\mu$ g/mL intervals, and detection (0.19  $\times$  0.51  $\mu$ g/mL) and quantification (0.63  $\times$  1.7  $\mu$ g/mL) limits for vincristine and doxorubicin, respectively.

## Introduction

Vincristine and doxorubicin (Figure 1) are two drugs used in oncology. Vincristine is an alkaloid obtained from *Catharanthus roseus* (1) whose cytostatic action is not fully understood, but apparently results from its capability to complex with tubulin, inhibiting its polymerization in the achromatic mitotic spindle and therefore inhibiting cell division (2). Doxorubicin or adriamycin is an anthracycline antibiotic, isolated from *Streptomyces coeruleorubidus* or *Streptomyces peucetius* (1). Its cytostatic effect results from various mechanisms like inhibition of replication and transcription of cell DNA, interaction with topoisomerase II with a consequent break of DNA, deregulation of calcium and sodium transports at cell membrane level, and generation of free radicals of oxygen (3). These two drugs are commonly used in the treat-

ment of multiple myeloma refractory to alkylating agents, in association with dexametazone. The chemotherapeutic protocol, known as vincristine, adriamycin, dexametazone (VAD) regimen, consists of vincristine sulphate (0.4 mg/day) and doxorubicin hydrochloride (9 mg/m<sup>2</sup>/day), administered as continuous endovenous infusion for 96 h (4 days) with dexametazone (20 mg/m<sup>2</sup>/day) given orally in 3 weekly courses of four days each (4–6). At the present time, patients are forced to stay in the hospital during a course treatment for VAD's delivery. However, the emergence of smaller, low weight, easier-to-use, and portable infusion devices has been enabling these patients to be treated in ambulatory or outside of the hospital. In turn, it is necessary to prove that drugs keep remain stable in admixture. Methods for the determination of doxorubicin or vincristine in biological fluids and plant extracts by liquid chromatography tandem mass spectrometry or UV detection are described in literature (7–12). Some stability studies in portable infusion devices were found using mostly high-performance liquid chromatography (HPLC)–UV methodology (13–16). Nevertheless, none of these techniques allow the simultaneous evaluation of both drugs or are able to quantify different drug concentrations, as the commercial formulations vary from country to country.

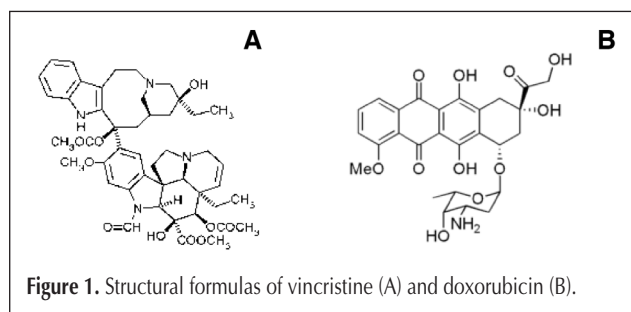


Figure 1. Structural formulas of vincristine (A) and doxorubicin (B).

\* Author to whom correspondence should be addressed: email fjramos@ci.uc.pt.

This study presents a novel and optimized HPLC–UV method for the simultaneous determination of vincristine and doxorubicin in pharmaceutical preparations using a reversed-phase column by isocratic elution. Nowadays, chromatographic methods are the most frequently used in the qualitative and quantitative evaluation of drugs. The method described was validated according to the International Conference on Harmonization (ICH) standards (17), in order to be routinely used in laboratory, supply document evidence that will perform the tasks for which it is indicated and ensure that it is, at very least, exact (recovery > 98%), precise [coefficient of variation (CV) < 15%], reproducible (CV < 20%) and robust within the specified variation (17–24).

## Experimental

### Reagents and standards

The reagents utilized were HPLC-grade acetonitrile, SDS (Peypin, France), potassium anhydrous hydrogenphosphate, p.a., Merck (Darmstadt, Germany), phosphoric acid 85%, RPE-ACS, Carlo Erba (Milan, Italy), sodium chloride 0.9%, B. Braun Medical (Melsungen, Germany), purified water obtained with a Milli-Q system (Millipore, Bedford, MA). The doxorubicin hydrochloride (2 mg/mL) in physiological saline 0.9% (pH = 3), the methyl ester of *p*-amino benzoic acid (methylparabene), and *n*-propyl-*p*-hydroxy-benzoate (propylparabene) were purchased from Sigma-Aldrich (St. Louis, MO) and, together with vincristine sulphate (Fluka Chemie, Buchs, Belgium), were used as standards. Vincristine Irex (2 mg/2 mL, PCH Pharmachemie, Haarlem, the Netherlands) and Adriblastine CS (50 mg/25 mL, Pharmacia Corporation, Peapack, NJ) were used to prepare the sample solutions.

All the remaining reagents used were p.a. quality and were purchased from Merck.

### Materials and equipment

Determination of vincristine and doxorubicin was made in a Gilson HPLC–UV system, consisting of a Model 321 pump, a Model 151 UV–vis detector, and an injector, with a loop of 100  $\mu$ L (Villiers-le-Bel, France). Separation was attained through a C<sub>18</sub> Waters Spherishorb ODS column 4.6  $\times$  250 mm, 5  $\mu$ m (Milford, MA) and data was processed by a UniPoint 2.10 software (Gilson, Villiers-le-Bel, France). The following items were also used: precision balances by Mettler Toledo AG285 (Greifensee, Switzerland), WPA CD7400 pH meter (Cambridge, UK); UniEquip vortex (Munich, Germany); B-169 vacuum pump by Büchi (Flawil, Switzerland); Sonorex RK/100 Banelin ultrasound bath (Berlin, Germany); micropipettes by Gilson and membrane filters by Schleicher & Schuell, 0.2  $\mu$ m, 50 mm (Dassel, Germany).

### Standard solutions

Standard solutions were prepared in sodium chloride (0.9%). For vincristine sulphate, a stock solution of 100  $\mu$ g/mL was prepared. For doxorubicin hydrochloride, a concentration of 2000  $\mu$ g/mL, in sodium chloride (0.9%), was used as stock

solution. Working solutions were prepared from stock solutions, with concentrations of 0.5–10  $\mu$ g/mL for vincristine and 2–100  $\mu$ g/mL for doxorubicin. For methylparabene and propylparabene, stock solutions of 100  $\mu$ g/mL were prepared, and from these five intermediate solutions of 40, 20, 5, 2, and 1  $\mu$ g/mL. Finally, working solutions with concentrations of 0.1–1.1  $\mu$ g/mL were prepared from the previously-mentioned solutions. The solutions were stored in amber glass bottles, in the refrigerator, at a temperature of 2–8°C.

### Sample solutions

The admixtures of vincristine and doxorubicin in sodium chloride 0.9% were prepared from the commercial dosage forms containing 1 mg/mL and 2 mg/mL of the drugs, respectively. Three samples were prepared with the following concentrations: 1.5  $\mu$ g/mL vincristine and 42.6  $\mu$ g/mL doxorubicin (sample A); 15.2  $\mu$ g/mL vincristine and 857.1  $\mu$ g/mL doxorubicin (sample B); and 30.3  $\mu$ g/mL vincristine and 1700  $\mu$ g/mL doxorubicin (sample C). These concentrations were established from the daily drugs' dosage, the body surface area (BSA) of patients, the period of infusion, and from the volume and flow rate of portable infusion pumps available in market. Before injection in the HPLC system, samples B and C were diluted 10 and 20-fold in sodium chloride (0.9%), respectively, to allow quantification.

### Chromatography

Analysis and sample drug quantifications were undertaken by reversed-phase LC, coupled to UV detection ( $\lambda$  = 230 nm). Elution was isocratically made by a flow rate of 1.5 mL/min. The mobile phase consisted of a mixture of acetonitrile (90%) in water and potassium hydrogenphosphate buffer 50mM (pH 3.2  $\pm$  0.1) adjusted with phosphoric acid at 85% (32:68, v/v), previously filtered and degassed. Drug identification was performed through retention times and their quantification from the peak area, intersecting the value read in a calibration curve constructed from the injected standards on the same day.

## Results and Discussion

### Development and optimization of the analytical methodology

Chromatographic conditions were optimized to improve the performance of the method. A 150-mm column was initially tested, but was unable to separate vincristine from propylparabene, both of which eluted together or with very close retention times. It was then observed that the 250 mm column enabled a better separation of the different components of the sample and gave origin to well-resolved peaks.

Previous selection of the solvents for the mobile phase was based on the information available in the literature (16,25) and on the solubility characteristics of the two drugs. The influence of the relative percentages of acetonitrile at 90% in water and of potassium hydrogenphosphate buffer 50mM (pH 3.2  $\pm$  0.1) in the mobile phase was studied, and it was noted that the 32:68 mixture was the most adequate. Higher percentages of acetonitrile (90%) reduced peak resolution and

lower percentages increased run time, without considerable improvement on the drugs' separation.

Detection wavelength was defined from the absorption spectrum of vincristine and doxorubicin in the UV-vis (Figure 2). The compromise wavelengths of 227, 230, and 233 nm were initially tested, but the second one proved to be more adequate to the simultaneous quantification of the two drugs.

Figure 3 represents a typical chromatogram, obtained in the chromatographic conditions defined for the method.

### Validation of the method

#### Specificity

Specificity (17,18) was evaluated from samples of vincristine and doxorubicin (10 µg/mL) submitted to conditions of forced degradation with H<sub>2</sub>SO<sub>4</sub> 1M and with NaOH 1M, in the presence of light. Samples were submitted to these conditions during four days and injected in the chromatographic system at times: 0, 24, 48, and 96 h. Results were compared with those obtained for the standard solutions. Under the degradation conditions tested, no peaks were detected in the chromatogram of the samples, due to the elution of the degradation products with the solvent front. Comparatively, peaks corresponding to vincristine and doxorubicin were identified in the chromatograms of the standards, with different retention times.

In parallel, standard solutions of methylparabene and propylparabene, present in the commercial formulations of vincristine, were injected in order to verify their interference in the analytical response of the method. The obtained chromatogram presents four perfectly resolved peaks with good separate retention times (Figure 3). These results demonstrate that the developed method does not suffer the interference of the parabenes present in pharmaceutical forms, being specific for vincristine and doxorubicin.

#### Linearity

Linearity (17,18) was evaluated in the interval of concentrations of 5–100 µg/mL for doxorubicin and 1–5 µg/mL for vincristine, using seven standard solutions. A standard calibration curve was

constructed, and linearity was evaluated by the correlation coefficient obtained through the treatment of the results. Each standard was analyzed in triplicate. Obtained results demonstrated that the method was linear in the concentration range of 1–5 µg/mL for vincristine and 5–100 µg/mL for doxorubicin, with average correlation coefficients ( $r^2$ ) of 0.9967 and

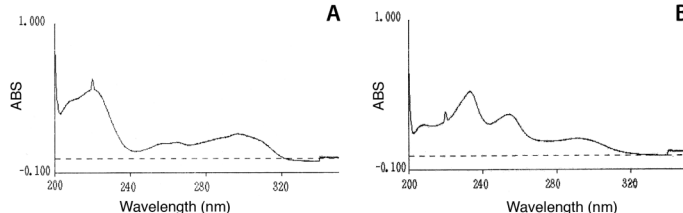


Figure 2. UV-VIS absorption spectra of vincristine (A) and doxorubicin (B) solutions in NaCl 0.9% with concentrations of 5 and 10 µg/mL, respectively.

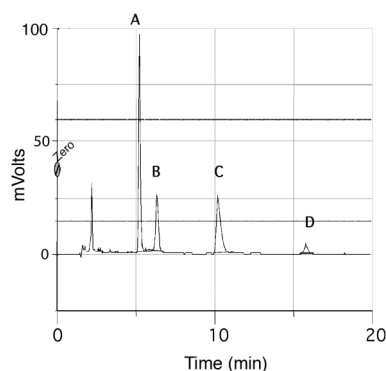


Figure 3. Typical chromatogram of the different analytes on a 5 µg/mL concentration (a = Methylparabene, b = Doxorubicin, c = Vincristine, and d = Propylparabene).

Table I. Repeatability of the Analytical Method ( $n = 3$ )

	Repeatability					
	Vincristine (µg/mL)			Doxorubicin (µg/mL)		
	1.5	2.2	3.0	42	64	84
Mean (µg/mL)	2.43	3.19	4.05	42.54	66.49	87.53
CV (%)	1.87	5.50	3.90	2.31	4.05	4.92

Table II. Intermediate Precision of the Analytical Method ( $n = 3$ )

	Intermediate precision									
	Vincristine (µg/mL)					Doxorubicin (µg/mL)				
	1	2	3	4	5	2	4	6	8	10
Mean (µg/mL)	1.32	2.38	3.48	4.68	6.03	1.92	4.22	5.87	8.16	10.81
CV (%) ± SD	6.47 ± 4.35	3.20 ± 1.04	2.53 ± 1.03	3.13 ± 2.90	9.10 ± 3.69	2.40 ± 1.86	1.70 ± 1.54	4.24 ± 1.64	2.36 ± 0.58	4.04 ± 0.34

0.9958, respectively. The mean values of regression equations for vincristine and doxorubicin were, respectively,  $m = 159333$ ,  $b = 81853$ , and  $m = 113893$ ,  $b = 386588$ , where  $m$  is the slope and  $b$  the intercept.

#### Precision

Precision was considered at two levels, repeatability and intermediate precision, according to the ICH recommendations (17,18). The repeatability was evaluated in triplicate with the concentrations of 1.5, 2.2, and 3.0  $\mu\text{g/mL}$  of vincristine and 42, 64, and 84  $\mu\text{g/mL}$  of doxorubicin. In the evaluation of the intermediate precision, five concentration levels (9 replicated) were used, determined on four different days, and/or by three different operators. Reproducibility, according to the ICH concept (17,18), was not evaluated, since the method still has to be applied in different laboratories.

Tables I and II sum up the results obtained regarding repeatability and intermediate precision. CV data obtained were considered acceptable, according to Bressolle and co-workers (20). These authors considered CV data of  $\pm 15\%$  and  $\pm 20\%$ , respectively, for repeatability and intermediate precision, as tolerable values for concentrations approaching the quantification limit, like tested concentrations.

#### Accuracy

Accuracy was evaluated through recovery testing; three blank samples were fortified with 1, 1.5, and 3 times the

smallest concentration of vincristine and doxorubicin in the samples.

Percentages of recovery of the three blanks spiked with vincristine and doxorubicin are shown in Table III. The percentages of recovery obtained are always  $> 98\%$ , which points to the accuracy of the method, according to the acceptability criteria for this kind of testing (19).

#### Limits of detection and quantification

Regarding detection and quantification limits (17,18), because no quantifiable results were obtained in the blank tests, the formula:

$$y_L = b + K \cdot s_{x/y}$$

developed by Miller (26) was used. Where  $b$  is the intercept value from the least squares calculation, and  $s_{x/y}$  is the standard deviation given by

$$s_{x/y} = \left[ \sum_{i=1}^n (y_i - y_i')^2 / (n-2) \right]$$

In this formula,  $y_i - y_i'$  expresses the distances of each value from the respective calibration point, and  $n$  is the number of measures. The constant,  $K$ , is 3 for the limit of detection (LOD) and 10 for limit of quantification (LOQ) (24,26).

The LOD of the method, calculated through the Miller formula, was 0.19  $\mu\text{g/mL}$  for vincristine and 0.51  $\mu\text{g/mL}$  for doxorubicin. The LOQ, determined by the same method, was 0.63  $\mu\text{g/mL}$  for vincristine and 1.7  $\mu\text{g/mL}$  for doxorubicin. These values were considered acceptable for the present study, and they demonstrate that it is possible to quantify samples with low concentrations of the referred drugs.

	Accuracy					
	Vincristine ( $\mu\text{g/mL}$ )			Doxorubicin ( $\mu\text{g/mL}$ )		
	1.5	2.2	3.0	42	64	84
Mean ( $\mu\text{g/mL}$ )	1.59	2.30	2.95	42.54	66.49	87.53
CV (%)	2.05	2.26	1.27	2.31	4.05	4.92
Recovery (%)	106.26	104.47	98.29	101.28	103.89	104.20

	Quantification of vincristine and doxorubicin admixtures	
	Nominal concentration ( $\mu\text{g/mL}$ )	% of drug Mean $\pm$ SD
<b>Sample A</b>		
VCR	1.5	109.52 $\pm$ 5.92
DOXO	42.6	96.69 $\pm$ 5.40
<b>Sample B</b>		
VCR	15.2	102.95 $\pm$ 8.36
DOXO	857.1	101.59 $\pm$ 4.10
<b>Sample C</b>		
VCR	30.3	106.13 $\pm$ 1.59
DOXO	1700	99.67 $\pm$ 4.84

\*The results are the mean  $\pm$  SD of three injections and are expressed as percentage.

#### Method application to vincristine and doxorubicin samples

Admixtures of vincristine and doxorubicin prepared from the commercial dosage forms were quantified. Each sample was analyzed in triplicate and the results are expressed on Table IV. The results are the mean  $\pm$  SD of three injections and are expressed as percentage.

#### Conclusion

The developed HPLC–UV method is simple, sensitive, specific, and adequate to the simultaneous quantification of vincristine and doxorubicin. The method was validated according to ICH guidelines and proved to be precise and accurate. The developed method can, thus, be used in the laboratory to routinely quantify vincristine and doxorubicin simultaneously and to evaluate the physico-chemical stability of referred drugs in mixtures for endovenous use. The method also

proved to have adequate characteristics to be applied to the simultaneous quantification of methylparabene and propylparabene.

## Acknowledgments

The authors thank the CIMAGO—Centre for Research of Environment, Genetics and Oncobiology (project No. 04/05) for the financing granted.

The authors also thank the Coimbra's University Hospitals (HUC), specially their pharmaceutical services, for the support given during the present study.

## References

- Martindale. *The Complete Drug Reference*, 34th ed. Pharmaceutical Press, London, UK, 2005.
- AHFS Drug Information. American Society of Health-System Pharmacists, Bethesda, MD, 2005.
- W. Osswald and S. Guimarães. *Terapêutica Medicamentosa e suas Bases Farmacológicas*, 4th ed., Porto Editora, Porto, 2001.
- B.G.M. Durie. *Multiple Myeloma—A Concise Review of the Disease and Treatment Options*. International Myeloma Foundation: USA, 2004. On-line accessed on 05.06.22 at <http://myeloma.org>
- New Front-Line Data at ASCO in Multiple Myeloma Support Important Role of VELCADE® (bortezomib) for Injection—Market-leading drug in relapsed setting also benefits high-risk patients. Millennium Pharmaceuticals. On-line accessed on 05.06.08 at <http://www.millennium.com>.
- K.C. Anderson, R.A. Kyle, J.R. Berenson, and W.S. Dalton. Recent advances in the biology and treatment of Multiple Myeloma. American Society of Haematology. On-line accessed on 04.06.25 at <http://www.hematology.org/>.
- C. Sottani, G. Tranfo, M. Bettinrlli, P. Faranda, M. Spagnoli, and C. Minoia. Trace determination of anthracyclines in urine: a new high-performance liquid chromatography/tandem mass spectrometry method for assessing exposure of hospital personnel. *Rapid Commun. Mass Spectrom.* **18**: 2426–2436 (2004).
- F. Lachâtre, P. Marquet, S. Ragot, J.M. Gaulier, P. Cardot, and J.L. Dupuy. Simultaneous determination of four anthracyclines and three metabolites in human serum by liquid chromatography–electrospray mass spectrometry. *J. Chromatogr. B* **738**: 281–291 (2000).
- M.S. Schmidt, R. Huang, R.J. Classon, and D.J. Murry. Determination of vincristine in infant plasma by liquid chromatography–atmospheric pressure chemical ionization–mass spectroscopy. *J. Pharm. Biomed. Anal.* **41**: 540–43 (2006).
- J.I. Lee, J.M. Skolnik, J.S. Barret, and P.C. Adamson. A sensitive and selective liquid chromatography–tandem mass spectrometry method for the simultaneous quantification of actinomycin-D and vincristine in children with cancer. *J. Mass Spectrom.* **42**: 761–70 (2007).
- J.M. Skolnik, J.S. Barret, H. Shi, and P.C. Adamson. A liquid chromatography–tandem mass spectrometry method for the simultaneous quantification of actinomycin-D and vincristine in children with cancer. *Cancer Chemother. Pharmacol.* **57**: 458–64 (2006).
- M.M. Gupta, D.V. Singh, A.K. Tripathi, R. Pandey, R.K. Verma, S. Singh, A.K. Shasany, and S.P. Khanuja. Simultaneous determination of vincristine, vinblastine, catharanthine, and vindoline in leaves of *catanranthus roseus* by high-performance liquid chromatography. *J. Chromatogr. Sci.* **43**(2): 450–53 (2005).
- E.K. Nyhammar, S.G. Johansson, and B.E. Seiving. Stability of doxorubicin hydrochloride and vincristine sulfate in two portable infusion-pump reservoirs. *Am. J. Health-Syst. Pharm.* **53**: 1171–73 (1996).
- J.H. Beijnen, C. Neef, O.J. Meuwissen, J.J. Rutten, H. Rosing, and W.J. Underberg. Stability of intravenous admixtures of doxorubicin hydrochloride and vincristine sulfate. *Am. J. Hosp. Pharm.* **43**: 3022–27 (1986)
- M.L. Stiles and L.V. Allen, Jr. Stability of doxorubicin hydrochloride in portable pumps reservoirs. *Am. J. Hosp. Pharm.* **48**: 1976–77 (1991)
- J.L. Wolfe, L.A. Thoma, C. Du, B.R. Goldspiel, J.F. Gallelli, G.J. Grimes, and G.K. Potti. Compatibility and stability of vincristine sulfate, doxorubicin hydrochloride, and etoposide in 0.9% sodium chloride injection. *Am. J. Health-Syst. Pharm.* **56**: 985–89 (1999).
- International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use. ICH Q2B: Validation of Analytical Procedures—Methodology, 1996.
- International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use. ICH Q2A: Text on Validation of Analytical Procedures, 1994.
- G.A. Shabir. Validation of high-performance liquid chromatography methods for pharmaceutical analysis. *J. Chromatogr. A* **987**: 57–66 (2003).
- F. Bressolle, M. Bromet-Petit, and M. Audran. Validation of liquid chromatographic and gas chromatographic methods—Applications to pharmacokinetics. *J. Chromatogr. B* **686**: 3–10 (1996).
- J. Ermer. Validation in pharmaceutical analysis. Part I: an integrated approach. *J. Pharm. Biomed. Anal.* **24**: 755–67 (2001).
- Eurachem Working Group. *The Fitness for Purpose of Analytical Methods—A Laboratory Guide to Method Validation and Related Topics*. 1998.
- The United States Pharmacopeia Convention. US Pharmacopeia 24, Validation of Compendial Methods. Rockville, 1999.
- M. Thompson, S.L.R. Ellison, and R. Wood. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). *Pure Appl. Chem.* **74**: 835–855 (2002).
- The Merck Index*. 13<sup>th</sup> ed.; Chapman and Hill, London, UK, 2001.
- J.N. Miller. Basic statistical methods for analytical chemistry. Part 2. Calibration and regression methods: a review. *Analyst* **116**: 3–14 (1991).

Manuscript received January 7, 2008;  
revision received March 27, 2008.