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Quantitation of a novel metalloporphyrin drug in plasma by atomic absorption spectroscopy

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Abstract

A bioanalytical method to quantify cobalt mesoporphyrin (CoMP), a novel therapeutic agent, in plasma has been developed and validated. The approach involves atomic absorption spectroscopy to determine total cobalt in a sample and a back-calculation of the amount of compound present. Endogenous plasma cobalt concentrations were small (<0.2 ng ml^{-1} Co in rat plasma) in comparison to the quantitation limit (4.5 ng ml^{-1} Co). The inter-day imprecision of the method was 10.0% relative standard deviation (RSD) and the inter-day bias was $\pm 8.0\%$ relative error (RE) over a standard curve range of 4.5–45.0 ng ml^{-1} Co. Because it quantifies total cobalt, the method cannot differentiate between parent drug and metabolites, but negligible metabolism allows reliable estimates of the actual parent drug concentration. A correlation study between the atomic absorption method and ^{14}C-radiometry demonstrated excellent agreement ($r = 0.9868$, slope $= 1.041 \pm 0.028$, intercept $= 223.7 \pm 190.0$) and further substantiated the accuracy of the methods. Methodology was successfully applied to a pharmacokinetic study of CoMP in rat, with pharmacokinetic parameter estimation. The elimination half-lives, after intra-muscular and subcutaneous administration, were 7.7 and 8.8 days, respectively.

Keywords: Cobalt mesoporphyrin (CoMP); Pharmacokinetics; Xenobiotics; Plasma; Atomic absorption spectroscopy; Correlation

1. Introduction

The vast majority of bioanalytical methods for determining xenobiotics in plasma or other biological samples involve the use of a highly selective chemical separation or several separations implemented in tandem, followed by a sensitive and selective means of detection. Analytical extractions coupled to chromatographic separations are representative of this direct approach to bioanalytical quantitation [1,2]. Although widely accepted as a powerful and reliable approach, chromatography is not universally applicable to all problems in bioanalysis, especially when the chromatographic properties of the compound are unfavorable. In these situations, the analytical chemist is compelled to face the challenge of bioanalysis from an alternative perspective.

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The determination of the antiobesity drug candidate, cobalt mesoporphyrin (CoMP, Fig. 1), in biological samples has proven to be one such unfavorable situation. Unlike other porphyrins [3–7], this compound exhibits a number of undesirable analytical properties, including extremely strong (nearly irreversible) protein binding [8], no native fluorescence, poor analytical extraction characteristics, high surface binding properties and only modest detectability using UV, electrochemical or mass spectrometric detection. It has been shown that the high protein binding associated with this compound can be significantly disrupted only by addition of a concentrated solution of sodium or potassium cyanide [8]. Without this reagent present to form a monocyano complex, chemical speciation makes clean chromatography extremely difficult. Although the compound contains two carboxylic acid-moieties that could be used for prosthetic attachment of derivatives, clearly the numerous analytical problems associated this molecule make a method based on derivatization cumbersome or unlikely.

Atomic absorption spectroscopy (AAS) is an important analytical technique for a variety of samples, including plasma and tissues [9]. It is extensively used for the quantitation of metals or elements, and is generally very convenient in that it can be implemented without a prior separation step. Samples are usually only diluted or, at most, digested with a mineral acid. Because the technique generally responds only to the elemental portion of a compound, however, AAS does not distinguish between a drug molecule containing a metal atom and related metabolites that could be present.

In this work, atomic absorption spectroscopy was used to indirectly quantify CoMP in plasma from Wistar rats by determining total cobalt and relating this to the amount of parent drug present. Metabolic profiling for [^{14}C]-CoMP in several animal models had previously demonstrated only slight metabolism of CoMP [8] and allows nearly exact correlation between the determined amount of cobalt and the amount of drug present. The method has been applied to a pharmacokinetic study in Wistar rats following single intra-muscular (IM) and subcutaneous (SC) doses of CoMP glycinate.

2. Experimental

2.1. Reagents

Cobalt mesoporphyrin, glycinate salt (PD 160384, CoMP) was synthesized at Parke–Davis Pharmaceutical Research, Ann Arbor, MI. Radiolabeled [^{14}C]-CoMP was synthesized in-house (lot 6b, radiochemical purity 97.0%, chemical purity 99.2% or lot 12c, radiochemical purity 95.6%, chemical purity 99.1%). All CoMP and [^{14}C]-CoMP concentrations, except where noted otherwise, are expressed as free-acid equivalents. Water and methanol (HPLC grade) were obtained from EM (Cherry Hill, NJ). Triton X-100 was obtained from Sigma (St. Louis, MO). Heparinized rat plasma was obtained from Parke–Davis (Ann Arbor, MI) or from Pel-Freez (Rogers, AK). Scintillation cocktail was ultima gold XR, (Packard, Meriden, CT).

2.2. Apparatus

An atomic absorption spectrophotometer (Varian, Sunnyvale, CA) equipped with a graphite furnace (GTA-95, Varian), integrator (DS-15, Varian), autosampler (PSD-95, Varian), deuterium lamp background correction, and a cobalt hollow-cathode lamp (PN 56-101232-00, Varian) was used for cobalt determinations. Graphite furnace tubes were obtained from Varian (p/n 63-100012-00). Conical bottom, polypropylene autosampler vials were obtained from Elkay (Boston, MA).

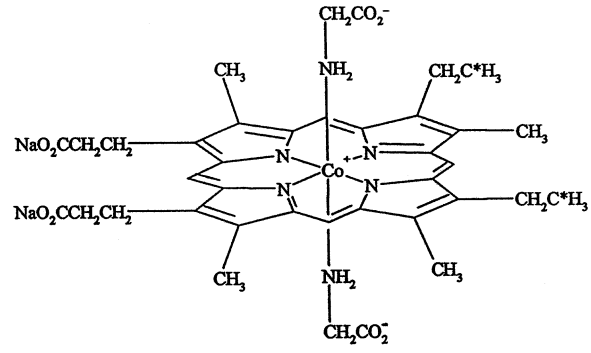

Fig. 1. Chemical structure of cobalt mesoporphyrin glycinate, with the position of the ^{14}C isotope indicated by *.

Table 1
Graphite furnace atomic absorption spectrophotometer temperature program for cobalt in rat plasma

Step	Final temperature (°C)	Time (s)	Argon flow (ml min^{-1})	Integration	Functional operation
1	85	3.0	3		Injection
2	95	40.0	3		Drying I
3	150	3.0	3		
4	150	60.0	3		Drying II
5	500	10.0	3		
6	500	30.0	3		Na/K removal
7	850	10.0	3		
8	850	30.0	0		Ashing
9	2300	1.2	0		Aspiration
10	2300	2.0	0	Active	Measurement
11	2400	3.0	3		Tube cleaning

The instrument was operated with argon purge gas flow of 3.0 ml min^{-1}, a detection wavelength of 241.2 nm, a hollow cathode lamp current of 7.0 mA and deuterium lamp baseline correction. The graphite furnace temperature began at 85°C (injection) and proceeded according to the program outlined in Table 1. The peak-height response for cobalt was recorded in step 9 of the program, with an integration time of 2.0 s. Replicate injections ($n = 2$) were averaged for each sample, standard or quality control. In graphite furnace atomic absorption, organic interferences were discriminated against by thermal decomposition at temperatures between 150 and 850°C. Ionic sodium and potassium, two common plasma components, were removed by ashing at 500°C. Other less volatile metals and some refractory organic compounds were removed at higher temperatures on the heating curve. Cobalt was aspirated at 2300°C and an absorption reading was taken immediately after the graphite furnace reached this temperature (integration). Any remaining background or broadbanded background absorption was corrected for by a deuterium background correction technique [10].

Scintillation counting was performed on a Minaxi Tricarb 4000 series counter (Packard, Meriden, CT).

2.3. Standards, quality controls and blanks

A stock solution of CoMP was prepared at 1 mg/10 ml by dissolving a weighed amount of CoMP in a small amount of aqueous ammonium hydroxide (1 N). This stock solution was diluted with methanol, serially diluted with plasma and spiked (10 μl) into plasma to produce five final standard concentrations of 50, 100, 150, 250 and 500 ng ml^{-1} of CoMP, corresponding to between 4.5 and 45 ng ml^{-1} of elemental cobalt.

Using a separate weighing of CoMP, quality controls containing 75.0, 200 and 400 ng ml^{-1}, corresponding to 6.75, 18.0 and 36.0 ng ml^{-1} of cobalt, were prepared. These controls contained a minimum of 95% plasma. Quality control samples were then stored as 0.5 ml aliquots at −20°C.

2.4. Atomic absorption sample preparation

Rat plasma samples and controls were assayed by aliquoting 100-μl of heparinized plasma samples into glass culture tubes, adding 10-μl of aqueous 1% Triton X-100 (an antifoam surfactant), and votexing (~3 s). Samples were then transferred to polypropylene autosampler vials. Standards were prepared by aliquoting 990-μl of plasma into glass-culture tubes, adding 100-μl

aliquots of Triton X-100 solution and vortexing prior to transferring to autosampler vials. Using the autosampler, a 10-μl aliquot of each sample, control or standard was injected into the furnace tube. All samples, controls and standards were injected in duplicate and the average absorbance was used for calculations. Before the start of each auto-run, a response close to zero (± 0.004) was obtained for the first two blank injections. This mean blank value was subtracted from all subsequent injections as a background correction.

2.5. Calibration

Five standards, containing cobalt in the form of CoMP, and a blank sample were injected in duplicate. Calibration curves were established using average ($n = 2$) peak-height response versus concentration of CoMP. Because of the well-documented curvilinear response of this analytical technique [10], the best-fit line was determined by a least-squares quadratic fit of the calibration data from each batch run using a weighting factor of $1/\text{concentration}^2$ ($1/c^2$). Peak heights were chosen over peak area to decrease the likelihood of interferences contributing to the response.

2.6. Method validation

For method validation purposes, typical batch runs consisted of a standard curve with duplicate injections, quality controls in triplicate at three levels (3×3 samples) with duplicate injections, and a blank injected in duplicate. A total of three batch runs were processed for this validation and from these data the performance of the method was assessed. Method selectivity, quality of calibration curve fits, and intra-/inter-day precision (% relative standard deviation) and accuracy (% relative error), based on quality controls were estimated and are reported here.

2.7. Comparison of methods

A correlation study was conducted to compare the quantitative accuracy of the atomic absorption method to that for a radiometric method based on scintillation counting of [^{14}C]-CoMP. Male Wistar rats were dosed at 1.2 or 2.5 mg kg^{-1}, IV, or at 2.5 mg kg^{-1}, IM with [^{14}C]-CoMP. Blood samples were drawn at time intervals from 0 h to 29 days, through jugular-vein cannula. For each sample, plasma was separated from the cells, diluted (100 μl) with 10 ml of scintillation fluid and counted for 10 min. After converting from counts min^{-1} to disintegrations min^{-1} and correcting for background, CoMP equivalent concentrations were calculated for each sample using the specific activity (60.26 or 62.18 μCi mg^{-1}) of the labeled compound. Samples from some rats were pooled to ensure adequate sample volume.

For the atomic absorption portion of the correlation study, 20 plasma samples were selected from those described above and assayed in duplicate. Samples were selected to encompass representative high, medium and low plasma levels over a ~2.5 order of magnitude range, based on data obtained from the scintillation-counting experiments. Plasma samples were diluted as necessary (dilution factors of 1, 10, 50 or 100) with pooled, blank Wistar rat plasma so that their concentrations would fall within the calibration range of the atomic absorption method.

To assess the accuracy of the method, correlation data were entered as x, y pairs (concentration by radiometry versus concentration by atomic absorption) into a spread-sheet-plotting program containing an unweighted linear regression feature (SIGMAPLOT, version 3.0). Because this program did not report the uncertainties associated with slope and intercept, these parameters were calculated separately using CALIBRAT, a general-purpose linear regression program available in our laboratory. The slope, intercept and correlation coefficient calculated using SIGMAPLOT agreed exactly with those calculated using CALIBRAT, to at least four significant figures.

2.8. Pharmacokinetic study

The pharmacokinetics of CoMP were assessed after single 30 μmol kg^{-1} IM or SC doses in Wistar rats ($n = 4$). Plasma samples were harvested over the course of 50 days and assayed by the atomic absorption method. Noncompartmental pharmacokinetic parameters C_{max}, t_{max}, $t_{1/2}$

and AUC were assessed for each portion of the study.

3. Results and discussion

A conventional HPLC approach to the determination of CoMP would require the use of potassium cyanide (~5% v/v) to disrupt to strong and extensive protein binding which takes place in plasma and tissues [8]. Without this treatment, recovery from a sample preparation procedure is typically less than 5%. Detection of the compound by absorbance of visible light (λ_{max} 415 nm), although possible, is not sensitive (250 ng ml^{-1} quantitation limit in plasma) or selective. The attachment of a prosthetic fluorophore to one of the carboxylic acid moieties in the molecule would make to an already cumbersome sample preparation procedure even more tedious. A brief evaluation of mass spectrometric detection was dominated by potassium adducts which resulted from potassium cyanide addition. This adduct was generally unreliable, making LC/MS unsuitable for quantitation. For these reasons, the use of graphite furnace atomic absorbance was selected for evaluation.

Table 2
Intra- and inter-run accuracy (%RE) and precision (%RSD) estimates for the atomic absorption method, based on quality controls containing CoMP

	Nominal quality-control concentration (ng ml^{-1})		
	75.0	200	400
Run 1			
Intra-run mean	76.0	205	348
Intra-run %RSD	10.5	5.1	0.7
Intra-run %RE	1.3	2.5	−13.0
Run 2			
Intra-run mean	69.8	222	433
Intra-run %RSD	3.6	2.3	3.9
Intra-run %RE	−6.9	11.0	8.3
Run 3			
Intra-run mean	70.6	222	378
Intra-run %RSD	1.0	4.8	2.5
Intra-run %RE	−5.9	11.0	−5.5
n	9	9	9
Inter-run mean	72.1	216	386
Inter-run %RSD	8.7	5.4	10.0
Inter-run %RE	−3.9	8.0	−3.5

3.1. Atomic absorption method performance

Graphite furnace tubes did not show any significant loss in response (8.6% relative standard deviation (RSD)) over the course of 80 sample injections (10 μl). Reproducibility of response, determined for nine replicate injections of diluted biological samples at low, medium, and high concentrations of CoMP, was ±8.7, 5.4, and 10.0% for CoMP, at concentrations of 75.0, 200, and 400 ng ml^{-1}, respectively.

Rat plasma contains a small amount of endogenous cobalt. Six sources of representative rat plasma gave variable, but very small blank readings of less than 0.003 absorbance units (AU), or $<12\%$ of a typical low standard reading (0.026 AU). Because these low levels of endogenous cobalt were well below the quantitation limit, they did not interfere with the accurate quantitation of drug. The quantitation limit for the method was defined as the lowest concentration on the standard curve. This level of cobalt (4.5 ng ml^{-1} Co) could be detected with a signal-to-noise (S/N) ratio of approximately 10, corresponding to 50.0 ng ml^{-1} for parent drug. Replicate standards produced a precision of 3.2% RSD at this level. Samples containing less than 50.0 ng ml^{-1} of CoMP were not quantified.

Assay calibration was validated with rat plasma standards containing 50.0–500 ng ml^{-1} of CoMP in three separate batch runs. Standard peak-height responses were related to the amount of CoMP added to rat plasma over these ranges using a quadratic function. With regression weightings of $1/c^2$, the best-fit quadratic line was determined for each batch run. Calibration curve reproducibility was evaluated by the variation of individual back-calculated standards from the regression line. Relative standard deviations for calibration standards ranged from 2.4 to 11.2% over three batch runs, with relative errors between

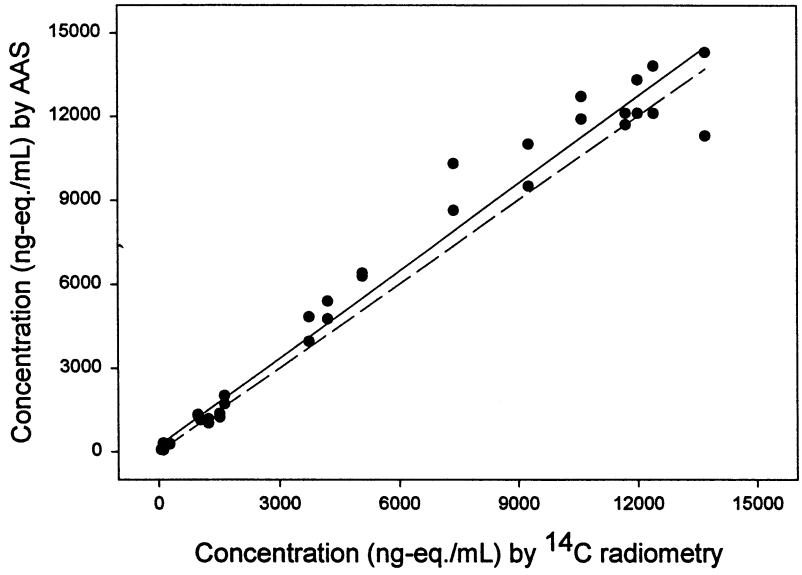

Fig. 2. Correlation plot of concentration of CoMP as determined by ^{14}C-radiometry and atomic absorption spectroscopy. The solid line is a determined linear regression line for the data (slope = 1.041 ± 0.028, intercept = 223.7 ± 190.0, correlation coefficient (r = 0.9868) and the dashed line is the line of unity slope.

−4.3 and 2.0%. These results were slightly better than those generated using $1/c$ weighting. Based on the accuracy (percent relative error (%RE)) and precision (%RSD) of calibration standards, the method demonstrated sufficient adherence to a quadratic model over the concentration range of 50.0 to 500 ng ml^{-1} of CoMP. For routine assays, samples containing greater than 500 ng ml^{-1} of CoMP were diluted with rat plasma and reassayed. An alternate standard preparation approach, using cobalt nitrate as a primary standard, did not produce accurate results (> 20% RE) for CoMP quality controls and was not pursued.

Intra-run precision and accuracy were determined by assaying quality controls in triplicate, at each of three levels, in three separate batch runs. Using the $1/c^2$ weighting, intra-run precision estimates for CoMP were less than 10.5, 5.1, and 3.9% (%RSD) for controls containing 75.0, 200, and 400 ng ml^{-1} of CoMP, respectively. Intra-run %RE was ± 13.0%, slightly better than those produced with $1/c$ weightings Table 2.

Inter-run precision and accuracy were determined by pooling determinations of triplicate quality controls over three batch-runs. Inter-run precision estimates were 8.7, 5.4, and 10.0% (%RSD) for controls containing 75.0, 200, and 400 ng ml^{-1} of CoMP, respectively. Inter-run RE was < 8.0%. Comparable results were obtained using a regression weighting of $1/c$, but this weighting was not pursued.

3.2. Comparison of methods

A graph of CoMP concentrations, as determined by AAS, versus those determined by ^{14}C-radiometry is presented in Fig. 2. In this plot, individual data points are depicted as solid black dots, the regression result is shown as a solid black line, and the line of unity (line with slope = 1) is depicted as a dashed line. The regression results yielded the linear equation:

$$Y = 1.041X + 223.7$$

where the uncertainties (standard deviations) in slope and intercept are 0.028 and 190.0. When compared to 95% confidence intervals from Student's t-test, slope and intercept are not statistically distinguishable ($p > 0.05$) from 1 and 0, respectively, suggesting that any real differences in the data collected by these orthogonal methods are obscured by random error.

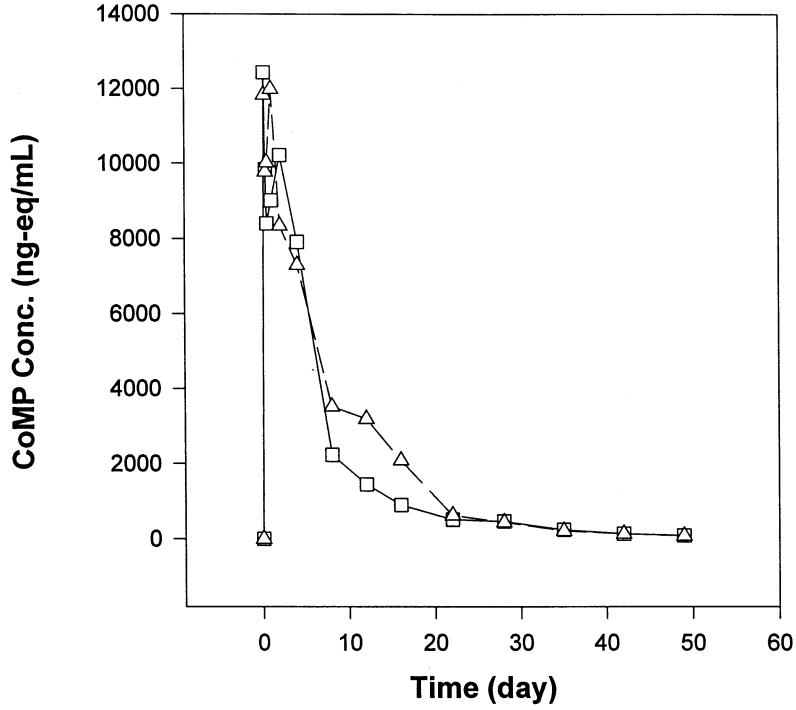

Fig. 3. Pharmacokinetic disposition profiles for single 30 μmol kg^{-1} intra-muscular (IM, □) or subcutaneous (SC, 7△) doses of CoMP in Wistar rats.

The correlation coefficient for the regression line has been calculated as 0.9868, indicating a strong correlation between the CoMP, as determined by the two methods. The correlation of concentration, as separately determined by AAS and by ^{14}C-radiometry gives additional credibility to the accuracy of each method. Although either approach would yield only equivalent concentrations of CoMP in the presence of metabolites, the absence of any significant metabolism makes the selective quantitation of parent drug possible. Because of a strong correlation between the concentration data determined by these two methods, and because the slope and intercept of the regression line for these data are not distinguishable from 1 and 0, respectively, quantitative results collected by either method can be directly compared.

Atomic absorption spectroscopy, although nonspecific, can accurately quantify CoMP in plasma because of minimal metabolism ($< 5\%$) in all species studied [8]. The AAS approach circumvented the need for conventional extraction/separation/detection methodologies commonly used for small drug molecules. Sample preparation was minimized to a few volumetric dilutions and a surfactant addition, which are easy to implement. Standardization of the AAS assay using CoMP as the primary standard in lieu of a conventional cobalt source added quantitative accuracy to the method by minimizing matrix- and compound-related effects. Although plasma from mammalian species does contain endogenous cobalt, the levels are generally negligible (< 0.2 ng ml^{-1} for rats) when corrected for by background subtraction technique used in this work.

3.3. Pharmacokinetic study

Following IM or SC administration of 30 μmol kg^{-1}, CoMP was slowly absorbed, with maximum plasma concentration (C_{max}) achieved at 1.1 and 1.3 days post-dose, respectively. The absorption may last for several days (Fig. 3). CoMP elimination $t_{1/2}$ values were 7.7 and 8.8 days for IM and SC routes, respectively. Plasma AUC values were similar after IM (97.8 μg-eq day ml^{-1}) and SC (99.3 μg-eq day ml^{-1}) doses.

4. Conclusions

An indirect method for quantifying the novel therapeutic agent CoMP in plasma, based on atomic absorption spectroscopy, has been developed and validated. The method is accurate ($<$ 8% RE) and precise ($<10\%$ RSD), based on replicate control determinations, and is easier to implement than proposed conventional procedures involving disruption of protein binding, protein precipitation and HPLC. The methodology has been applied to a methods comparison study, versus ^{14}C radiometry, with good results and has been used to obtain information regarding the pharmacokinetics of this potential drug.

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References

[1] T.D. Parker, D.T. Rossi, D.S. Wright, Anal. Chem. 68 (1996) 2437–2441.
[2] D.T. Rossi, D.S. Wright, J. Pharm. Biomed. Anal. 15 (1997) 495–504.
[3] R.M. Smith, D. Doran, M. Mazur, B. Bush, J. Chromatogr. 181 (1980) 319–327.
[4] W.E. Schreiber, V.A. Raisys, R.F. Labbe, Clin. Chem. 29 (1983) 527–530.
[5] H.C. Freidmann, E.T. Baldwin, Anal. Biochem. 137 (1984) 473–480.
[6] S.W. Kennedy, D.C. Wigfield, G.A. Fox, Anal. Biochem. 157 (1986) 1–7.
[7] W. Wu, A. Stalcup, J. Liq. Chromatogr. 17 (1994) 1111–1124.
[8] M.R. Feng, D.T. Rossi, C. Strenkoski, A. Black, P. DeHart, M. Lovdahl, W. McNally, Xenobiotica 28 (1998) 413–426.
[9] D.W. Rosenberg, Drug Metab. Dispos. 21 (1993) 846–849.
[10] D.C. Harris, Quantitative Chemical Analysis, ch. 21, W.H. Freeman, New York, 1991.