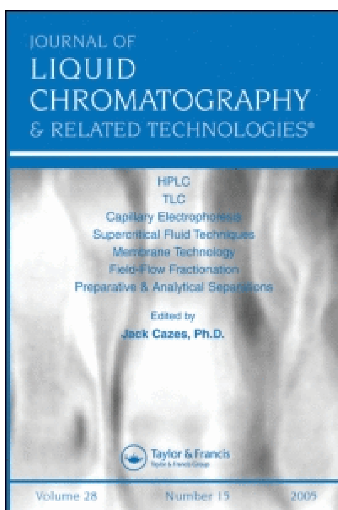
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



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

A VALIDATED ANALYSIS OF PEMOLINE IN URINE BY HPLC/DAD AFTER SOLID PHASE EXTRACTION

Nicholas Parissis[a]; Weidong He[a]; Trifon Kiratzidis[b]
[a] University of Ghent, Laboratory of Toxicology, Ghent, Belgium [b] Veria Medical Centre, Veria, Greece

Online publication date: 17 November 1999

To cite this Article Parissis, Nicholas , He, Weidong and Kiratzidis, Trifon(1999) 'A VALIDATED ANALYSIS OF PEMOLINE IN URINE BY HPLC/DAD AFTER SOLID PHASE EXTRACTION', Journal of Liquid Chromatography & Related Technologies, 22: 20, 3151 — 3175

To link to this Article: DOI: 10.1081/JLC-100102082
URL: http://dx.doi.org/10.1081/JLC-100102082

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J. LIQ. CHROM. & REL. TECHNOL., 22(20), 3151–3175 (1999)

Downloaded At: 10:12 24 January 2011

A VALIDATED ANALYSIS OF PEMOLINE IN URINE BY HPLC/DAD AFTER SOLID PHASE EXTRACTION

Nicholas Parissis,[1] Weidong He,[1,]* Trifon Kiratzidis[2]

[1] University of Ghent
Laboratory of Toxicology
Hospitaalstraat 13
9000 Ghent, Belgium

[2] Veria Medical Centre
Paster Street 8
59300 Veria, Greece

ABSTRACT

A rapid bioanalytical method has been developed for the determination of pemoline in urine, using 4-methyl-primidone as the internal standard. The analyte was isolated from urine with a C_{18} reverse solid-phase extraction column and analysed by high performance liquid chromatography with a photo diode array detector. The method was linear in the studied range of 0.20 to 1.40 µg/mL urine. For the standard curve a weighted ($1/x^2$) linear regression line (y = - 0.006 + 0.976x) was computed and validated (R^2 = 0.998). The limit of quantitation was 0.04 µg/mL urine. Recovery studies for the accuracy gave a mean percent recovery of 93.9 and a predicted percent recovery in the range of 91.5 to 96.0. The precision, expressed as coefficient of variation, was in the range of 2.03 to 3.67 µg/mL for the intraday and from 1.96 to 4.35 µg/mL for the inter-day. The ANOVA (analysis of variance) tables were also given to validate the day to day reproducibility. Application of the method to clinical samples was demonstrated.

Copyright © 1999 by Marcel Dekker, Inc. www.dekker.com

Downloaded At: 10:12 24 January 2011

INTRODUCTION

Pemoline, Stimul®, 2-imino-5-phenyl-4-oxazolidinone, a CNS stimulant with positive effects on psychomotor performance,[1] is structurally dissimilar to the amphetamines and is indicated as an adjunctive therapy in children with attention deficit hyperactivity disorder (ADHD); it decreases impulsive behaviour and hyperactivity and increases attention.[2,3] It has been used in the treatment of mental depression,[4] as a mild stimulant in geriatric patients,[5] in narcolepsy,[6,7] and to increase alertness and relieve fatigue in multiple sclerosis patients.[8,9] It's also used in cancer[10,11] and AIDS patients[12,13] suffering from depressed moods, apathy, decreased energy, poor concentration, and weakness.

Pemoline is also an ingredient of an oral preparation, also containing yohimbine and methyltestosterone, given with the intention of managing failure of sexual desire and functioning in males and females.[14] It should not be used for prevention or treatment of normal fatigue.[15] When pemoline is misused to enhance athletic performance (doping),[16] there is a risk of dangerous physical overexertion. Because of the absence of a sense of fatigue, a drugged athlete may be able to mobilize ultimate energy reserves and in extreme situations, cardiovascular failure may result.[17]

General screening methods for stimulants, narcotic analgesics, or beta-blockers used in doping control or toxicological analysis do not include pemoline because of its difficulty of extraction and analysis.[18,19,20,21] This is solved by giving special attention and developing a separate analytical method for its detection in urine samples.[22]

Clinical studies concluded that since the optimum therapeutic serum concentration shows wide variation between patients, the dosing regimen must be determined individually. Routine monitoring in the clinical laboratory of the pemoline serum concentrations is not useful because of this apparent variation in optimum serum concentration and because of the linear relationship between dose and concentration.[23]

In the USA, for children 6 years and over, and with ADHD, the initial oral dose is 37.5 mg (20 mg in UK) given as a single dose each morning and it may be increased by 18.75 mg a day at weekly intervals until the desired clinical response is obtained. The effective dose for most patients is 56.25 to 75 mg a day and the maximum recommended dose is 112.5 mg a day.[14,15,24] The similarity of the structures of Pemoline and 4-methyl-primidone (IS) are given in Figure 1.

Methods for the determination of pemoline in biological fluids have been published since 1969 by UV spectrophotometry,[25] gas chromatography with NPD,[26] GC-FID,[27] GC-NSD,[28] GC-ECD,[29,30] high performance liquid chromatography with UV detector,[31-36] mass-spectrometry[37] and thin-layer chromatography.[38]

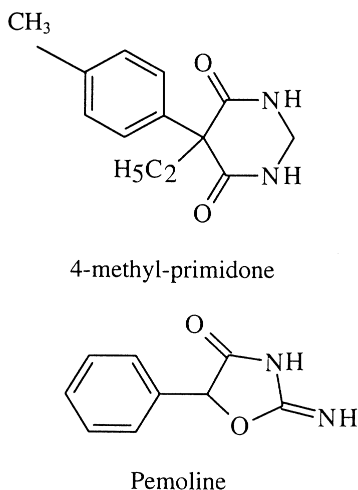


Figure 1. Chemical structures of 4-methyl-primidone and pemoline.

Downloaded At: 10:12 24 January 2011

This paper describes a gradient HPLC method with a photo diode array detector (DAD) which facilitates quantification of the analyte. For the sample preparation, a solid phase extraction (SPE) method was developed, which allows extraction of a large amount of sample and obtaining an increased sensitivity of determination.

EXPERIMENTAL

Liquid Chromatography System and Chromatographic Conditions

A Hewlett Packard model 1090M liquid chromatograph, equipped with a HP 1040A diode array detector (DAD) was used. The system was controlled by a HP 79994A HPLC work station which consists of a HP 9000-300 computer, a HP 9133 disc driver, a HP 2225AB Thinkjet printer, and a HP 7440A Colour Pro Graphics plotter. A Rheodyne injector with a 10 μL loop was fixed on the instrument.

The analytical chromatographic column was a 100 mm × 3 mm i.d. Chromsep glass column packed with Lichrosorb RP-18, 7 μm (Cat. no. 28297) protected by a 10 mm × 2.1 mm i.d. reversed phase guard column (cat. no. 28141), all from Chrompack (Antwerp, Belgium). The sample clarification kit was the ACRO LC13 disposable filter assembly from Gelman Sciences, Michigan, USA.

The gradient elution consisting of water containing acetonitrile(A) was applied with the following profile: 0 to 4 min, from 5 to 10%(v/v) A; 4 to 5 min, from 10 to 15% A; 5 to 9.50 min, from 15 to 25% A and 9.50 to 12 min, 25% A at a flow rate of 0.7 mL/min resulting in an average back pressure of 100-130 bar. The monitoring wavelength was set at 216 nm, bandwidth 4 nm, for the pemoline detection with a reference wavelength at 550 nm, bandwidth 100 nm. The degassing was with helium and the separation was performed at ambient temperature. Ultraviolet spectra in 210-400 nm were memorized in peak-controlled mode. That means at the apex, at the up- and downslopes of each peak detected on the 216 nm signal, and also at the base after the peak. After an equilibration time of about 60 min, the first injection could be applied.

Solid-phase extraction (SPE) C_{18} BondElut extraction columns (200 mg / 3.0 mL capacity) obtained from Varian (Belgium).

Downloaded At: 10:12 24 January 2011

Reagents and Samples

Drug Standard

The pemoline was a gift from Abbott Laboratories Ltd. (Queenborough, Kent, England, ME11 5EL). The internal standard, 4-methylprimidone, was obtained from Aldrich Chemical Co (Belgium). Both were analysed by mass spectrometry in order to confirm the absence of impurities, before used in our laboratory.

Stock Solutions

Stock pemoline was prepared by dissolving the required amount of the drug standard in methanol to give a 2 mg/mL solution. Stock 4-methylprimidone was also prepared in methanol to give a 0.5 mg/mL solution. Initially, two identical stock standards were prepared from each chemical, so that they could be checked against each other for stability. UV spectrometry was used to assay these gravimetrically prepared standard solutions. To calculate the maximum storage time, the standards were analysed monthly in duplicate. This allowed the calculation and estimation of the sample standard deviation s (s = difference between duplicates / $\sqrt{2}$). The mean of the duplicate measurements were plotted with respect to time and the least-squares line drawn. The smallest difference in 2 measured values that is significant at the 95% confidence level is approximately 3s. For this reason the maximum storing time is given by the point at which the regression line reaches a concentration which is 3s less than the initial concentration value of the stock standard.

The two above-mentioned stock solutions were stored at 4°C in dark amber glass vials with tightly fitting, teflon-lined screw caps and controlled monthly for stability, were found stable for 6 months.

Solvents and Chemicals

All solvents and chemicals were of HPLC grade or of analytical-reagent grade, obtained from Arco Chemical Products, (Gent, Belgium) and Merck, (Darmstadt, Germany). The enzyme β-glucuronidase (EC 3.2.1.31 G0876) crude solution was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and was stored frozen at -22°C in the dark. Water was de-ionized, doubly distilled, and stored in a glass container. A computer file was kept for all chemicals which reflected the date of receipt, the amount received, the molecular formula, molecular weight, and the date that purity was confirmed.

Preparation of Pemoline-Free Urine

Pemoline free urine was collected from pemoline free volunteers. Urine was pooled, extracted, and after analysis, no peaks corresponding to pemoline were observed.

Urinary Sample Collection and Storage

Urine samples were collected from each subject in 250 mL polyethylene containers. As soon as possible, 5 mL aliquots were separated and stored in polyethylene tubes at -22°C until analysis.

Calibration Standards

Pemoline working standard solution was prepared daily by diluting 1 mL stock solution in 50 mL distilled water giving a concentration of 40 μg/mL of pemoline. Calibration standards were prepared by adding 25, 100, 125, 150, and 175 μL working standard in 5 mL drug free urine corresponding to 0.2, 0.8, 1.0, 1.2, and 1.4 μg/mL of pemoline in urine. All reagents and standards, refrigerated for storage were allowed to equilibrate for at least 2 hours at room temperature before use.

Quality Control Sample

An additional stock standard, independent of that used for preparing the working standards, was used to make the quality control (QC) samples.

QC samples at three concentration levels were prepared by pooling drug-free urine and spiking with the separately prepared stock standard solution of pemoline. The low quality control (LQC) and medium quality control (MQC) were prepared by diluting the high quality control (HQL) of 1.40 μg/mL with drug free urine in a ratio of 1:1.4 and 1:3.5 (v/v) respectively. The QC samples were stored in glass vials at -22°C until they were analysed.

Downloaded At: 10:12 24 January 2011

To evaluate the long-term stability of pemoline in urine under the specified freezing conditions, blank urine was spiked with pemoline at 2 concentrations and stored under required conditions. These were the stability samples. Following the designated storage period, fresh urine was spiked with pemoline at the same two concentrations as the stability samples; these were the comparison samples. Ten replicates of each of the two stability samples were simultaneously analyzed as a single batch, and the 40 responses are determined.

The pemoline was considered adequately stable if the ratios of the mean response for the stability samples to that of the comparison samples, for a) the combined high and low b) the low and c) the high concentration samples, all laid within the range 90-110%.

The above three QC samples were used to validate the accuracy of the method. They were also used in routine pemoline analysis, were analyzed in duplicate immediately after the calibration standards, and their results provided the basis of accepting or rejecting the run. At least four of the six QC samples had to be within 20% of their respective nominal values. Two of the six QC samples (not both at the same concentration) may be outside the ± 20% respective nominal value.

For the assessment of the precision of the method, 6 validation pools were used, being authentic samples from a dosed healthy volunteer.

Downloaded At: 10:12 24 January 2011

Sample Pretreatment

Calibration and quality control samples were thawed in a water bath at 37°C, 30 μL of internal standard stock solution was added and further analyzed by LLE or SPE procedure.

To 5 mL of study urine sample, the pH was adjusted to 5 with 1M of acetic acid or 1M of sodium acetate solutions. A 0.2 mL of β-glucuronidase crude solution was added and the sample was stoppered, mixed well, and incubated at 37°C for 12 hours. Thereafter, 30 μL of I.S. stock solution was added and analyzed by LLE or SPE procedure.

Liquid-Liquid Extraction (LLE)

To 5 mL urine sample, a 0.1 g solid buffer $NaHCO_3/K_2CO_3$ (2:1) was added to adjust the pH to 9.6. To remove non-polar interference, the sample was first extracted with 3 mL n-pentane. After vortexing for 1 min, centrifugation and separation, 2 mL saturated Na_2SO_4 solution in water was added in the water layer and the sample was extracted with 2×5 mL dichloromethane.

The organic layer was transferred to a second tube, filtered through the sample clarification kit (pore size 0.2 μm), and evaporated to dryness under vacuum. The residue was dissolved in 150 μL methanol and then in 50 μL water. A 10 μL aliquot was injected into the HPLC system.

Solid Phase Extraction (SPE)

Bond Elut C_{18} reverse phase SPE columns were mounted on a Vac-elut (Analytichem International) vacuum manifold and each was conditioned successively with 5 mL chloroform, 5 mL acetonitrile, 10 mL water, and 5 mL water containing 0.1 g $NaHCO_3/K_2CO_3$ (2:1) buffer pH 9.6, by applying the vacuum. The same buffer was also added to the 5 mL urine sample and loaded onto the column at a flow of 1-2 mL/min or a vacuum of 2-4 in Hg.

Preconditioning allows the solvation of chains of the sorbent material and produces a water-miscible layer with large surface area to allow an interaction to take place with the urine sample. The flow must be controlled because the columns are not densely packed and high flows may produce channeling effects and consequently low column performance.

After the column was dried for 2 min, by increasing the vacuum up to 15 in Hg, the pemoline and IS were eluted with 2 × 3 mL solution of acetonitrile: dichloromethane (10:90). The collected extract was centrifuged and the water layer was discarded with a pasteur pippet. The rest was filtered through the sample clarification kit (pore size 0.2 μm) and evaporated to dryness under a stream of nitrogen.

Downloaded At: 10:12 24 January 2011

The residue was dissolved in 150 μL methanol and then in 50 μL water. A portion of 10 μL was injected into the HPLC system or the tubes were closed before stored at -22°C.

RESULTS

Chromatography

Typical chromatogram of spiked urine extract is shown in Figure 2. Under the desired chromatographic conditions pemoline, 4-methyl-primidone and other compounds are well separated. The approximate retention times for pemoline and 4-methyl-primidone are 6.1 and 10.2 min respectively. The chromatographic analysis was rapid allowing separation within 12 min. Pemoline has a λ_{max} of 216 nm.

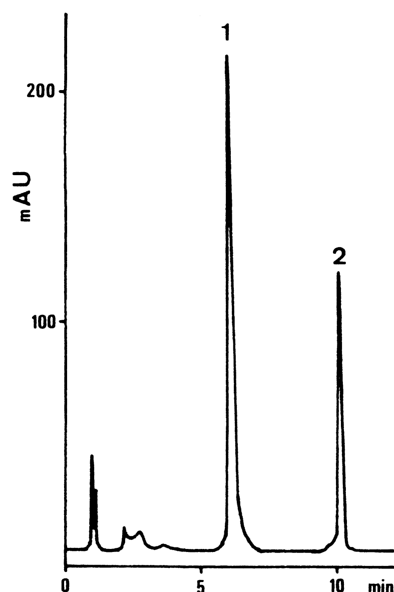


Figure 2. Chromatogram by DAD, of a 5 mL spiked urine extract after SPE. 1. Pemoline, 1.40 μg/mL urine; 2. 4-methyl-primidone, 3 μg/mL urine.

Downloaded At: 10:12 24 January 2011

Method Validation

Statistical Analysis

The statistical analysis of the results was generated using the SPSS programme for Microsoft Windows, version 7.5 (Microsoft, WA, USA) and Excel for Microsoft Windows, version 97 (Microsoft, WA, USA).

The following acceptance criteria were used to evaluate the data; (a) p-values for all statistical tests be < 0.05; (b) the within/between assay variability be within 15%; (c) 75% of all QC samples analysed be within 15% of their respective nominal values; (d) the correlation coefficient of calibration curves be 0.98 or the coefficient of determination (r^2) be 0.96.[39]

Linearity Range

The linearity for pemoline was checked in the concentration range of 0.20 to 1.40 μg/mL urine. Consideration was taken that the response ratio of peak areas (pemoline/I.S.) did not span over 150%.

Choice of the Model: Weighted or Unweighted

For the calibration of an instrument, since the response of a higher standard concentration preparation usually has a larger variability, the ordinary least-squares approach may not be appropriate. In this case, a weighted least-squares method is often considered to remove the heterogeneity of the variability. The weight is selected so that the variance (s^2) of the response at each standard concentration preparations is stabilized [i.e. the variance (s^2) of Y_i at each standard preparation remains a constant]. Selection of an appropriate weight depends on the pattern of the standard deviation (s) of the response at each standard concentration. For example, if the standard deviation (s) of the response at standard concentration preparation X is proportional to X, an appropriate choice for the weight is $1/x^2$, if is proportional to $\sqrt{x}$ the weight is 1/x and if it remains constant the weight is 1. These are the three possible weights, commonly adopted in practice, for various situations.[40]

For the selection of weights, if there are replicates at each standard concentration preparation X, Chow[40] suggested fitting the following linear regression model to study the relationship between the standard deviation of the instrument response Y and the standard X: $SD(Y_i) = \beta_o + \beta_1\sqrt{x} + \beta_2 x$. Let q be the p value for testing the following hypotheses: H_o: $\beta_1 = \beta_2 = 0$ vs H_a: at least one β is not zero. Also, let Δ be the level of significance for weight selection. The criterion for weight selection can be summarized as follows:

1. If $q > \Delta \Rightarrow H_o$ is not rejected and
   $SD(Y_i) = \beta_o = \text{constant}$,

then no weight is necessary.

2. If $q < \Delta$ and $SS(b_2 / b_o,b_1) > SS(b_1 / b_o,b_2)$

then weight = $1/x^2$.

3. If $q < \Delta$ and $SS(b_2 / b_o,b_1) < SS(b_1 / b_o,b_2)$

then weight = 1/x.

$SS(b_2 / b_o,b_1)$ denotes the extra regression sum of squares due to the inclusion of the term $\beta_2 x$ provided that β_o and $\beta_1\sqrt{x}$ are already in the model.

Similarly, the contribution of the regression sum of squares due to $\beta_1\sqrt{x}$ after the two terms β_o and $\beta_2 x$ being included in the model is expressed by $SS(b_1 / b_o,b_2)$.

Downloaded At: 10:12 24 January 2011

Downloaded At: 10:12 24 January 2011

The calibration curve for pemoline was obtained from spiked urine in one day at 5 standard concentration preparations (x = 0.20, 0.80, 1.00, 1.20, and 1.40 μg/mL) with n = 5 replicates for each point. The response ratio of peak areas between pemoline and internal standard versus the theoretical concentration was fitted by a least-squares linear regression. The standard deviations of the response ratios at each level of standard concentration preparation were [SD(Y): 0.004, 0.017, 0.023, 0.032, and 0.045 respectively]. It can be seen that the standard deviation at higher levels of standard concentration tends to be higher. Therefore, a weighted least-squares method is necessary for determining the standard curve.

We take $\Delta = 0.05$ to be the level of significance for weight selection. Since the calculated p value for testing the H_o: $b_1 = b_2 = 0$ is found to be $p = 0.008$ and $p < \Delta$ then H_o is rejected and weighting is necessary. This implies that the standard deviation of the response ratios is highly correlated to the concentration and a weight that is a function of X is needed, to stabilize the variance of the response ratios.

Since $SS(b_1 / b_o, b_2) < SS(b_2 / b_o, b_1)$, or $6\times10^{-5} < 12\times10^{-5}$, weight = $1/x^2$ is selected. To select an appropriate statistical model for determining the standard curve, Chow[40] proposed an ad hoc criterion. He recommended the following selection procedure:

Starting with the linear model 3:

$$Y_i = a + \beta_1 x_i + \beta_2 x_i^2 + \beta_3 x_i^3$$

let p_3 be the p value for testing H_{03}: $\beta_3 = 0$.

If p_3 is smaller than Δ (a predetermined level of significance), select model $Y_i = a\, x_i^{\beta}$ or $\log(Y_i) = \log(a) + \beta \log(x_i)$, otherwise the model above reduces to:

Model 2: $Y_i = a + \beta_1 x_i + \beta_2 x_i^2$

let p_2 be the p value for testing H_{02}: $\beta_2 = 0$.

If p_2 is less than Δ, the above model is chosen, otherwise it is reduced to:

Model 1: $Y_i = a + \beta_1 x_i$

The results from the model selection procedure were: for model 1(H_{01}: $\beta_1 = 0$), a p-value of <0.001; for model 2(H_{02}: $\beta_2 = 0$), a p-value of 0.133; and for model 3(H_{03}: $\beta_3 = 0$), a p-value of 0.928. Model 1 is selected since hypothesis H_{03} and H_{02} are both not rejected at the 5% level of significance sequentially.

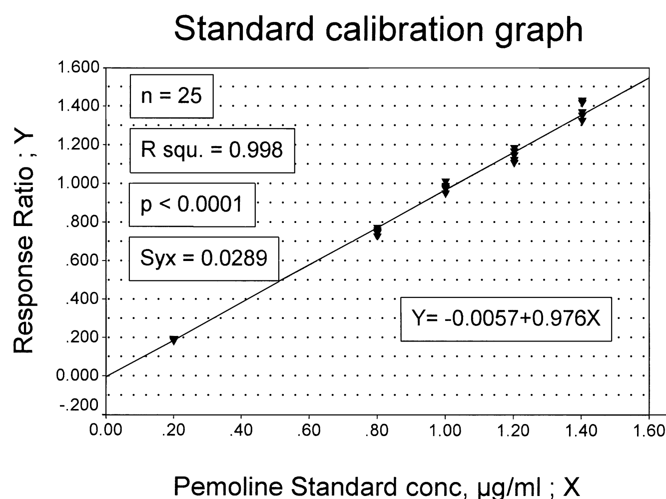


Figure 3. A typical weighted ($1/x^2$) linear regression calibration line.

Downloaded At: 10:12 24 January 2011

The typical equation describing the standard calibration line, as determined by the weighted ($1/x^2$) linear regression, was y = - 0.006 + 0.976 x, (Figure 3). A coefficient of determination (R^2) of 0.998, a standard error of the estimate (s_{yx}) of 0.0289, a standard error of the slope (SEb) of 0.008 and a standard error of the intercept (SEa) of 0.004 were obtained.

Assay Detection Limits

The limit of decision (qualitative), L_c, and the limit of quantitation, L_q, were obtained by use of the slope (b) and the standard error of the intercept (SEa) of the regression line.[41] The limit of decision, calculated from y-a = 3 SEa and y-a = b L_c, for pemoline was L_c = 0.01 µg/mL in urine. The limit of quantitation, calculated from y-a = 10 SEa and y-a = b L_q, was L_q = 0.04 µg/mL in urine. This is the lowest concentration that can be determined with a 10% maximum allowed relative standard deviation.

Accuracy / Recovery

The accuracy of the method is defined as the degree of agreement of test results generated by the method to the true value.[42] The analytical recovery was used to assess the accuracy and it was measured by spiking drug-free urine with known concentrations of the standards (the 3 quality control samples: LQC, MQC and HQC).

Table 1

Recovered and Added Concentrations of Pemoline from a Recovery Study

Day	Added µg/mL	Recovered	Percent Recovery	Absolute Bias	Percent Bias
1	0.40	0.36	90.0	0.04	10.0
		0.37	92.5	0.03	7.5
2		0.37	92.5	0.03	7.5
		0.36	90.0	0.04	10.0
3		0.35	87.5	0.05	12.5
		0.37	92.5	0.03	7.5
1	1.00	0.95	95.0	0.05	5.0
		0.97	97.0	0.03	3.0
2		0.96	96.0	0.04	4.0
		0.94	94.0	0.06	6.0
3		0.96	96.0	0.04	4.0
		0.97	97.0	0.03	3.0
1	1.40	1.32	94.3	0.08	5.7
		1.35	96.4	0.05	3.6
2		1.36	97.1	0.04	2.9
		1.30	92.9	0.10	7.1
3		1.31	93.6	0.09	6.4
		1.34	95.7	0.06	4.3

Downloaded At: 10:12 24 January 2011

After the extraction of the analyte from the matrix and injection onto the analytical instrument, its response was compared with the response of the standard injected directly to the column, which gave the analytical recovery. The internal standard was added to the final injection solvent just before injection.

Since the added amount, X, is a known quantity it is assumed fixed. However the recovered amount, Y, is a random variable.

Table 1 gives the data from the recovery study, consisting of 18 determinations at 3 different added concentrations, in duplicate, on 3 different days. This table also provides the percent recovery, Z, the absolute bias, B and the percent bias, Pb, defined respectively as: Z = 100 (Y/X)%, B = Y-X, and PB = 100 (B/X)%.

Also we get as the mean percent recovery, $\overline{Z}$ = 93.9 and s_z^2 = 7.53, the mean absolute bias, $\overline{B}$ = 0.05, and s_B^2 = < 0.001 the mean percent bias, $\overline{PB}$ = 6.1 and s_{PB}^2 = 7.53.

Table 2

Estimates of the Intercept and Slope for the Recovered Concentration

	Intercept	Slope
Estimate	- 0.02018	0.9686
Standard error	0.01003	0.00984
t statistics	- 2.012	98.489
p value	0.061	< 0.0001

Hence the 95% confidence interval for percent recovery and percent bias are given by $(L, U) = \bar{x} \pm t_{(0.025,\ 17)} \times (s / \sqrt{n})$.[45]

$(L_z, U_z) = 93.9 \pm 2.11 \times 0.647$ or (92.5%, 95.3%)

$(L_{PB}, U_{PB}) = 6.1 \pm 2.11 \times 0.647$ or (4.7%, 7.5%)

Table 2 summarizes the estimates of the intercept and slope and their estimated standard errors from fitting a simple linear regression model of the recovered concentration on the known added concentration. The estimated regression line is given by Y = - 0.0202 + 0.969 X, $R^2 = 0.9984$ and $s_{yx} = 0.01715$. (The p value for the null hypothesis of zero slope is less than 0.0001, so the null hypothesis is rejected at the 5% level of significance). The null hypothesis of the slope being equal to 1 is rejected at the 5% level of significance if:[43]

$$|T| = \left| \frac{b-1}{SE(b)} \right| > t_{(0.025,\ n-2)}$$

This gives:

$$|T| = \left| \frac{0.969-1}{0.00984} \right| = 3.15$$

which is larger than $t_{(0.025,\ 16)} = 2.12$. Hence we reject the null hypothesis of the slope being 1 at the 5% level of significance.

Figure 4 displays the scatter diagram of the recovered and added concentrations and the estimated regression line. Since $R^2 = 0.9984$ and the null hypothesis of zero coefficient ($\beta_2 = 0$) for a quadratic term in the model ($Y = a + \beta_1 X + \beta_2 X^2$) was not rejected at the 5% level of significance (p = 0.0814 for β_2), the simple linear regression model is an appropriate statistical model.

Downloaded At: 10:12 24 January 2011

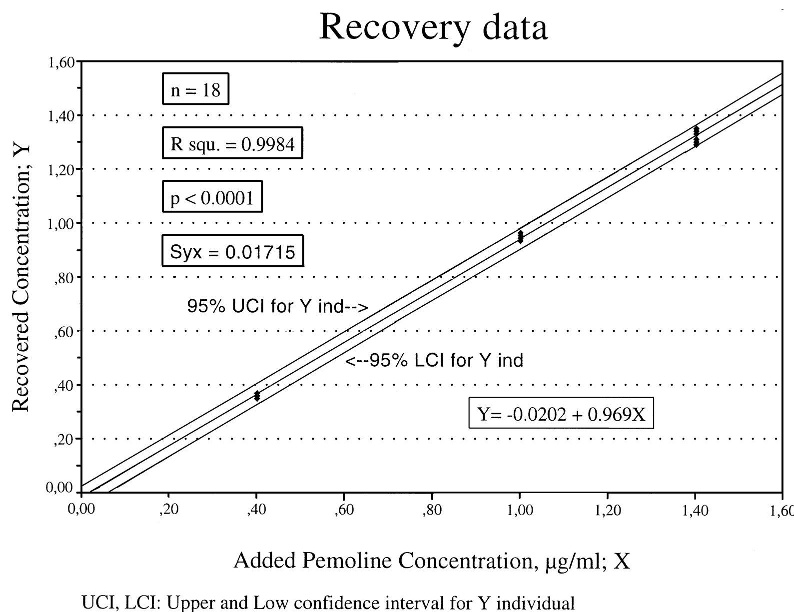


Figure 4. Scatter plot and regression line of recovery data.

Downloaded At: 10:12 24 January 2011

Table 3 gives the predicted percent recoveries, predicted percent biases and their corresponding 95% confidence intervals. To test the validity of the simple regression model, the test of lack of fit can be applied which gives the following ANOVA Table 4 for lack of fit (LOF).[44]

The sum of squares due to lack of fit is 0.00088 and its p value is 0.085 since $F = 3.52 < F_{(0.05, 1, 15)} = 4.54$.

As a result, we fail to reject the null hypothesis of no lack of fit. Based on the evidence of no lack of fit and $R^2 = 0.9984$, the simple linear regression is an adequate model for describing the relationship.

Precision

Precision of a quantitative method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings. It is measured by analyzing repeatedly a ready-made sample pool and expressed as the coefficient of variation of the results. From the previous recovery experiment and Table 1, we can use the percent recovery values to quantify the overall precision of the assay method. (Table 4).

Downloaded At: 10:12 24 January 2011

Table 3

Predicted Percent Recovery and Percent Bias by Simple Linear Regression Model

Day	Added μg/mL	Recovered	Predicted Percent Recovery	95% CI of Recovery	Predicted Percent Bias	95% CI of Bias
1	0.40	0.36	91.5	(89.8%, 93.2%)	8.5	(6.8%, 10.2%)
		0.37				
2		0.37				
		0.36				
3		0.35				
		0.37				
1	1.00	0.95	94.2	(93.2%, 95.2%)	5.8	(4.8%, 6.8%)
		0.97				
2		0.96				
		0.94				
3		0.96				
		0.97				
1	1.40	1.32	96.0	(94.4%, 97.5%)	4.0	(2.5%, 5.6%)
		1.35				
2		1.36				
		1.30				
3		1.31				
		1.34				

Table 4

Anova Table for LOF for the Recovery Data

Source of Variation	df	Sum of Square	Mean Square	F Value	p Value
Regression	1	2.85232	2.85232	9700.078	> 0.0001
Residual	16	0.00470	0.00029		
Lack of fit	1	0.00088	0.00088	3.52	0.085
Pure error	15	0.00382	0.00025		
Total	17	2.85702			

By replacing the values in the equation: $CV_z\% = (S_z / \overline{Z}) \times 100$, we get a 2.92% CV. The within-run CV's for pemoline analysis in 3 different urine sample pools (n = 8 each) were 3.67, 2.03, and 2.17% for the respective concentrations of 0.52 ± 0.019, 1.00 ± 0.020 and 1.29 ± 0.028 ($x \pm s$)μg/mL.

Table 5

Interday Precision for Pooled Urine 4

	Day 1	**Day 2**	**Day 3**
$\bar{x} \pm s$, μg/mL n = 5	1.31 ± 0.040	1.31 ± 0.057	1.33 ± 0.026
CV, %	2.97	4.35	1.96
Immediate Precision CV, % n = 15		3.09	
95% upper confidence limit for Intermediate precision[45]		6.06	

Table 6

Results of Day-to-Day Reproducibility

	Pool 5	**Pool 6**
Day 1	0.88	1.29
	0.82	1.37
	0.80	1.30
	0.85	1.33
	nd[a]	1.27
Day 2	0.79	1.24
	0.83	1.27
	0.80	1.35
	0.83	1.38
	0.78	1.31
Day 3	0.78	1.31
	0.79	1.33
	0.84	1.36
	0.83	1.30
	0.80	1.35

[a] nd = not determined.

The analysis was carried out in one laboratory by one operator, using the same reagents and instruments over one day for each sample pool. The interday precision (or reproducibility) is defined as the long-term variability of the measurement process.

Downloaded At: 10:12 24 January 2011

Table 7

ANOVA Table for Pooled Urine 5*

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F Ratio	p-Value
Between Days	2	0.0027	0.0013	1.7314	0.2220
Within Days	11	0.0085	0.0008		
Total	13	0.0111			

* Unequal sample sizes.

Table 8

ANOVA Table for Pooled Urine 6*

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F Ratio	p-Value
Between Days	2	0.0012	0.0006	0.3358	0..7213
Within Days	12	0.0217	0.0018		
Total	14	0.0229			

* Equal sample sizes.

Downloaded At: 10:12 24 January 2011

One pooled urine sample (Pool 4), analyzed in quintuplicate on 3 different days over a two-week period, gave the results and the between-run CV's shown in Table 5. Table 6 gives the results and Tables 7 and 8 give the ANOVA of the day to day reproducibility for two different levels of pemoline in urine pools 5 and 6.

For pooled urine 6, the p-value is 0.7213. This means that if the null hypothesis, H_o, is true (and the day means don't differ), there is 72.13% chance of getting day means that differ so much. In other words the null hypothesis is very credible and there is statistically no difference between the different day means.[46] For pooled urine 5, the p-value is 0.2220 or the H_o is credible.

Selectivity

Selectivity of an analytical method is its ability to measure accurately an analyte in the presence of interference that may be expected to be present in the sample matrix.[47]

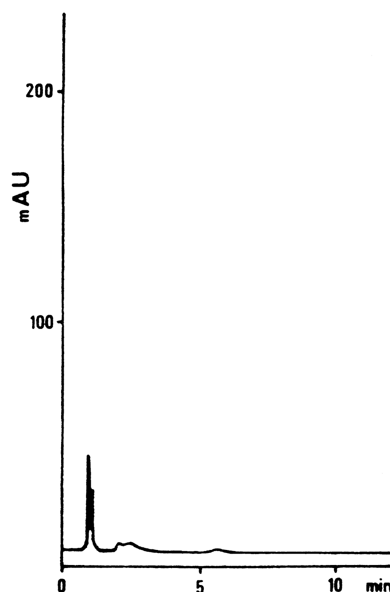


Figure 5. Chromatogram of an extracted blank urine.

Downloaded At: 10:12 24 January 2011

In Figure 5, where we examine the chromatographic blank from urine, in the expected time windows of the analytes we found a lack of response for n = 5 independent sources of the same matrix. Also peak homogeneity could be demonstrated by plotting the absorbance ratio (or by calculating the area ratio) of two signals acquired at two different wavelengths.

Comparison Between LLE and SPE Methods

In order to cross-validate the developed methods, several spiked urine samples (n = 10), were analyzed using the two methods. The mean percent extraction recoveries of pemoline were: 94 and 26, and the coefficient of variation: 2.9 and 10.2, for the SPE and LLE methods, respectively. The results are demonstrating the low extraction efficiency of the LLE method.

DISCUSSION

Approximately 75% of an oral dose is excreted in the urine within 24 hours, about 43% is excreted unchanged. From the total single oral dose of pemoline, more than 50% is metabolised to pemoline glucuronide, 4% pemoline

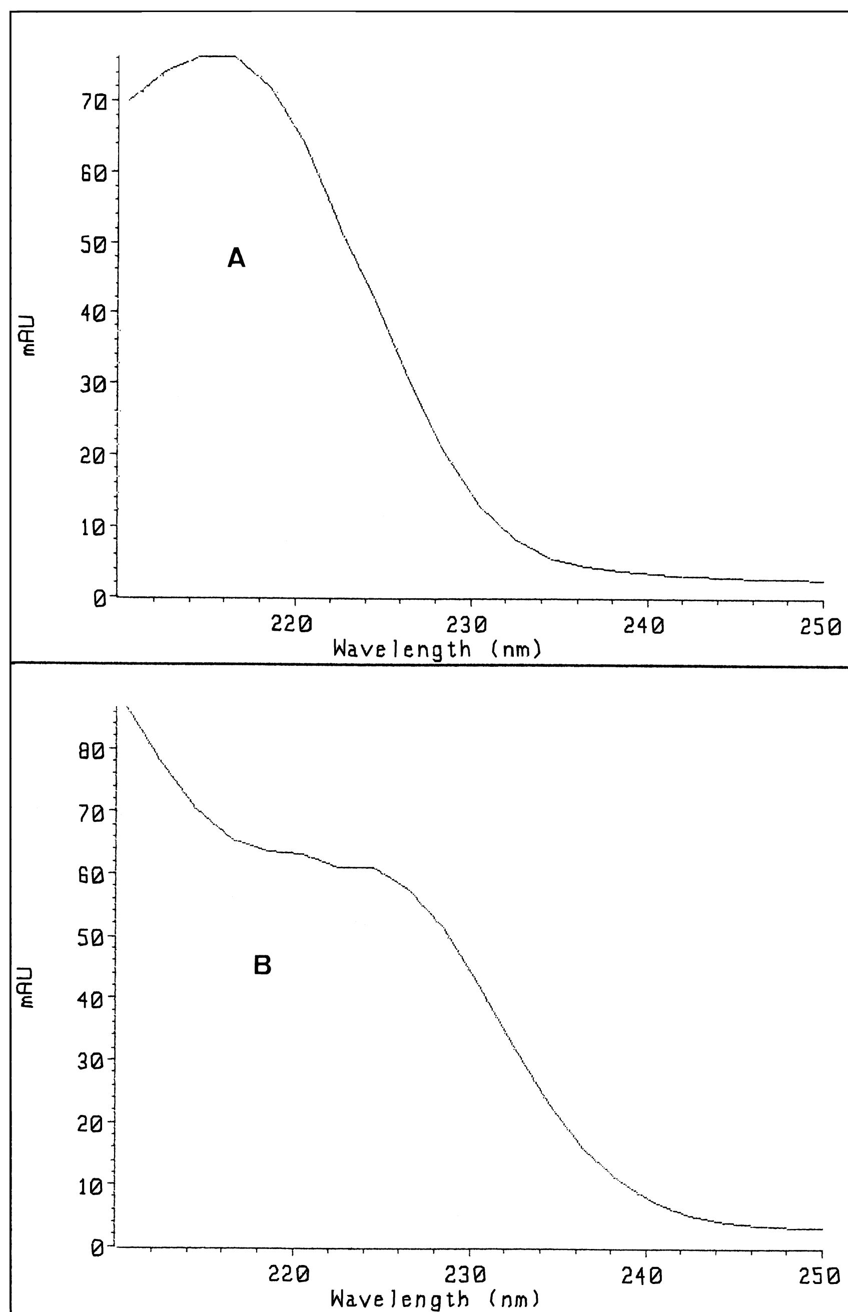


Figure 6. UV spectra of pemoline (A) and 4-methyl-primidone (B) at 216 nm under the described conditions.

Downloaded At: 10:12 24 January 2011

dione and unidentified polar metabolites. Only negligible amounts are excreted in faeces.[5,48] Pemoline dione is active (although much less active than pemoline) and may contribute to the CNS stimulant activity of the main drug. Pemoline glucuronide, which has high polarity, can be hydrolyzed enzymatically with β-glucuronidase at the optimum conditions of pH 5 and at 37°C for about 12 hours.

When 4-methyl-primidone, theophylline, and 8-chloro-theophyline were chromatographed and compared to serve as internal standards, the first was chosen since it gave a retention time larger than that of pemoline which was not the case with the other two.

The low extraction recovery of pemoline by the LLE method is due to its very low solubility in non-polar solvents. The addition of Na_2SO_4 in order to decrease the solubility of the drug in water did not improve significantly the results. It is known[32] that at pH higher than 11, a decrease in the percentage of drug extracted is observed, due probably to a decomposition of the drug.

The combination of HPLC with diode-array detection (DAD) is considered as a highly effective screening method. It gives the advantage of identifying the analytes both by retention time and UV spectrum. It allows the acquisition of UV spectral data during the elution of the peak without stopping the solvent flow. The UV spectra of pemoline and 4-methyl-primidone under the described chromatographic conditions are shown in Figure 6.

HPLC-DAD offers the advantage to separate and quantify pemoline without derivatization and at low temperature, that is without risk of decomposition.

We preferred for SPE the acetonitrile: dichloromethane solvent mixture to that of acetonitrile: diethylether since the former involved no interfering peaks in the blank samples. After the evaporation of the solvent, the sample residue was dissolved first in methanol to ensure a good solubilization and then diluted with water to provide better injection quality. The use of acetonitrile is known to increase the peak widths, creating a loss in efficiency and selectivity.[18]

After every 100 injections, it is necessary to change the guard column to maintain good injection quality and peakwidth and to prevent excessive pressure.

SPE technique is more selective than LLE and gives much cleaner extracts. The reason is, that in SPE, 2-3 clean-up steps are included while in LLE only one. One clean-up step occurs during loading the sample on the column under controlled flow, one possible washing step with an intermediate polarity solvent, and one clean-up during elution and selective desorption of the analyte, leaving the interference behind on the column.

Downloaded At: 10:12 24 January 2011

ACKNOWLEDGMENTS

The authors gratefully thank Mr. R. Vereecke and Mr. J. Vanderverren for their technical help in this study.

Address correspondence and reprint requests to Weidong He at Department of Organic Chemistry, University of Ghent, Coupure Links 653 B4, B9000 Ghent, Belgium, Tel: +329 2645952, Fax: +329 2646243, e-mail: weidong.he@rug.ac.be; or for Dr. N. Parissis: nparissis@medscape.com.

REFERENCES

1. **The New Encyclopaedia Britannica. Human Learning and Cognition: Environmental factors, Vol. 22**, 15th ed., 1994, p. 880.

2. J. H. Jaffe, ed., **Encyclopaedia of Drugs and Alcohol, Simon and Schuster, Vol. 2,** Macmillan Publishers, 1995, p. 786.

3. G. M. Levin. "Attention-Deficit Hyperactivity Disorder: The Pharmacist's Role," Am. Pharm., **NS35(11)**, 10-20 (1995).

4. A. Metz, R. I. Shader, "Combination of Fluoxetine with Pemoline in the Treatment of Major Depressive Disorder," International Clinical Psychopharmacol., **6(2)**, 93-96 (1991).

5. **AHFS Drug Information**, The American Society of Hospital Pharmacists, Inc., Bethesda MD,1992, pp. 1308-1310.

6. M. M. Mitler. "Evaluation of Treatment with Stimulants in Narcolepsy," Sleep, **17(8)**, S103-S106 (1994).

7. **Drug Evaluations Annual 1991, 1992,** American Medical Association, p. 214.

8. **Drug Evaluations Annual 1991, 1992,** American Medical Association, pp. 382-383.

9. B. G. Weinshenker, M. Penman, B. Bass, G. C. Ebers, G. P. Rice, "A Double-Blind, Randomised, Crossover Trial of Pemoline in Fatigue Associated with Multiple Sclerosis," Neurology, **42(8)**, 1468-1471 (1992).

10. W. Breitbart, H. Mermelstein, "Pemoline. An Alternative Psychostimulant for the Management of Depressive Disorders in Cancer Patients," Psychosomatics, **33(3**), 352-356 (1992).

Downloaded At: 10:12 24 January 2011

11. P. Masand, P. Pickett, G. B. Murray, “Psychostimulants for Secondary Depression in Medical Illness,” Psychosomatics, **32**, 203-208 (1991).

12. V. F. Holmes, F. Fernandez, J. K. Levy, “Psychostimulant Response in AIDS-Related Complex Patients,” J. Clin. Psychiatry, **50**, 5-8 (1989).

13. S. Ferrando, “Substance Use Disorders and HIV Illness,” AIDS Reader, **7(2)**, 57-64 (1997).

14. J. E. F. Reynolds, ed., **Martindale, The Extra Pharmacopoeia,** 30th ed., Pharmaceutical Press, London, UK, 1993, pp. 1229-1230.

15. **The Science and Practice of Pharmacy**, J. Remington, ed., Mack Printing and Publishing Company, Easton, PA, 1995, pp. 1234.

16. F. T. Delbeke, “Doping in Cyclism: Results of Unannounced Controls in Flanders (1987-1994),” Int. J. Sports Med., **17(6)**, 434-438 (1996).

17. **Pocket Atlas of Pharmacology**, H. Lüllmann, K. Mohr, A. Ziegler, D. Bieger, eds., Georg Thieme Verlag Press, Stuttgart, New York, 1993, pp. 88-89.

18. A. Turcant, A. Premel-Cabic, A. Cailleux, P. Allain, “Toxicological Screening of Drugs by Microbore HPLC with Photodiode-Array Detection and UV Spectral Library Searches,” Clin Chem, **37(7)**, 1210-1215 (1991).

19. Lai Chi-Kong, Lee Ting, Au Kam-Ming, A. Chan Yan-Wo, “Uniform Solid-Phase Extraction Procedure for Toxicological Drug Screening in Serum and Urine by HPLC with Photodiode-Array Detection,” Clin Chem, **43(2)**, 312-325 (1997).

20. A. Tracqui, P. Kintz, P. Mangin, “Systematic Toxicological Analysis Using HPLC/DAD,” J. of Forensic Sciences, **40(2)**, 254-262 (1995).

21. Chen Xiao-Hua, J. Wijsbeek, J. Franke, R. De Zeeuw, “A Single-Column Procedure on Bond Elut Certify for Systematic Toxicological Analysis of Drugs in Plasma and Urine,” J. of Forensic Sciences, **37(1)**, 61-71 (1992).

22. D. H. Catlin, R. C. Kammerer, C. K. Hatton, M. H. Sekera, J. L. Merdink, “Analytical Chemistry at the Games of the 23rd Olympiad in Los Angeles, 1984,” Clin. Chem, **33(2)**, 319-327 (1987).

23. C. P. Collier, S. J. Soldin, J. M. Swanson, S. M. MacLeod, F. Weinberg, J. G. Rochefort, “Pemoline Pharmacokinetics and Long Term Therapy in Children with Attention Deficit Disorder and Hyperactivity,” Clin. Pharmacokinet., **10(3)**, 269-278 (1985).

Downloaded At: 10:12 24 January 2011

24. **Drug Evaluations Annual 1991, 1992**, American Medical Association, 1992, p. 312.

25. L. M. Cummins, J. E. Perry, "Spectrophotometric Determination of Pemoline and Mandelic Acid in Biological Fluids," J. Pharm. Sci., **58**, 762-763 (1969).

26. D. J. Hoffman, "Sensitive GLC Assay for Pemoline in Biological Fluids Using Nitrogen Specific Detection," J. Pharm. Sci., **68**, 445-447 (1979).

27. J. C. Libeer, P. Schepens, "GLC Determination of Pemoline in Biological Fluids," J. Pharm. Sci., **67**, 419-421 (1978).

28. N. P. E. Vermeulen, M. W. E. Teunissen, D. D. Breimer, "Pharmacokinetics of Pemoline in Plasma, Saliva and Urine Following Oral Administration," Brit. J. Clin. Pharmac., **8**, 459-463 (1979).

29. O. J. Igwe, J. W. Blake, "Gas-Liquid Chromatographic Analysis of Pemoline in Biological Fluids Using Electron Capture Detection," J. Chromatogr. Sci., **19(12)**, 617-624 (1981).

30. F. T. Delbeke, M. Debackere, N. Desmet, "Use of the Electron-Capture Detector in Human and Equine Doping Analysis by Gas Chromatography," Chromatographia, **17(7)**, 381-386 (1983).

31. C. Tomkins, S. Soldin, S. MacLeod, J. G. Rochefort, J. Swanson, "Analysis of Pemoline in Serum by High Performance Liquid Chromatography: Clinical Application to Optimise Treatment of Hyperactive Children." Ther. Drug Monit., **2**, 255-260 (1980).

32. G. P. Cartoni, F. Natalizia, "Determination of Pemoline by High-Pressure Liquid Chromatography," J. Chromatogr., **123**, 474-478 (1976).

33. G. P. Cartoni, M. Ciardi, A. Giarrusso, F. Rosati, "Reversed Phase High-Performance Liquid Chromatographic Detection of Pemoline in Doping Control," J. Chromatogr. **202**, 131-133 (1980).

34. K. Nishihara, Y. Kohda, Y. Saitoh, F. Nakagawa, Y. Honda, "Determination of Pemoline in Plasma, Plasma Water, Mixed Saliva and Urine by High-Performance Liquid Chromatography," Ther. Drug Monit., **6(2)**, 232-237 (1984).

35. T. Aoyama, H. Kotaki, Y. Saitoh, F. Nakagama, "Determination of Pemoline in Plasma, Urine and Tissues by Reversed Phase High-Performance Liquid Chromatography," J. Chromatogr., **430(2)**, 351-360 (1988).

Downloaded At: 10:12 24 January 2011

36. K. Slais, M.W. Nielen, U. A. Brinkman, R. W. Frei, "Screening of Amphetamines by Gradient Microbore Liquid Chromatography and Pre-Column Technology," J. Chromatogr., **393(1)**, 57-68 (1987).

37. M. Van Boven, P. Daenens, "Combined Gas-Liquid Chromatographic-Mass Spectrometric Analysis of Pemoline in Biological Samples," J. Chromatogr., **134**, 415-421 (1977).

38. W. Gielsdorf, "Determination of the Psychostimulants Pemoline, Fenozolone and Thozalinone by Gas Chromatography-Mass Spectrometry and Thin-Layer Chromatography," J. Clin. Chem. Clin. Biochem., **20(2)**, 65-68 (1982).

39. E. O. Aboagye, M. A. Graham, A. D. Lewis, P. Workman, A. B. Kelson, M. Tracy, "Development and Validation of a Solid-Phase Extraction and High-Performance Liquid Chromatographic Assay for a Novel Fluorinated 2-Nitroimidazole Hypoxia Probe (SR-4554) in Balb/c Mouse Plasma," J. Chromatogr. B, **672**, 125-132 (1995).

40. **Statistical Design and Analysis in Pharmaceutical Science**, S. C. Chow, J. P. Liu, eds., Marcel Dekker, Inc., New York, 1995, pp. 25-71.

41. **Statistics for Analytical Chemistry, IV**, J. C. Miller, J. N. Miller, eds., Horwood, Chichester, UK, 1986, p. 96.

42. **Quality in the Analytical Chemistry Laboratory**, E. Newman, ed., John Wiley and Sons, 1995, pp. 172.

43. R. C. Kohberger, "Manufacturing and Quality Control," in **Biopharmaceutical Statistics for Drug Development**, Chapter 14, K. Peace, ed., Marcel Dekker, New York, 1988, pp. 605-629.

44. **National Committee for Clinical Laboratory Standards, Evaluation of the Linearity of Quantitative Analytical Methods; Proposed Guideline**, NCCLS publication EP6-P, NCCLS, Villanova, PA, 1986, pp. 524.

45. **Introductory Statistics, 5th Edition**, T. Wonnacott, R. Wonnacott, eds., John Wiley and Sons, 1990, pp. 261-263.

46. **Introductory Statistics, 5th Edition**, T. Wonnacott, R. Wonnacott, eds., John Wiley and Sons, 1990, pp. 331-334.

47. **United States Pharmacopeia XXII**, **National Formulary XVII**, "Validation of Compendial Methods," General chapter <1225>, The United States Pharmacopeial Convention, Inc., Rockville, MD, 1990, pp. 1610-1612.

Downloaded At: 10:12 24 January 2011

48. **Clarke's Isolation and Identification of Drugs in Pharmaceuticals, Body Fluids and Post-Mortem Material**, 2nd ed., A. C. Moffat, J. V. Jackson, M. S. Moss, B. Widdop, eds., The Pharmaceutical Press, London, 1986, pp. 856.

Received December 1, 1998
Accepted April 4, 1999
Manuscript 4953

Downloaded At: 10:12 24 January 2011

Downloaded At: 10:12 24 January 2011

Request Permission or Order Reprints Instantly!

Interested in copying and sharing this article? In most cases, U.S. Copyright Law requires that you get permission from the article's rightsholder before using copyrighted content.

All information and materials found in this article, including but not limited to text, trademarks, patents, logos, graphics and images (the "Materials"), are the copyrighted works and other forms of intellectual property of Marcel Dekker, Inc., or its licensors. All rights not expressly granted are reserved.

Get permission to lawfully reproduce and distribute the Materials or order reprints quickly and painlessly. Simply click on the "Request Permission/Reprints Here" link below and follow the instructions. Visit the U.S. Copyright Office for information on Fair Use limitations of U.S. copyright law. Please refer to The Association of American Publishers' (AAP) website for guidelines on Fair Use in the Classroom.

The Materials are for your personal use only and cannot be reformatted, reposted, resold or distributed by electronic means or otherwise without permission from Marcel Dekker, Inc. Marcel Dekker, Inc. grants you the limited right to display the Materials only on your personal computer or personal wireless device, and to copy and download single copies of such Materials provided that any copyright, trademark or other notice appearing on such Materials is also retained by, displayed, copied or downloaded as part of the Materials and is not removed or obscured, and provided you do not edit, modify, alter or enhance the Materials. Please refer to our Website User Agreement for more details.

Order now!

Reprints of this article can also be ordered at

http://www.dekker.com/servlet/product/DOI/101081JLC100102082