Kinetic spectrophotometric determination of hydrocortisone acetate in a pharmaceutical preparation by use of partial least-squares regression

The Analyst

M. Blanco, J. Coello, H. Iturriaga, S. Maspoch* and N. Villegas

Departament de Química, Unitat de Química Analítica, Facultat de Ciències, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain. E-mail: iqan7@blues.uab.es

Received 1st February 1999, Accepted 5th May 1999

A kinetic spectrophotometric method for the determination of hydrocortisone acetate based on its condensation with isonicotinic acid hydrazide is proposed. The method is applied to the determination of hydrocortisone acetate in a commercially available pharmaceutical preparation, presented as a pomade, that also contains another corticosteroid and additional active compounds. The operating procedure involves dissolving the pomade in chloroform and the addition of the reagent solution directly to the cuvette, in this way avoiding the previous extraction of analytes from the insoluble pomade matrix required by the alternative HPLC procedure. Calibration is performed by partial least-squares regression, using absorbance or first derivative spectra values recorded each minute during the first 30 min of reaction. Use of first derivative spectra overcomes possible scattered light problems produced by excipients precipitating, and produced slightly better results than absorbance data. The relative standard deviation obtained for 11 replicates analysed on different days was approx. 1.5%. The proposed method improves both accuracy and precision of the classical initial rate method and the precision of the HPLC procedure.

Introduction

Kinetic methods of analysis possess several potential advantages over equilibrium methods. Prominent among them are the ability to resolve systems involving alternative reactions taking place before the process of interest has finished and mixtures of very similar compounds on the basis of their differential rate of reaction with a common reagent. However, the poor reproducibility of traditional kinetic methods has so far hindered more extensive use.

Notwithstanding their shortcomings, kinetic methods have grown in use in recent years[1] as a result of the interaction of a new generation of analytical instruments and the development of powerful computers and chemometric software that enable the acquisition and processing of the information produced by such instruments. Specially prominent among instrumental novelties in this context are fluorimetric and UV–vis spectrophotometric diode array detectors, which allow signals to be simultaneously recorded at many different wavelengths in a very short time and provide three-dimensional data matrices of the form (A_{wj}, λ_w, t_j, w = wavelength, j = time). In this way, the information required to discriminate the analytes can be supplied by small differences in reaction rate and/or spectral differences between analytes or products; as a result, the resolving power of traditional univariate methods is substantially boosted. A fairly large number of available procedures for processing multivariate kinetic signals have been reported.[2,3] Among these procedures underline those based on principal components analysis,[4] which provide advantages such as the resolving of mixtures without a prior knowledge of the kinetic model followed by the system, the ability to process first-order,[5,6] second-order[7] and even more complex systems,[8,9] and also to model, to a certain extent, interactions between analytes.[6]

Although these methods have primarily been used in multicomponent determinations, they are also advantageous for studying individual kinetics.[10] In fact, in addition to the improved precision typical of multivariate methods,[2,11] they provide the ability to model the effects of slight interferences from reactions slower than the analytical process or potential perturbations of the sample matrix; moreover, they are applicable to non-linear systems (*i.e.* for those cases where the kinetics of interest is neither first- nor pseudo first-order in the analyte).

In this work, we have developed a kinetic spectrophotometric method for the determination of hydrocortisone acetate based on the condensation of this Δ^4-3-ketosteroid with isonicotinic acid hydrazide (isoniazid, INH). The specific purpose was to analyse the steroid in the commercially available preparation Hemorrane®, which contains hydrocortisone acetate, prednisone and benzocaine as active compounds. The proposed kinetic method is selective for hydrocortisone acetate; despite its high chemical similarity, prednisone reacts more slowly and contributes little to the analytical signal. Calibration was perfomed by using partial least-squares (PLS) regression and the results were compared with those provided by the initial rate method as applied to the same kinetic runs. PLS was found to provide much more accurate and precise values.

The accuracy of the results obtained in the analysis of different samples of the pharmaceutical preparation was confirmed by comparison with those provided by high performance liquid chromatography (HPLC) as an alternative validation method. The two methods were found to be comparably accurate; on the other hand, the proposed kinetic method proved more precise, no doubt as a result of working in the same solvent phase, avoiding in this way the step of extraction of the analyte from the polar solvent insoluble components of the pomade, required by the HPLC procedure.

Experimental

Apparatus and software

UV–vis spectra were recorded on a Hewlett–Packard (Avondale, PA, USA) HP-8451A diode array spectrophotometer equipped with an HP 89055A internal agitation system for mixing of the reagents inside the cuvette by means of a teflon-coated magnet. Cuvettes of 1 cm light path were used. All measurements were made with the thermostated cell at 25.0 ± 0.1 °C by means of a Frigiterm (Selecta, Abrera, Spain) S-382 thermostatic bath, and the room at 24 ± 1 °C. A cut-off filter (190–300 nm) was used to avoid photodecomposition of hydrocortisone acetate as the reaction developed. Spectra were recorded at 2 nm intervals, using an integration time of 1 s.

The chromatographic determination was carried out by using a system consisting of Shimadzu (Kyoto, Japan) LC-10AD pumps, a Hewlett–Packard 1040A HPLC diode array UV–vis spectrophotometer and a Model 9153 C data station, also from Hewlett–Packard. A Spherisorb ODS-2 C_{18} column (15 cm long × 0.46 cm id, 5 μm particle size, Tracer, St. Cugat del Vallés, Spain) was employed.

A Selecta ultrasonic bath and an Alresa (Madrid, Spain) centrifuge were also used.

All reagents were poured into the cuvette with the aid of Biogen Científica (Biohit, Helsinki, Finland) micropipettes.

Analytical models were constructed by using the PLS1 algorithm included in Unscrambler v. 6.1 (CAMO A/S, Trondheim, Norway). First-derivative spectra were obtained by using the Savitsky–Golay algorithm with a second-order polynomial and an overall window size of 21 points.

Reagents

All reagents and solvents used were analytical-grade. A fresh 2.5×10^{-3} mol l^{-1} stock solution of hydrocortisone acetate (Sigma, St. Louis, MO, USA) was prepared on a daily basis by accurately weighing about 0.10 g of reagent and dissolving it in 100 ml of chloroform. The stock was used to make a 2.5×10^{-4} mol l^{-1} working solution by dilution in chloroform, the container being wrapped in aluminium foil and stored refrigerated at 4–5 °C as the contents were found to gradually decompose at room temperature.

Hydrochloric acid solution at 0.05 mol l^{-1} in methanol was prepared by appropiate dilution and was standardized titrimetrically against TRIS.

An isonicotinic acid hydrazide solution of approximate concentration 5×10^{-3} mol l^{-1} was made by accurately weighing about 0.07 g of the reagent (Fluka, Buchs, Switzerland), dissolving it in 20 ml of 0.05 mol l^{-1} HCl and making to 100 ml with methanol.

Samples

The samples studied belonged to two different production batches of the pharmaceutical preparation Hemorrane® (from Laboratorios Nycomed Leo, Madrid, Spain) and were purchased at a chemist. The preparation is available as a pomade containing 15 mg of hydrocortisone acetate, 2.5 mg of prednisone and 10 mg of benzocaine per gram as active compounds. Fig. 1 shows its UV spectrum. The pomade excipients are insoluble in polar solvents but its active compounds are very readily soluble in both chloroform and ethanol.

Kinetic spectrophotometric procedure

Calibration solutions were prepared in the spectrophotometric cuvette by pouring appropriate volumes of the different solutions using micropipettes. Thus, a volume of 0.05–1 ml of hydrocortisone acetate solution was placed in the cuvette and made to 2 ml with chloroform. The cuvette was then accommodated in the spectrophotometer and stirred; after 1 min, a reference spectrum was recorded. Then, 0.5 ml of the reagent solution (isonicotinic acid hydrazine) was added, the time at which the first drop was released being taken as the initial reaction time. The UV–vis spectrum was recorded at 2 nm intervals over the wavelength range 300–500 nm for 1 min over a 30 min period. This spectral region contains the absorption band for the reaction product (λ_{max} = 388 nm), but also the tail of the reagent band. Stirring of the cuvette contents was maintained throughout the measurement process. The initial rate was determined from the kinetic curve obtained at the maximum absorption wavelength for the reaction product.

Calibration models were constructed from 11 samples evenly spanning the hydrocortisone acetate concentration range (0.5–10) × 10^{-5} mol l^{-1}. The samples were prepared in duplicate in a fully random manner on different days. The predictive capacity of the different models tested was assessed by using 17 samples containing analyte concentrations within the calibration range. The reproducibility of the kinetic method was calculated from the kinetic curves for a set of 11 samples containing identical concentrations of hydrocortisone acetate (3.0×10^{-5} mol l^{-1}) but prepared on different days.

Hemorrane® samples were prepared by weighing about 0.27 g from each pharmaceutical batch and dissolving it in 50 ml of chloroform. The reaction kinetics was recorded in duplicate, using the same procedure as for the calibration mixtures; the cuvette was loaded with 0.5 or 0.75 ml of sample solution, which were equivalent to theoretical hydrocortisone acetate concentrations of 4×10^{-5} and 6×10^{-5} mol l^{-1}, respectively. The solution was stirred throughout the measurement process.

Chromatographic procedure

Prior to chromatographic analysis, the active components must be removed from the pomade. To this end, 3 portions of about 0.12 g of each batch of the pharmaceutical preparation were weighed and dissolved in 5 ml of chloroform. A portion of 4 ml of each solution was placed in a centrifuge tube immersed in an ultrasonic bath and 45 ml of hot methanol were slowly added. The mixture was allowed to cool down in an ice bath and centrifuged. A 20 μl portion of the supernatant was passed through a nylon filter of 0.45 μm pore size and eluted with an acetonitrile–water mobile phase at a constant flow-rate of 1.0

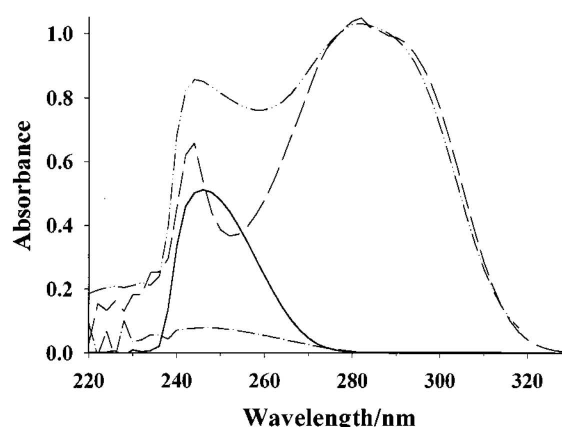

Fig. 1 UV-spectra for Hemorrane® (-···-), hydrocortisone acetate (—), prednisone (-·-) and benzocaine (---), at the nominal concentration.

ml min^{-1}. A mobile phase elution gradient was used involving passage of a 40:60 acetonitrile–water mixture during the first minute and gradually altering this proportion to 70:30 in 4 min. Under these conditions, well-defined, well-resolved, tailless peaks were obtained with retention times of 2.3, 3.7 and 5.3 min for prednisone, benzocaine and hydrocortisone acetate, respectively. Samples were injected in triplicate and their chromatograms recorded at the maximum absorption wavelength for each species (*viz.* 245, 290 and 240 nm, respectively), using a bandwidth of 4 nm.

The concentration range where the integrated peak area was linearly related to the concentration was examined and calibration curves were run from solutions containing variable concentrations of the active compounds in such a way that the central concentration was very close to the theoretical concentration in the pharmaceutical.

Data acquisition and processing

The kinetic–spectral data recorded for each sample were transferred to an IBM PC-486 compatible computer *via* an RS232C/HP-829339A interface with a view to their subsequent mathematical processing. The PLS1 algorithm included in the Unscrambler software package was used to build up the different calibration models. In order to achieve the best predictive capacity, different spectral modes (absorbance and first derivative) and working wavelenth ranges were assayed.

The different PLS models assayed were constructed by using the cross-validation method[4] and as many cross-validation segments as samples, each segment comprising the duplicates of each sample.

The number of significant principal components (PCs) chosen for each calibration model was that for which the mean squared error of cross validation (MSECV) was not significantly different from the lowest MSECV value.[12,13] The significance criterion used was that proposed by Haaland and Thomas,[12] based on an F-test at $\alpha = 0.25$.

$$\mathrm{MSECV} = \frac{\sum_{i=1}^{I_\mathrm{C}} (\hat{c}_i - c_i)^2}{I_\mathrm{c}} \quad (1)$$

where c_i and $\hat{c}_i$ are the experimental and calculated concentrations, respectively, and I_c the total number of calibration samples.

For easier comparison and interpretation of the results of each model, the relative standard error of prediction (RSEP) for the calibration and prediction sets was calculated from

$$\mathrm{RSEP}\,(\%) = \sqrt{\frac{\sum_{i=1}^{M} (c_i - \hat{c}_i)^2}{\sum_{i=1}^{M} c_i^2}} \times 100 \quad (2)$$

where c_i and $\hat{c}_i$ have the same meaning as in eqn. (1) and M is the total number of samples included in the different sets.

Results and discussion

Reaction of hydrocortisone acetate with isonicotinic acid hydrazide

The condensation of isonicotinic acid hydrazide to form yellow-coloured hydrazones in weakly acidic solutions is typical of all steroids containing a carbonyl group and is widely documented and applied for analytical purposes.[14–16] The reaction is reversible and conforms to second-order kinetics with a pH-dependent rate. Δ^4-3-ketosteroid groups such as in hydrocortisone acetate react quantitatively at room temperature in less than an hour. Other carbonyl groups require a long time or much stronger reaction conditions.

The most suitable experimental conditions to quantify hydrocortisone acetate were studied. The chief experimental constraint in this respect arose from the physical state of the preparation studied. Thus, the sample was only soluble in chloroform and precipitated in polar solvents. The best working conditions were found to be those provided by a 4:1 mixture of chloroform and methanol, which dissolved both the polymers in the pomade, the isoniazide hydrochloride and the water formed in the reaction. The other variable to be optimized was the hydrochloric acid concentration. The acid acts as a catalyst for the reaction and affects the spectrum of the hydrazone formed. An HCl concentration about twice that of reagent, consistent with reported choices,[14] was adopted as optimal.

The fact that the methanol content in the mixed solvent could not exceed a given threshold limited the maximum usable reagent concentration. In this work, a constant reagent concentration of 1×10^{-3} mol l^{-1} was employed. Under these conditions, excess reagent resulted in pseudo first-order kinetics for the analyte with a half-time of 11 ± 1 min in dilute solutions—more concentrated solutions deviated somewhat from this behaviour.

Despite its structural similarity to hydrocortisone, prednisone reacts at a much lower rate, so the contribution of its reaction to the recorded signal during the first 30 min is virtually zero. If the process is allowed to develop for long enough, this $\Delta^{1,4}$-3-ketosteroid also condenses with isonicotinic acid hydrazide to give the corresponding hydrazone, which exhibits an absorption maximum at 406 nm. Fig. 2 shows the absorption spectra for both hydrazones after about 24 h of reaction.

Determination of hydrocortisone acetate

Initial rate method. A plot of initial rate (in A min^{-1}) against the initial hydrocortisone acetate concentration for the different calibration samples was found to be linear; the corresponding regression parameters and confidence limits at a significance level of 95% were as follows:

$$\mathrm{rate}_0 = (-0.5 \pm 1.7) \times 10^{-3} + (810 \pm 40)\,[\text{hydrocortisone acetate}]_0 \; (r = 0.996)$$

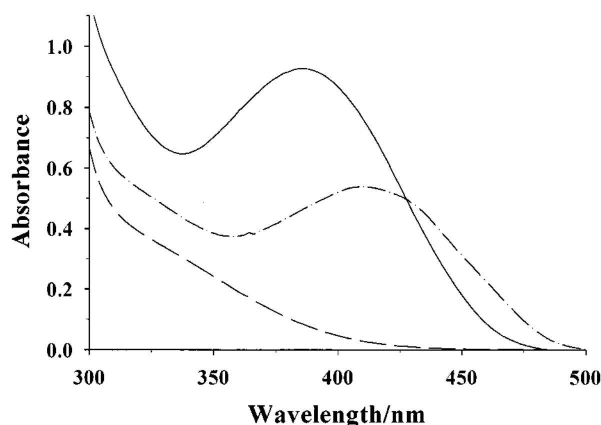

Fig. 2 UV-spectra of 1×10^{-3} mol l^{-1} isonicotinic acid hydrazide (---) and reaction products of hydrocortisone acetate (—) and prednisone (-·-), both at a 6×10^{-5} mol l^{-1} concentration, after 2 and 24 h, respectively.

Table 1 shows the predicted values for the different sample sets studied. Note the relatively low reproducibility obtained (RSD $\simeq$ 15%).

PLS calibration. PLS calibration allows one to use all the information contained in the change of the absorption spectrum with time. Fig. 3 shows the changes in the 300–500 nm region for a 6×10^{-5} mol l^{-1} hydrocortisone acetate solution during the first 30 min of reaction.

The reproducibility of the results might be affected if one of the pomade components precipitated inadvertently with time. Although this was avoided as far as possible by using a high chloroform content and continuous stirring, if it occurred at all, it would introduce turbidity in the medium; this in turn would change the solution absorbance by a virtually wavelength-independent factor. This effect can be readily corrected[17] by using the derivatives of the original spectra rather than direct absorbance measurements. Accordingly, the calibration models were constructed from both absorbance and first-derivative spectra. Also, although PLS is a whole-spectrum calibration system, if the information contained in the different variables is highly correlated, it may be useful to exclude those contributing with no analytically significant information. The selection was made by choosing various wavelength ranges and times. However, no significant differences among models were obtained and the results were only slightly better if the absorption tail in the reagent spectrum was excluded. Only the results obtained using spectral data recorded during the first 30 min over the wavelength ranges 350–460 (absorbance) and 370–480 nm (first-derivative), respectively, are presented. The ensuing models contained 3 and 5 factors, respectively. The need for more than one factor can be ascribed to PLS correcting the deviation of the more concentrated samples from the pseudo first-order kinetics. Table 1 shows the relative standard error of prediction obtained in the quantitation of the calibration and prediction sets using these PLS1 models, as well as the average hydrocortisone acetate concentration for the 11 samples containing 3×10^{-5} mol l^{-1} of the analyte used in the reproducibility study and the relative standard deviation computed from these samples. As can be seen, the results provided by absorbance and first-derivative spectra were virtually identical, which confirms that the pomade remains dissolved throughout the reaction and that the PLS results are much more accurate and reproducible than those provided by the initial rate method.

Table 2 shows the individual results for the 17 prediction samples studied. Although some samples contained identical concentrations, they were prepared from different weighings on different days. Note the high precision, similar throughout the concentration range studied. Because both procedures were similarly precise, that based on absorbance spectra was chosen for simplicity.

Table 1 Figures of merit for the differents methods. Relative standard error of prediction (%) for calibration (RSEPC) and prediction sets (RSEPV); mean and relative standard deviation found for 11 replicates of a 3.00×10^{-5} mol l^{-1} hydrocortisone acetate solution

	Initial rate	Absorbance (350–460 nm)[a]	First derivative (370–480 nm)[b]
RSEPC (%)	4.02	1.24	0.52
RSEPV (%)	9.1	1.46	1.01
Mean/mol l^{-1}	3.2×10^{-5}	3.01×10^{-5}	3.00×10^{-5}
RSD (%)	15	1.5	1.8

[a] Number of PCs used in the model = 3. [b] Number of PCs used in the model = 5.

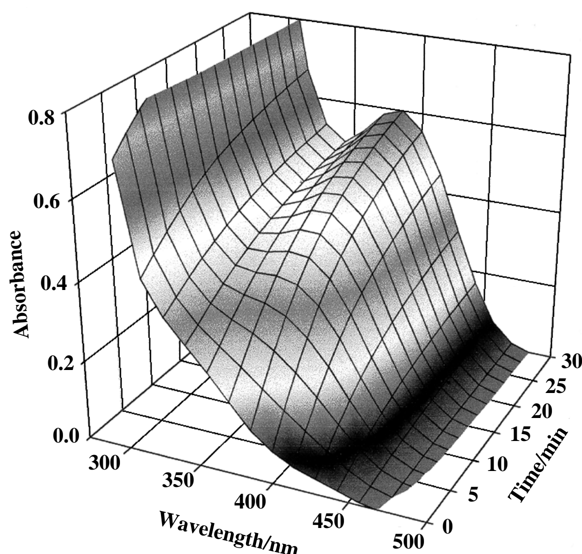

Fig. 3 Absorbance–kinetic spectra for 6×10^{-5} mol l^{-1} hydrocortisone acetate over the wavelength range 300–500 nm during the first 30 min of reaction.

Determination of hydrocortisone acetate in Hemorrane®

Once the proposed method was checked to provide such good results with laboratory-made samples, it was applied to the analysis of the pharmaceutical preparation Hemorrane® in samples from two different production batches. The results, together with those provided by the initial rate method, are compared in Table 3 with those obtained by HPLC (average values and their corresponding confidence intervals at α = 0.05). As can be seen, the average values obtained with the PLS

Table 2 Results obtained in the quantitation of the prediction samples by using the PLS1 models

		Absorbance (350–460 nm)[a]		First derivative (370–480 nm)[b]	
Sample	Added/ 10^5 mol l^{-1}	Found/ 10^5 mol l^{-1}	Relative Error (%)	Found/ 10^5 mol l^{-1}	Relative Error (%)
1	1	0.99	0.9	1.01	−0.7
2	2	2	−0.2	2.01	−0.5
3	3	2.97	1	2.99	0.4
4	3	3.01	−0.5	3	−0.1
5	3	3.04	−1.2	3.06	−1.8
6	3	3.06	−2.1	3.04	−1.4
7	4	4.01	−0.2	3.95	1.3
8	4	3.99	0.2	4	0.1
9	4	4.05	−1.2	3.98	0.5
10	6	5.9	1.6	5.92	1.3
11	7	6.84	2.3	6.84	2.4
12	7	7.18	−2.5	7.09	−1.2
13	7	7.2	−2.9	6.99	0.2
14	9	8.92	0.9	9.01	−0.1
15	9	8.98	0.2	8.98	0.2
16	9	9.04	−0.4	8.99	0.1
17	9	9	0	8.89	1.2

[a] Number of PCs used in the model = 3. [b] Number of PCs used in the model = 5.

Table 3 Average hydrocortisone acetate content (mg g^{-1} pomade) and their corresponding confidence intervals at $\alpha = 0.05$ (n = 6), obtained by applying each of the methods tested to two different Hemorrane® batches

	Kinetic method		
	Initial rate	PLS	HPLC
Batch K 04	14.4 ± 0.8	14.3 ± 0.3	14.3 ± 0.9
Batch K 51	13.2 ± 1.3	14.4 ± 0.3	14.3 ± 1.0

model coincide with the HPLC results; however, the kinetic method is much more precise, as confirmed by an *F*-test at the 95% confidence level. On the other hand, the results of the initial rate method for one of the sample batches are significantly different from those of the HPLC method.

Conclusions

As shown in this paper, the joint use of multiparametric recordings of a kinetic signal and multivariate calibration by PLS provides a powerful analytical tool. Even in such apparently simple systems as those studied in this work, the use of the whole recorded information results in substantially improved accuracy and precision with conventional kinetic methods, which are thus made competitive with HPLC in those cases where, as in the studied example, the analyte must be previously removed from a complex matrix. The ability to analyse the sample in a homogenous phase, with minimal manipulation, results in considerably increased precision.

Acknowledgements

The authors are grateful to Spain's Dirección General de Investigación Científica y Técnica (DGICyT) for financial support received through Project PB94–0693. Núria Villegas wishes to acknowledge additional funding from the DGU of the *Generalitat de Catalunya* in the form of an FPI scholarship.

References

1 S. R. Crouch, *Anal. Chim. Acta*, 1993, **283**, 453.
2 B. M. Quencer and S. R. Crouch, *CRC Crit. Rev. Anal. Chem*., 1993, **24**(3), 243.
3 T. F. Cullen and S. R. Crouch, *Mikrochim. Acta*, 1997, **126**, 1.
4 H. Martens and T. Naes, *Multivariate Calibration*, Wiley, New York, 1989.
5 J. Havel, F. Jiménez, R. D. Bautista and J. J. Arias León, *Analyst*, 1993, **118**, 1355.
6 M. Blanco, J. Coello, H. Iturriaga, S. Maspoch and M. Redon, *Anal. Chim. Acta*, 1995, **303**, 309.
7 M. Blanco, J. Coello, H. Iturriaga, S. Maspoch, M. Redón and J. F. Rodríguez, *Quím. Anal.,*1996, **15***,* 266.
8 M. Blanco, J. Coello, H. Iturriaga, S. Maspoch and J. Riba, *Anal. Chem.*, 1994, **66**, 2905.
9 M. Blanco, J. Coello, H. Iturriaga, S. Maspoch, M. Redon and N. Villegas, *Analyst*, 1996, **121**, 395.
10 G. López-Cueto, J. F. Rodríguez-Medina and C. Ubide, *Analyst*, 1997, **122**, 519.
11 T. Owen, *Int. Lab*., 1987, **17**, 68.
12 D. M. Haaland and E. V. Thomas, *Anal. Chem*., 1988, **60**, 1193.
13 M. Blanco, J. Coello, H. Iturriaga, S. Maspoch and M. Redón, *Appl. Spectrosc*., 1994, **48**(1), 37.
14 E. J. Umberger, *Anal. Chem*., 1955, **27**, 768.
15 L. L. Smith and T. Foell, *Anal. Chem.*, 1959, **31**, 102.
16 I. Ringler and J. Perrine, *Endocrinology*, 1961, **69**, 1095.
17 P. Levillain and D. Fompeydie, *Analusis*, 1986, **14**, 1.

Paper 9/00856J