The Analyst

Validated determination of total arsenic species of toxicological interest (arsenite, arsenate and their metabolites) by atomic absorption spectrometry after separation from dietary arsenic by liquid extraction: toxicological applications

L. Benramdane[ab], M. Accominotti[ab] and J. J. Vallon*[ab]

[a] *Laboratoire de Biochimie, Pharmacotoxicologie et Analyse des Traces, Hôpital Edouard Herriot, Place d'Arsonval, 69437 Lyon 03, France*
[b] *Laboratoire de Chimie Analytique III, Faculté de Pharmacie, Université Claude Bernard, 8 Avenue Rockefeller, 69373 Lyon 08, France*

A validated method for the selective extraction of total As species of toxicological interest (arsenite, arsenate and mono- and dimethylated arsenic species) from urine, followed by atomic absorption spectrometric determination, is described. The mechanisms involved in extraction were studied and the extraction method was optimized. The urine sample was acidified with concentrated HCl and KI and sodium hypophosphite were added. Under these conditions, As species were reduced to their corresponding iodide arsines, extracted with toluene and back-extracted with 1 mmol l^{-1} NaOH solution. Only inorganic arsenic and its metabolites in humans (monomethylarsonic and dimethylarsinic acid) were extracted. Arsenobetaine of dietary origin was not extracted. This method can detect if any As increase in urine originates from inorganic As intoxication or only from dietary non-toxic As species such as arsenobetaine.

Keywords: *Inorganic arsenic; inorganic arsenic metabolites; arsenobetaine; arsenic reduction; arsenic extraction; atomic absorption spectrometry; urine*

Because of its ubiquity in the environment , arsenic is present in all environmental compartments in numerous inorganic and organic chemical forms. The distribution of As in the environment results from natural processes (*e.g.*, marine sedimentary rocks, erosion and weathering of soils and minerals) and human activities (*e.g.*, mining operations, smelting, pesticide manufacture).[1,2] The chemical form of As depends on its source: inorganic As from minerals, industrial discharges and pesticides and organic As from industrial discharges, insecticides and biological action on inorganic As.[2,3] In humans, inorganic As [arsenite (As^{III}) and arsenate (As^{V})] is by far the most toxic of the arsenic species: for an adult individual, the lethal dose is probably between 100 and 200 mg of arsenious acid.[2,4] In the organism, inorganic As is metabolized in its methylated forms [monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA)]. The ingestion of large amounts of inorganic arsenicals causes acute intoxication characterized mainly by serious abdominal, cardiovascular and neurological symptoms.[5–7] Chronic intoxication by inorganic As is observed under continuous exposure even to very low doses over a few years. Chronic exposure is associated with dermatological, neurological and vascular effects.[2,4,6] After some years of exposure, As causes skin cancer, lung cancer and other types of malignant neoplasms.[2,8,9]

From a metabolic point of view, inorganic As is methylated in the organism and the major metabolite is DMA. In workers exposed chronically to inorganic As, Farmer and Johnson[10] found that the total urinary As level is increased up to hundreds of $\mu g\ l^{-1}$ with the following distribution of the different species: As^{V} 3.5%, As^{III} 12.5%, MMA 16% and DMA 66.5%. Urine excretion forms the major pathway for the elimination of As compounds from the body; hence urinary As determinations are important for assessing a subject's exposure to toxic As species, because a good correlation is observed between absorption and urinary excretion. In urines, average concentrations are between 20 and 50 $\mu g\ l^{-1}$.[11,12]

In biological analysis the total urinary As concentration is still used for the assessment of As exposure. However, the measurment of total As may not be appropriate for the biological monitoring of occupational exposure. Indeed, numerous studies have shown that total urinary As can show an important rise within the 10 h following seafood ingestion.[11,13] Moreover, these elevated levels are without any consequence on health because As of dietary origin is exclusively in organic and therefore non-toxic forms.[13–15] The major organic form of As in marine organisms is arsenobetaine (Asbet); it represents 96% of total extracted As from the tissue, the remaining 4% being arsenocholine (Aschol), tetramethylarsonium (TMAs) and arseno sugars.[15–17] The As species of toxic origin are inorganic As (As^{III}, As^{V}) and its metabolites (DMA and MMA). In unexposed subjects these species are present in the urine in small amounts and their levels remain unchanged after marine food consumption.[11]

These observations show that it is important to apply a method selective enough to determine the As of toxic origin separately from Asbet, which is the major As form of dietary origin. With this aim, several speciation methods have been reported. They involve the determination of the individual concentrations of As^{III}, As^{V}, DMA, MMA, Asbet, Aschol and sometimes TMAs and arseno sugars[17–19] in urine.

Speciation is generally accomplished in three steps: sample preparation, species separation and their detection. HPLC is the most common separation technique used in ion-exchange[19,20] and ion-pairing[17] modes, coupled with various systems of detection: ICP-AES,[21,22] AAS[23,24] and ICP-MS.[18–20] These methods are selective and sensitive but require a very fastidious pre-treatment to eliminate the matrix (proteins, salts and interferent elements), which alters the chromatographic separation and interferes in detection by ICP-MS.

In this paper, we propose a useful, simple, rapid and sensitive method for the unique determination of inorganic As (As^{III}, As^{V}) and its metabolites (DMA, MMA), based on solvent extraction and determination by AAS; organic As from dietary intake (Asbet) is not extracted. Similar methods have been reported. One method is based on reduction of As^{V} with KI in 40 min after acidification with HCl, extraction with toluene followed by back-extraction with dilute HNO_3.[20] In another modified

method toluene and dilute HNO_3 solution were replaced with chloroform and de-ionized water, respectively.[25]

This paper demonstrates the mechanism of extraction of each As species and the role of each reagent implicated in the reaction preceding the extraction step. This study allowed the optimization of a very simple, fast and reproducible method, with a high yield of As species of inorganic origin; sodium hypophosphite added to the medium drastically improved the performance of the method.

When total As is found at elevated levels in urine, it is important to determine the percentage of As of inorganic origin. In this case the proposed method can be applied with great facility.

Experimental

Reagents

All reagents were of analytical-reagent grade. Stock standard solutions of each arsenic compound (1 g l^{-1}) were prepared by dissolving in water appropriate amounts of As_2O_3 (Sigma, St. Louis, MO, USA), $Na_2HAsO_4{\cdot}7H_2O$ (Sigma), $CH_3AsO_3Na_2{\cdot}6H_2O$ (Carlo Erba, Milan, Italy) and $(CH_3)_2AsO_2Na{\cdot}3H_2O$ (Sigma). A mixture of these four As species was also prepared (see Sample preparation). The Asbet solution (0.412 g l^{-1} As) was provided by CNRS Solaize (Lyon, France). For the reduction and extraction step, 37% HCl (Merck, Darmstadt, Germany), KI (Fluka, Buchs, Switzerland), $NaH_2PO_2{\cdot}H_2O$ (Prolabo, Paris, France) and 1 mmol l^{-1} solution NaOH (Fluka) were used.

For ETAAS, 0.05% Triton X-100 (Merck) was used for sample dilution. A 10 g l^{-1} $Ni(NO_3)_2{\cdot}6H_2O$ (Merck) solution was used as a chemical modifier.

A 50 μg l^{-1} working standard solution, prepared daily from a 1 g l^{-1} As stock standard solution (Spex Industries, Edison, NJ, USA) and Lyphocheck urine controls levels 1 and 2 with As concentrations of 50 and 150 μg l^{-1}, respectively (Bio-Rad Labs., Richmond, CA, USA) were used for checking the AAS instrument.

Urines selected with a low total As content (< 10 μg l^{-1}) were spiked with the mixture of As species (see Sample preparation) for calibration of the extraction step.

Instrumentation

The AAS instrument used was a SpectrAA Zeeman 220 (Varian, Palo Alto, CA, USA) equipped with a pyrolytic graphite-coated graphite furnace and a Zeeman-effect background corrector.

Extraction method

Qualitative study

Basis of the method. Our method for the extraction of As^{III}, As^{V}, DMA and MMA from urine was adapted from an early method described by Charlot:[26] As^{III} is extracted with benzene from a concentrated HCl medium; As^{V} is extracted only in the presence of KI and hypophosphite. Our adaptation to the four As species of inorganic origin was developed as follows.

Study of mechanisms involved in extraction. Concentrated solutions (0.1 g l^{-1}) of As^{III}, As^{V}, MMA and DMA were used because of their color properties, allowing rough observation of the extraction process. Benzene was replaced with the less toxic toluene.

(i) Arsenite. Arsenite is extracted by the organic solvent from a concentrated HCl medium and in the presence of I^- according to the following reaction:

$$H_3AsO_3 + 3IH \rightleftharpoons AsI_3 + 3H_2O \quad (1)$$

The reaction is complete and immediate in concentrated HCl; AsI_3 is yellow in water and orange–red in toluene. If I^- is absent, As^{III} can also be extracted, probably by formation of $AsCl_3$ which is an uncolored species.

Back-extraction of As^{III} in water is then explained by the following equation:

$$(AsI_3)_{toluene} + 3H_2O \rightleftharpoons (H_3AsO_3)_{water} + 3HI \quad (2)$$

The equilibrium involves both the organic and aqueous phases. Its deplacement to the right is greatly facilitated by alkalinization, and the yield is greatly improved compared with an acidic or neutral medium.

(ii) Arsenate. Arsenate can only be extracted if it is reduced to As^{III}. I^- is responsible for this reduction together with the formation of AsI_3, which is then extracted. The reactions involved are as follows:

$$H_3AsO_4 + 2HI \rightleftharpoons H_3AsO_3 + I_2 + H_2O \quad (3)$$

$$H_3AsO_3 + 3HI \rightleftharpoons AsI_3 + 3H_2O \quad (4)$$

(iii) MMA and DMA. MMA and DMA are extracted by the same mechanism as arsenate because in these species arsenic is pentavalent (As^{V}). MMA and DMA are reduced with the formation of the methylated arsines CH_3AsI_2 and $(CH_3)_2AsI$.[27] Both of these species are brown in both HCl and toluene media. The corresponding reactions are

$$CH_3AsO(OH)_2 + 4HI \rightleftharpoons CH_3AsI_2 + I_2 + 3H_2O \quad (5)$$

$$(CH_3)_2AsO(OH) + 3HI \rightleftharpoons (CH_3)_2AsI + I_2 + 2H_2O \quad (6)$$

The back-extraction from toluene is performed according to eqn. (2).

(iv) The role of sodium hypophosphite. During the qualitative study of extraction we observed that in the absence of hypophosphite, AsI_3, CH_3AsI_2 or $(CH_3)_2AsI$ is slowly destroyed with the appearance of a yellow species in HCl (I_3^-) and a purple species in toluene (I_2). This destruction can be avoided if the medium is added with the very reducing hypophosphite ion. If KI is also present, its reducing power makes the reaction immediate.

Optimization of the extraction method

Optimization of the method was studied on each species (As^{III}, As^{V}, DMA, MMA) in 300 μg l^{-1} solutions. Several parameters can modify the nature of extraction, *e.g.*, concentration of HCl, KI, NaH_2PO_2, nature of extraction solvent, pH of the aqueous solution for back-extraction of As and reaction and mixing times. At concentrations of HCl ⩾6.5 mol l^{-1} (Fig. 1), KI ⩾75

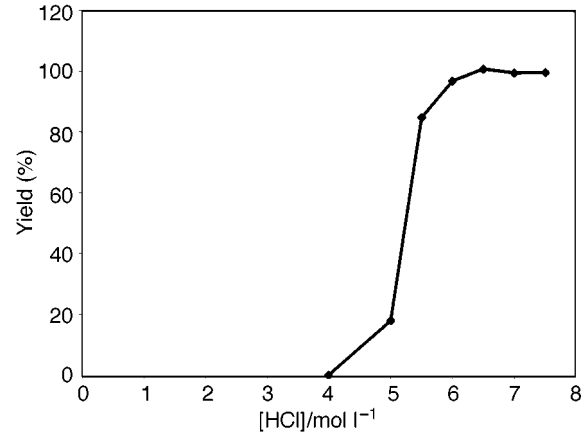

Fig. 1 Extraction yield as a function of HCl concentration in the medium.

mmol l^{-1} and NaH_2PO_2 ⩾325 mmol l^{-1} (Fig. 2), total and immediate reduction is observed. The yields are close to 100% if toluene extraction is accomplished in two steps (2 ml each) with 2 min of vortex mixing. Back-extraction from toluene is complete with 1 mmol l^{-1} NaOH. Assays with identical conditions showed that arsenobetaine is unextractable.

Validation of the method

Sample preparation

Calibration standards (15, 20, 30, 40, 60 and 120 μg l^{-1}) were prepared by spiking six 1 ml urine aliquots with appropriate volumes of concentrated arsenic mixture. This mixture contained As^{III}, As^{V}, DMA and MMA, at 0.250 g l^{-1} equivalent As (the total As concentration is 1 g l^{-1}). The volume added was always ⩽2% (5–20 μl) of total volume of the sample in order to keep the matrix constant.[28]

Linearity

The extraction was tested from 0 to 300 μg l^{-1}. Atomic absorption showed linearity up to 100 μg l^{-1}. The linearity of the method was confirmed using classical tests, *i.e.*, comparison of the intercept with zero and correlation coefficients. The intra-assay reproducibility was determinated for replicate calibration curves prepared on the same day (n = 6). The inter-assay calibration curves were determined on six successive days. All data were obtained using the same urine spiked with the same mixture of As^{III}, As^{V}, DMA and MMA.

Precision and accuracy

The intra- and inter-day precision and the accuracy of the method were determined by performing replicate analyses of urine spiked with the As mixture (As^{III}, As^{V}, DMA and MMA) to prepare low, medium and high concentration levels (10, 50 and 150 μg l^{-1}, respectively). The procedure was repeated using the same spiked standards on the same day (n = 6) and on different days (n = 6) to determine the intra- and inter-day repeatability, respectively. The accuracy, expressed as the percentage deviation of the observed concentration from the theoretical concentration, with the relative error, was evaluated.

Recovery

The extraction efficiency was determined for all compounds by comparing the signal from urine spiked with a known amount of As species, in the range of the calibration curves, assayed accordingly, *versus* the signal for the same concentrations prepared in the NaOH–Triton X-100 mixture. Each sample was analysed in duplicate.

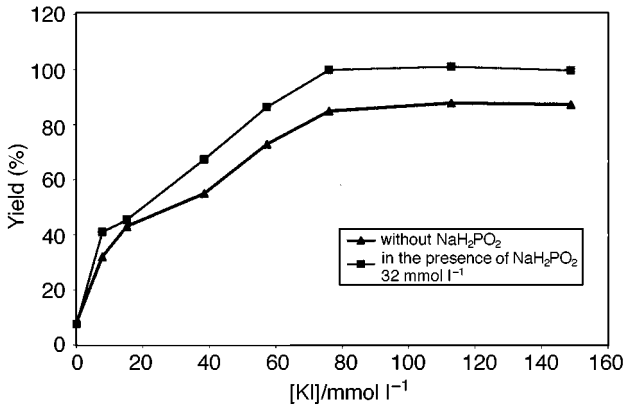

Fig. 2 Extraction yield as a function of KI final concentration in the medium.

Limit of detection (LOD) and limit of quantification (LOQ)

As the urine sample used for calibrations is not free from any arsenical, it could not be taken as a blank. Therefore, for AAS measurements, 1 mmol l^{-1} NaOH diluted twofold in 0.05% Triton X-100 was used to determine the LOD and the LOQ. This procedure was validated by comparison of the slopes (B) of calibration curves for NaOH–Triton X-100 with curves for urine extracts. Twelve curves for each medium showed that the mean slopes were identical (B = 0.0024 ± 0.0002 and 0.0025 ± 0.0001, respectively) and that the procedure was valid.

The LOD was determined by measuring 30 times the signal of the NaOH–Triton X-100 mixture and determining the concentration from the calibration curve in NaOH. The LOD was taken as three times the standard deviation of this concentration. The LOQ was taken as 10 times the standard deviation of this concentration.

Results

Optimized method

A 1 ml aliquot of urine in a polypropylene tube containing 100 μl of 3.5 mol l^{-1} KI, 0.5 ml of 3 mol l^{-1}, NaH_2PO_2, and 3 ml of 37% HCl was extracted with 2 ml of toluene. Following vigorous shaking for 2 min, the mixture was centrifuged for 2 min at 3000 rpm. The organic supernatant was removed and retained and a second extraction step was performed. The total volume (4 ml) of organic phase was then back-extracted in 1 ml of 1 mmol l^{-1} NaOH with 5 min of vortex mixing. The aqueous alkaline phase was diluted twofold with 0.05% Triton X-100 solution and then measured by AAS. In this step, the sample and the nickel modifier were co-injected into the furnace according to the procedure given in Table 1. Sample analyses were made by reference to calibration curves.

Validation

Linearity

The correlation coefficients (r) for the calibration curves were 0.9988 and 0.9973 for intra-assay and inter-assay linearity,

Table 1 AAS instrument and furnace parameters

Instruments parameters—	
Wavelength	193.7 nm
Slit width	0.2 nm
Lamp current	10 mA
Tube	Pyrolitic graphite coated
Background correction	On
Measurement mode	Peak area
Sample volume	10 μl
Sample modifier	5 μl

Furnace parameters—

Step	Temperature/ °C	Time/s	Gas flow rate/ 1 min^{-1}	Read
1	85	5.0	3.0	No
2	95	40.0	3.0	No
3	120	10.0	3.0	No
4	300	10.0	3.0	No
5	1200	5.0	3.0	No
6	1200	1.0	3.0	No
7	1200	2.0	0.0	No
8	2700	0.6	0.0	Yes
9	2700	2.0	0.0	Yes
10	2600	2.0	0.5	No
11	2600	1.0	3.0	No

Table 2 Reproducibility of the method

Added/μg l^{-1}	Found/μg l^{-1}* Intra-assay (n = 6)	Inter-assay (n = 6)
0	8 ± 0.7 (8.8%)	8 ± 0.8 (10.0%)
15	23 ± 1.7 (7.4%)	23 ± 1.9 (8.3%)
20	29 ± 1.8 (6.2%)	28 ± 2.2 (7.9%)
30	38 ± 1.9 (5.0%)	38 ± 2.5 (6.6%)
40	48 ± 1.9 (4.0%)	49 ± 2.1 (4.3%)
60	69 ± 2.8 (4.1%)	69 ± 3.7 (5.4%)
120	129 ± 3.0 (2.3%)	128 ± 3.7 (2.9%)

* RSD in parentheses.

Table 3 Intra- and inter-assay linearity

Parameter	Intra-assay linearity	Inter-assay linearity
Slope (mean ± s)	0.0025 ± 0.0001	0.0024 ± 0.0002
Intercept (mean ± s)	0.0280 ± 0.0019	0.0293 ± 0.0013
Coefficient of linear regression (r)	0.9988 ± 0.0008	0.9973 ± 0.0019

Table 4 Precision and accuracy of the method

Added/μg l^{-1}	Found/μg l^{-1}* Intra-day (n = 6)	Inter-day (n = 6)
0	8 ± 0.8 (10.0%)	8 ± 0.7 (8.8%)
10 (low concentration)	18 ± 1.7 (9.4%)	18 ± 1.8 (10.0%)
50 (medium concentration)	58 ± 2.4 (4.1%)	58 ± 2.9 (5.0%)
150 (high concnetration)	157 ± 4.1 (2.6%)	158 ± 4.6 (2.9%)

* RSD in parentheses.

respectively. For each point of the calibration, the concentrations were recalculated from the equation of the linear regression curves (experimental concentrations) and the relative standard deviations (RSD, %) were calcualted. The results are presented in Table 2. The small percentage differences between the nominal and found concentrations of the standards on the calibration curves confirmed that the assays were linear over the concentration ranges investigated. The mean slopes, intercepts and correlation coefficients are presented in Table 3. Concentrations of toxic As in unknown samples were determined using the calibration curves.

Precision and accuracy

The intra- and inter-day precision and the accuracy of the method are given in Table 4. The precision around the mean values did not exceed 10% (RSD).

Table 5 Extraction of As^{III}, As^{V}, DMA and MMA in the presence of arsenobetaine and analysis by AAS

As concentration in urine/μg l^{-1}			
As^{III} + As^{V} + DMA + MMA	Asbet	Total	As extracted/ μg l^{-1}
15	21	36	14.4
25	42	67	25.6
50	42	92	50.3
120	210	330	119.0
125	105	230	124.5
200	42	242	200.4

Recovery

The recoveries obtained for As^{III}, As^{V}, DMA, and MMA were 100.8, 101.9, 99.8 and 100.3%, respectively with corresponding RSDs of 6.40, 6.85, 7.35 and 8.32% (n = 18).

LOD and LOQ

The LODs and the LOQs determined as previously defined were 2 and 7 μg l^{-1}, respectively.

Specificity

Arsenobetaine is of dietary origin and non-toxic. In order to evaluate a possible interference, we spiked the same batch of urines with Asbet. The results (Table 5) indicate that Asbet is not extractable by the proposed method, at any concentration, and in the range of concentrations of inorganic As on the calibration curves.

Discussion and conclusions

The study of the mechanisms of the reactions involved in the reduction and extraction of iodide arsines formed by toluene showed that reduction of pentavalent As species (arsenate, DMA, MMA) is greatly improved by hypophosphite; hypophosphite is a better reductive species than HI, which was used alone in all previous methods proposed in the literature. Addition of hypophosphite also increases the yields of reduction–extraction together with shorter reduction times, owing to its reducing power, which improves the reducing action of HI and preserves the iodide arsines from destruction by oxygen. Another improvement is obtained by back-extraction in dilute NaOH, which displaces the equilibrium of extraction towards the aqueous phase with better yield.

The recovery of all As species was 100% and the LOQ was 7 μg l^{-1}. Arsenobetaine, which is the major non-toxic organic species of As, appeared unextractable by the proposed method.

The described validated method is very simple, specific and very fast. It allows the rapid investigation of any inorganic As intoxication without the use of the much more expensive, long and tedious separation methods of arsenic speciation.

References

1 Fitzgerald, L. D., in *Arsenic, Industrial, Biomedical and Environmental Perspectives*, ed. Lederer, W. H., and Fensterheim, R. J., Van Nostrand, New York, 1983, pp. 3–8.
2 Hindmarsh, J. T., and McCurdy, R. F., *Crit. Rev. Clin. Lab. Sci.*, 1986, **23**, 315.
3 Braman, R. S., *Top. Environ. Health*, 1983, **6**, 141.
4 *Clinical Toxicology*, ed. Polson, C. J., and Tattersal, R. N., Pitman, London, 1969, pp. 181–184.
5 Franzblau, A., and Lilis, R., *Arch. Environ. Health*, 1989, **44**, 385.
6 Lovell, M. A., and Farmer, J. G., *Hum. Toxicol.*, 1985, **4**, 203.

7 Takahashi, W., Pfenninger, K., and Wong, L., *Arch. Environ. Health*, 1983**,** **38**, 209.
8 Hopenhayn, R. C., Biggs, M. L., Fuchs, A., Bergolio, R., Telo, E. E., and Smith, A. H., *Epidemiology*, 1996, **7**, 117.
9 Morton, W., Starr, G., Pohl, J., and Stoner, S., *Cancer*, 1976, **37**, 2523.
10 Farmer, J. G., and Johnson, L. R., *Br. Med. J.*, 1990, **47**, 342.
11 Buratti, M., Calzaferri, G., Caravelli, G., Colombi, A., Maroni, M., and Foa, V., *Int. J. Environ. Anal. Chem.*, 1984, **17**, 25.
12 Yamato, N., *Bull. Environ. Contamin. Toxicol.*, 1988, **40**, 633.
13 Arbouine, M. W., and Wilson, H. K., *J. Trace Elem. Electrolytes Health Dis.*, 1992, **6**, 153.
14 *Food Contamination from Environmental Sources*, ed. Nriagu, J. O., and Simmons M. S., Wiley, New York, 1990, pp. 121–139.
15 Goessler, W., Maher, W., Irgolic, K. J., Kuehnelt, D., Schlagenhaufen, C., and Kaise, T., *Fresenius' J. Anal. Chem.*, 1997, **359**, 434.
16 Beauchemin, D., Bednas, M. E., Berman, S. S., McLaren, M. W., Siu, K. W. M., and Sturgeon R. E., *Anal. Chem.*, 1988, **60**, 2209.
17 Mingsheng, M., and Le, X. C., *Clin. Chem.*, 1998, **44**, 539.
18 Heitkemper, D., Creed, J., Caruso, J., and Fricke, F. L., *J. Anal. At. Spectrom.*, 1989, **4**, 279.
19 Shibata, Y., and Morita, M., *Anal. Sci.*, 1989, **5**, 107.
20 Bavazzano, P., Perico, A., Rosendahl, K., and Apostoli, P., *J. Anal. At. Spectrom.*, 1996, **11**, 521.
21 Morita, M., Uehiro, T., and Fuwa, K., *Anal. Chem.*, 1981, **53**, 1806.
22 Rubio, R., Padro, A., Alberti, J., and Rauret, G., *Microchim. Acta*, 1992, **109**, 39.
23 Rubio, R., Padro, A., and Rauret, G., *Fresenius' J. Anal. Chem.*, 1995, **351**, 331.
24 Zhang, X., Cornelis, R., De Kimpe, J., and Mees, L., *Anal. Chim. Acta*, 1996, **319**, 177.
25 Lebouil, A., Notelet, S., Calleux, A., Tutcant, A., and Allain, P., *Toxicorama*, 1997, **9**, 171.
26 Charlot, G., *Les Réactions Chimiques en Solution Aqueuse et Caractérisation des Ions*, Masson, Paris, 7th edn., 1983, pp. 272–276.
27 Pascal, P., *Nouveau Traité de Chimie Minérale*, Masson, Paris, 1958, vol. 9, pp. 474–494.
28 Shah, V. P., Midha, K. K., Dighe, S., McGilveray, I. J., Skelly J. P., Yacobi, A., Layloff, T., Viswamanda, C. T., Cook, C. E., McDowall, R. D., Pittman, K. A., and Spector, S., *J. Pharm. Sci.*, 1992, **81**, 309.

Paper 8/03842B
Received May 21, 1998
Accepted June 22, 1998