Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 893–897
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

A validated cold vapour-AAS method for determining mercury in human red blood cells

O.S. Ertas[a,*], H. Tezel[b]

[a] Faculty of Pharmacy, Department of Analytical Chemistry, Ege University, 35100 Bornova, Izmir, Turkey
[b] Faculty of Dentistry, Department of Restorative Dentistry and Endodontics, Ege University, 35100 Bornova, Izmir, Turkey

Received 8 March 2004; received in revised form 5 August 2004; accepted 5 August 2004
Available online 12 October 2004

Abstract

A cold vapour-atomic absorption spectrometry (CV-AAS) method for determining mercury in dental students and clinical teaching staff red blood cells at a dental school using amalgam as a restorative material has been validated. A number of blood samples (n = 122) from dental students in years I to V, clinical teachers in restorative dentistry and controls were collected and analysed. Accuracy, linearity, precision (repeatability and reproducibility) and robustness of the method have been determined, and detection and quantification limits have been calculated. Linearity of response was verified for concentrations ranging from 5 to 40 $\mu g L^{-1}$ of mercury. Correlation coefficient of the calibration straight lines was always ≥0.99. Intra-day precision of the method gave coefficient of variation (CV) of 5.51%. Inter-day precision of the method calculated after analysis of five different concentrations of mercury standard solutions by the same analyst in different days and by two different analysts in different days gave coefficient of variation 4.89 and 5.44%, respectively. The accuracy of the method was calculated a CRM NIST 966 (toxic metals in bovine blood) total amount of mercury was found a concentration of 28.83 ± 2.2 $\mu g L^{-1}$. Recovery was 89.27%. Robustness of the method evaluated by changing different experimental conditions under which analyses performed, fractional factorial design was done for assessing robustness of the method. Root mean square error was found out as 1.56.

Limits of detection and quantification were 1.84 and 4.03 μg of Hg per litre of sample, respectively. Results show the suitability of the method for direct measurement of mercury in red blood cells and the importance of the working conditions for people dealing with amalgam at a dental school.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Method validation; Cold vapour-AAS; Mercury; Human red blood cells

1. Introduction

Validation of an analytical method is a necessary step in controlling the quality of quantitative analysis. Method validation is an established process which is the provision of documentary evidence that a system fulfils its pre-defined specification or the process of providing that an analytical method is acceptable for its intended purpose [1]. Thus, with the background knowledge of linearity, detection and quantification limits precision and specificity of the analytical method. It is relatively easy to derive the confidence and the reliability of the analytical data obtained with it [2].

Mercury intake of human beings, excluding occupational exposure, comes mainly from two sources diet and amalgam restorations. Small amount of inorganic mercury also enter the body by inhalation, smoking and drinking alcohol [3,4].

The determination of mercury in various biological matrices, particularly in blood, urine and saliva, is becoming increasingly important in assessment of mercury contamination in the environment and at the workplace [5]. There have been many discussions concerning the correlation between amalgam restorations and mercury concentrations in blood, saliva and urine [6–11]. Interest in the possible toxicity of

* Corresponding author. Tel.: +90 533 7109 771; fax: +90 232 388 5258.
E-mail addresses: soguto@pharm.ege.edu.tr (O.S. Ertas), tezel@dent.ege.edu.tr (H. Tezel).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.08.014

mercury in dentistry has resulted in a rapidly increasing of articles.

The most often used method for Hg determination at present is the cold vapour-atomic absorption spectrometry (CV-AAS). It is generally agreed that oxidative conversion of all forms of mercury in the sample to Hg (II) is necessary prior to reduction to elemental Hg [2].

Preliminary treatment of sample is often required the metal to the analytical methodology in an appropriate form. A bewildering variety of combinations of strong acids (HCl, H_2SO_4, HNO_3), oxidants (H_2O_2, $KMnO_4$, $K_2S_2O_8$, KBr and $KBrO_3$) have been used.

The purpose of the present work was both to validate the analytical method for the determination of mercury and to determine the mercury levels of dental students' and faculty dentists' red blood cells.

2. Materials

2.1. Instrumentation

The determination of Hg was carried out by 'cold vapour'-atomic absorption technique using, mercury evaporation kit (Varian 4S) at Varian 10plus AA with Hg hollow cathode lamp.

2.2. Reagents and solutions

All chemicals used were of analytical reagent grade (Merck). Water used in the preparation of standard solutions was obtained from a Millipore Milli-Q-System. $KMnO_4$ solution (2%, m/v) was prepared from $KMnO_4$, make sure that all of the $KMnO_4$ crystals are dissolved and keep this solution in a dark brown bottle to prevent it from decomposing. Reducing solution was prepared by dissolving 10 g hydroxylamine sulphate and 15 g $SnCl_2{\cdot}2H_2O$ in 40 mL 1:6 H_2SO_4 and brought the volume to 100 mL. Three millilitres of total amount of a nitric acid 65% and perchloric acid 70% (5:1) were used during the digestion procedures. Mercury stock solution was prepared by dissolving 0.1354 g $HgCl_2$ in 100 mL ultrapure water. Calibration mercury standards were prepared daily by diluting the stock in 5% (v/v) sulphuric acid.

2.3. Samples

Dental students from first year to the fifth and clinical teachers working in the department of restorative dentistry of Ege University who are being exposed to amalgam more than those in other departments were examined. For experimental groups, individuals who had similar food consumption habits were selected. As well as alcohol and cigarette were selected to minimise the deviations that might result from diets. A total of 90% of the experimental and the control group had never consumed sea products. The rest of them consumed once or twice a month in their diet. No amalgam restorations were made in these individuals during the study.

The control group was composed of 14 clinical teachers who worked in the Department of Periodontology full time and never worked with amalgam but sometimes shared clinical areas. The controls were selected to be similar to be exposed group in terms of working conditions, alcohol and cigarette consumption and dietary habits.

The background of the groups and the usage of the clinics are shown in Table 1.

2.4. Procedures

Ten millilitres of venous blood was collected in metal-free polyethylene tubes. The blood samples were centrifuged at $1500 \times g$ for 20 min at 5 °C. Erythrocytes and plasma were then separated by means of a serum separator. All samples stored at −20 °C and analysed within 4 months.

Three millilitres of each sample was wet digested with the mixture of 3 mL nitric and perchloric acids (5:1) at 25–35 °C. Then the samples, filtered through Whatman Ashless Filter Paper Ø 90 mm, were added to ultrapure water to the total amount of 10 mL [12,13]. Three millilitres of sample solution transferred into reaction vessel and added 1 mL reducing solution and one drop of $KMnO_4$ (2%) as oxidant into vessel and stirred 90 s and measured the peak height at 253.7 nm and under air flow rate 2 L/min.

To avoid the contamination, glassware and polyethylene containers, before and after use, were washed with double-distilled water, then soaked in nitric acid solution at 30% (v/v) during 24 h, rinsed several times in doubled-distilled deionised water and dried in air. Items were kept in a clean place. An exhaustive cleaning of glassware material and

Table 1
The distribution of 122 individuals according to study groups

	Groups						
	1	2	3	4	5	6	7
Of individuals	First year students	Second year students	Third year students	Fourth year students	Fifth year students	[a]	[b]
Number of individuals	28	17	15	19	13	16	14
Working place	Student laboratory			Clinic 1	Clinic 2	Clinic 3	Clinic 4
Groups who works with Hg	−	+	+	+	+	+	−

[a] The clinical teachers working in the Department of Restorative Dentistry at the faculty.
[b] The clinical teachers working in the Department of Periodontology at the faculty.

polyethylene sample containers is very important. This fact has been carefully considered in this study and blank levels obtained are very low. Disposable polyethylene tips were cleaned as above and then used on the micropipettes.

The annual average of daily air mercury levels during the period of work in student laboratory, Clinic 1, Clinic 2, Clinic 3, Clinic 4 were, 0.027 mg/cm^3, 0.025, 0.025, 0.048, and 0.008 mg/m^3, respectively. The vapour concentration of mercury was measured by using $PdCl_2$ disks. The darkening of the test papers was evaluated with spectrophotometer. The average concentrations were derived from the results of numerous measurements carried out throughout the academic year [12].

3. Results and discussion

3.1. Validation of the method

To assess the validity of the proposed method, analytical performance characteristics for determination of mercury in dental students' and dentists' red blood cells were estimated.

Although nonlinear calibration is widely available, it is still considered mandatory that across the range of likely use (80–100%, or more widely 50–150%) of target concentration the calibration line to establish the relationship between the measured quantity and the concentration of the analytes should be linear. Linearity of response was studied by using mercury standard solutions containing 5, 10, 15, 20 and 40 $\mu g\,L^{-1}$. By plotting absorbance for each solution versus its mercury concentration, a linear relationship was obtained until 40 $\mu g\,L^{-1}$. The Hg level in red blood cells is low and, thus, in order to obtain the calibration straight line, five levels of standard solutions containing 5, 10, 15, 20, 25 $\mu g\,L^{-1}$ of Hg were employed. Equation of the calibration straight line, correlation coefficient (r) and standard error of calibration curve, after regression analysis with 99% confident level were $y = 0.0046X - 0.0018$ and $r^2 = 0.9965$ and S.E. 0.0029, respectively.

Precision is usually measured as the standard deviation of a set of data. Precision of the instrument was checked in order to show if instrument response Hg standard solution was always the same. This parameter considers only the error attributable to the operating system and not the error attributable to sample handling and preparation. The instrumental precision was calculated from 10 consecutive measurements of a 10 $\mu g\,L^{-1}$ Hg standard solution. A good precision, expressed as R.S.D.%, was obtained since relative standard deviation was equal to 1.6%.

To evaluate the precision of the method, measurements were performed under conditions of intra-assay precision and intermediate precision. Intra-assay precision of the method was estimated from the analysis of 10 sample solutions under repeatability conditions (short time, one analyst, one instrument, same sample). Coefficient of variation was 5.51%. These indicated a good repeatability of the procedure. Intermediate precision of the method was studied by carrying out analysis on five samples from two different groups and different individuals (a) and five samples from the same person (b) under various analysts. As it can be seen in Table 2, when five samples from group 1 were analysed (by duplicate) by the same analyst on 5 different days, CV obtained as equal to 4.31%. A slightly higher R.S.D. = 5.44% was obtained when five samples from group 2 were analysed by duplicate in different days by two different analysts.

The intermediate precision of calibration straight lines obtained on 7 different days (over a period of a month) by using different Hg standard solutions was also investigated. R.S.D.% is for slope and after linear regression analysis, correlation coefficient (r) for the straight lines was always $\geq$0.9984.

Recovery studies were made in order to evidence the lack of Hg losses or contamination during sample treatment and matrix interferences during the measurement step. For the determination of the recovery, known amounts of Hg were added to the blood, and the resulting spiked samples were analysed and compared to the known added value. All analyses were carried out in six replicate five Hg concentration of 50, 75, 100, 125 and 150%. The recovery was in the range of 89.27%.

To validate the accuracy of the method, a certified reference material NIST 966 (toxic metals in bovine blood) containing $31.4 \pm 1.7\ \mu g\,L^{-1}$ total amount of mercury was analysed and it was found a concentration of $28.83 \pm 2.2\ \mu g\,L^{-1}$.

Robustness was evaluated in order to know how sensitive the method is to small changes introduced in the procedure

Table 2
Intermediate precision in the determination of Hg in two different groups of dental students' blood

Group 1			Group 2		
Analyst	Days	Hg ($\mu L\,L^{-1}$)	Analyst	Days	Hg ($\mu L\,L^{-1}$)
A	1	2073 ± 0.023	B	6	6791 ± 0.015
A	2	3812 ± 0.028	B	7	20733 ± 0.076
A	3	5053 ± 0.011	B	8	9275 ± 0.105
A	4	3067 ± 0.032	B	9	4805 ± 0.034
A	5	7537 ± 0.014	B	10	3315 ± 0.054
Mean		4308	Mean		5252
R.S.D.%		4891	R.S.D.%		5434

Results are the mean of three determinations.

Table 3
Robustness in the determination of mercury in dentists' red blood cells

Slit width		HNO_3 volume added		$HClO_4$ volume added		$SnCl_2$ volume added		Waiting period	
nm	C[a]	mL	C[a]	mL	C[a]	mL	C[a]	s	C[a]
0.2	22.6	2.5	26.68	0.5	36.25	1	40.21	90	38.42
0.5	23.9	3	25.92	0.75	35.86	1.5	39.18	60	37.39
Mean	23.25	Mean	26.30	Mean	36.06	Mean	39.70	Mean	37.91
R.S.D.%	3.95	R.S.D.%	2.04	R.S.D.%	0.76	R.S.D.%	1.83	R.S.D.%	1.92

C[a] = Hg μg L^{-1} results are the mean of three determinations.

[1]. Changes introduced in the method were (a) changes in the slit width used for mercury measurement by CV-AAS, (b) changes in the volume of HNO_3 added for digestion, (c) changes in the volume of $HClO_4$ added for digestion of the samples, (d) changes in the volume of $SnCl_2$ added as reducing agent and (e) changes in waiting period. To HNO_3, $HClO_4$ and $SnCl_2$ added during the analyses procedure, analyses were carried out by demonstrating the robustness of the method to changes in the volume using 2.5, 0.5 and 1 mL solutions, respectively, and 3, 0.75 and 1.5 mL of HNO_3, $HClO_4$ and $SnCl_2$, respectively. To assess the influence of the slit width in mercury measurement by cold vapour-AAS, measurements were carried out by using two different slit widths: 0.1 and 0.5 nm. Robustness of the method was also demonstrated by analysing the waiting period. Measurements were carried out by using two different waiting times: 90 and 60 s. Relative standard deviations of obtained results were given in Table 3. Fractional factorial design was done for assessing robustness of the method. Root mean square error was found out as 1.56.

The limit of detection of analyte in a sample may be described as the concentration that gives an instrument signal significantly different from the blank or background signal. The limit of quantification is the lowest concentration of analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions. In order to obtain the detection and quantification limits, the blank signal was measured 20 times. The detection limit was calculated as $[(Y_b + 3\,\text{S.D.}) - b]a^{-1}$ and the quantification limit as $[(Y_b + 10\,\text{S.D.}) - b]a^{-1}$, where Y_b is the mean of the blank signal, S.D. is the standard deviation of the blank signal, b is the intercept of the calibration straight line, and a is the slope of the calibration straight line [1,14,15]. Results obtained were 1.8374 μg Hg per litre of blood for detection limit and 4.0319 μg Hg per litre of blood for the quantification limit.

3.2. *Measurement of mercury in dentists' and dental students' red blood cells*

Frequency and duration of amalgam work were different for all groups. For the second and the third groups, average weekly active amalgam work period was 8 h. The first group worked in the same laboratory within the same period, nearly 6 h per week. The fourth and fifth groups worked with amalgam for 25 h per week during a 5-week period. For the sixth group, the average weekly working time was 10 h. The temperature for all the rooms varied between 21 and 24 °C and the relative air humidity was between 14 and 39%.

Red blood cells were analysed using the method described in this study. When groups were analysed separately, the difference of mercury concentration in red blood cells measured at the beginning and at the end of the academic year was sig-

Table 4
Statistical evolution of the groups

Group	n	Mean ($\times 10^{-3}$)	Standard deviation ($\times 10^{-3}$)	R.S.D.%
1	28	5.9757[a]	10.0764[a]	16.8623[a]
		9.4551[b]	11.3899[b]	12.0463[b]
2	17	5.882	5.2117[a]	8.8604[a]
		8.9988[b]	11.7995	13.1123[b]
3	15	12.3874[a]	19.1991[a]	15.4988[a]
		5.5649[b]	10.0142	17.9952[b]
4	19	6.5172[a]	5.6081[a]	8.6051[a]
		2.8777[b]	5.5380[b]	19.2448[b]
5	13	7.4220[a]	6.0885[a]	8.2032[a]
		4.7454[b]	7.9087[b]	16.6658[b]
6	16	9.7872[a]	12.4072[a]	12.677[a]
		5.4351[b]	13.5465[b]	24.924[b]
7	14	5.7096[a]	3.9788[a]	6.9686[a]
		6.4636[b]	4.9284[b]	34.3465[b]

[a] Measurements at the beginning of academic year (μg L^{-1}).
[b] Measurements at the end of the academic year (μg L^{-1}).

nificant. The results of the groups were shown in Table 4. Increases were in the first, second and control groups and decreases were in the other groups. It should take into consideration that the third, forth, fifth and the sixth groups were dealing with the amalgam more than the other groups.

Exposure to mercury vapour within the confines of the dental office is the major source of high blood concentrations of mercury in dentists. About 80% of inhaled mercury will rapidly cross the pulmonary membranes and dissolve in the circulating blood [11]. This circulating mercury is partitioned between packed cells and plasma [16]. Chang et al. [6] showed that organic mercury levels in the blood of the individuals who did not consume sea products did not change. The mercury trapped within red blood cells is eventually eliminated in bile salts when the red blood cells disintegrate (half-life of 120 days).

In this study, other sources of mercury had been excluded in the selection of participants. This included provision of dental amalgam restorations to the members of the study groups. Nilsson and Nilsson [17] showed that amalgam-practising style was very important even if dentists were ultra-careful about mercury hygiene.

4. Conclusions

A simple method for the determination of mercury in human red blood cells by cold vapour-AAS described and validated. Linear range of the calibration, precision, repeatability, reproducibility, accuracy robustness and detection and quantification limits obtained for the proposed method show its suitability for determining mercury in human blood cells. Working with the same analyst is important for the reliability of the study. At least the analyst should be in the same experience level.

This study shows that dealing with the amalgam does not have any effect on red blood cells if it is given enough attention mercury hygiene in the laboratories. The increase of mercury level in groups 1 and 2 may be due to the lack of experience.

References

[1] D.B. Hibbert, Accred. Qual. Assur. 4 (1999) 352–356.
[2] A. Abarca, E. Canfranc, I. Sierra, M.L. Marina, J. Pharm. Biomed. Anal. 25 (2001) 941–945.
[3] G. Kohn, J. Am. Dent. Assoc. 125 (1994) 125–129.
[4] L.J. Brown, J. Am. Dent. Assoc. 122 (1991) 73–77.
[5] T. Guo, J. Baasner, M. Gradl, A. Kistner, Anal. Chim. Acta 320 (1996) 171–179.
[6] S.B. Chang, C. Siew, S.E. Gruninger, J. Dent. Res. 71 (1992) 66–74.
[7] J.R. Marckert, J. Am. Dent. Assoc. 122 (1991) 54–61.
[8] M. Marek, Adv. Dent. Res. 6 (1992) 100–109.
[9] J.W. Osborne, Adv. Dent. Res. 6 (1992) 135–138.
[10] S.B. Corbin, W.G. Kohn, J. Am. Dent. Assoc. 125 (1994) 381–388.
[11] T.W. Clarkson, J. Am. Coll. Toxicol. 8 (1989) 1291–1295.
[12] H. Tezel, O.S. Ertas, F. Ozata, C. Erakin, A. Kayali, Br. Dent. J. 191 (2001) 449–452.
[13] K.R. Snapp, D.B. Boyer, L.C. Peterson, C.W. Svare, J. Dent Res. 68 (1989) 780–785.
[14] L.A. Pasteenick, in: T.L. Bernard, R.A. Nash (Eds.), Drug Pharmaceutical Sciences, 23, Marcel Dekker, New York, 1993.
[15] S.D. Brown, T.B. Blank, S.T. Sum, L.G. Weyer, Anal. Chem. 66 (1994) 315R–322R.
[16] M.G. Cherian, J.B. Hursh, T.W. Clarkson, J. Allen, Arch. Environ. Health 33 (1978) 109–114.
[17] B. Nilsson, B. Nilsson, Swed. Dent. J. 10 (1986) 1–14.