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SPECTROPHOTOMETRIC DETERMINATION OF 0–50 ng OF CHROMIUM IN 1 ml OF HUMAN SERUM

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Summary—A method is described for the determination of chromium in 1 ml of human serum or plasma. It is based on a wet decomposition and a spectrophotometric determination with diphenylcarbazide after extraction with methyl isobutyl ketone. A 40-mm cuvette with less than 1 ml sample volume is described. Results are lower than expected from the literature, most samples from healthy persons containing less than 2 or 3 ng/ml. The reproducibility is about 1 ng/ml.

IN MANY BIOLOGICAL systems chromium is present, generally in extremely low concentrations. In the last two decades the functions and occurrence of chromium in these systems have been extensively studied. A comprehensive survey of this work has been given recently by Mertz.[1] It shows that chromium probably plays a role in biological processes in human beings. Some points are that the element is accumulated in high concentrations in certain areas of the brain, in nucleic acids and in hair, and that during the first weeks or months of life, the chromium concentration in many organs is higher than for the rest of life. Correlation may exist between certain diseases and the chromium content of human tissues and organs. Plasma undergoes significant changes in chromium content after an oral glucose load.

Mertz[1] reports values for the chromium concentration in blood (or serum or plasma) ranging from 0 to 520 ng/ml, and concludes that most of the values are between 20 and 50 ng/ml, and that plasma chromium levels considerably below 20 ng/ml may be suggestive of a low dietary intake, but do not necessarily mean chromium deficiency. Niedermeier *et al.*[2] recently described a direct-reading emission spectrographic method. They report that 50% of the 105 sera analysed have a chromium content below their detection-limit of 10 ng/ml. They report a mean value of 30 ng/ml.

In order to make further studies of the subject, further development of analytical methods is necessary, however. If these methods have to be applied in clinical laboratories they have to be performed with apparatus generally available there. In the present work the possibility of the development of such a method, applicable to the determination of chromium in 1 ml of human serum, was studied. Of the techniques used for the determination of metals at such levels, emission spectrography is not sensitive enough (except perhaps if special techniques[2] are used) and it is not available at most clinical laboratories. The latter objection may also be made against X-ray fluorescence. This technique was used by Beyermann *et al.*[3] for the determination of chromium in urine (down to 15 ng) after extraction with 8-quinolinol into chloroform. The use of atomic absorption for purposes similar to ours was described by Feldman *et al.*[4,5] but it seems not sensitive enough (down to 30 ng) for the present problem. Volatile metal chelates have recently been used for the determination of

metals in the subnanogram range. Measurements were made with gas chromatographs (with electron-capture detectors) and with mass spectrometers. The latter method has not so far been applied to practical problems of a similar nature to ours. The chromium content of the plasma of rats has been measured by GLC, but the animals had been injected with Na_2CrO_4 and the chromium content was about 50 ng/ml (Hansen *et al.*[6]).

A technique readily available in clinical laboratories is spectrophotometry, and therefore we tried to find a procedure using this technique. Except for some catalytic procedures which we did not study, the diphenylcarbazide reaction with chromium(VI) combined with methyl isobutyl ketone (MIBK) extraction seemed the most sensitive. Its application has often been described, *e.g.*, by Koch and Koch-Dedic,[7] Beyermann,[8] and Morsches and Tölg.[9] The method of decomposition entailed some modifications to the method of determination, which may be useful in other applications, too. Suitable apparatus is present in many clinical laboratories, except perhaps the cuvettes and the slit-attachment which may, however, be obtained commercially.

EXPERIMENTAL

Apparatus and reagents

Absorption measurements were made with the Uvispek H 700 (Hilger and Watts) with a slit-attachment H 742. A cuvette was made from a 50 × 34 × 12 mm Teflon block. A groove was milled, 17 mm deep, 36 mm long, and 2 mm wide. In both side-walls at the end of the groove a circular hole was drilled, the holes were threaded, and a perforated screw was placed in each hole (perforation 5 mm diameter). Quartz windows were placed in the holes and Buna-N O-rings used for sealing. The optical path was about 37 mm, the volume used for measurements about 0·9 ml. The H 742-slit was used with an aperture 2 mm high and 1 mm wide. Small volume 40-mm cuvettes are commercially available.

Pyrex and quartz tubes with glass stoppers were used both for sample decomposition and centrifugation. They were 15 mm in bore and 100 mm long and were heated in a copper block, in holes (6 in the block) with the proper diameter and about 20 mm deep. The block contained a small hole for a thermometer and was heated on an electric plate. A quartz tube, 3 mm outer and 1 mm inner diameter and 120 mm long, was sealed 2 mm above one of its ends and placed in the decomposition tube as a "stirring rod" to prevent bumping.

Doubly distilled demineralized water, distilled acetone, distilled methyl isobutyl ketone and "Suprapur" (Merck) acids were used, except for hydrochloric acid which was prepared by isothermic distillation.[10] The dichromate standard (100 μg/ml) was made from solid $K_2Cr_2O_7$; more dilute solutions were prepared daily. A chromium(III) solution was prepared from this standard by reduction with alcohol and sulphuric acid. Diphenylcarbazide solution (50 mg/25 ml) in MIBK was prepared fresh every week. MIBK–hydrochloric acid mixture was prepared by diluting 1 ml of 10*M* hydrochloric acid to 100 ml with MIBK. The decomposition mixture was prepared from nitric acid (65%) perchloric acid (70%) and sulphuric acid (96%) in the volume-ratio 3:1:1.

Procedure

Take a 1·0-ml sample in the decomposition tube, add 1·0 ml of the decomposition mixture, mix, add a stirring rod. Place the tubes in the block (at 60–70°) and after 5 min mix the foam formed with the rest of the contents. Heat the block to 90° in 25 min, then in 20 min more to 120° for the dissolution, in another 30 min to 150° for the predecomposition, in the next 45 min to 275° for the final decomposition and in 15 min to 330° for the removal of perchloric acid. Remove the tubes from the block and cool them. If the contents are yellow, heat again at 330° for 5 min.

Wash the wall and the stirring-rod each with 0·5 ml of 0·12*M* perchloric acid, add 0·2 ml of 0·01*M* potassium permanganate and place for 5 min on a water-bath (100°). Cool in ice, add 0·10 ml of 10*M* hydrochloric acid, keep at 0° until the permanganate colour disappears, add 1·00 ml of MIBK–hydrochloric acid solution (at 0°), extract for 1 min and then immediately centrifuge for 1 min at 2000 rpm. Take 0·85 ml of the organic phase in another centrifuge tube, add 0·10 ml of diphenylcarbazide solution, 0·01 ml of MIBK–hydrochloric acid solution and 0·10 ml of acetone. Mix, place in the dark, and measure the absorbance against water at 540 nm in a 40-mm microcuvette between 20 and 30 min after addition of the diphenylcarbazide. Substract the value of a blank obtained in the same way without the addition of serum.

Calibration curves without preliminary decomposition are prepared by the same procedure. Aliquots of a dichromate solution containing 0–100 ng of chromium are used, 0·2 ml of 96% sulphuric acid and 1 ml of 0·12*M* perchloric acid are added and the volume is adjusted to about 1·2 ml before the oxidation with permanganate.

DISCUSSION

The decomposition mixture described was also used by Feldman *et al.*[5] Its advantage over some other mixtures tried is that a small and constant amount is used. This eliminates the influence of impurities in the reagents. Moreover the decomposition does not take much work. There was some indication that blanks obtained with Pyrex tubes were slightly higher than those with quartz tubes but the difference—if any—was small. New tubes may cause loss of some chromium when first used. The oxidation of chromium(III) to chromium(VI) and the extraction with hydrochloric acid and MIBK (as $HCrO_3Cl.2MIBK$) have been described with slight variations by several others.[4,5,7,8,9,11]. Some of these authors[7,8,9] also describe the photometric determination. The method is very selective[8] and therefore not much work was done to investigate interferences. An exception was made for iron but at the levels normally present in serum the absorbance due to the iron contribution was found to be roughly equivalent to that of 0·1 ng of chromium.

Preliminary experiments gave some irregular results which suggested that perchloric acid left on the walls of the decomposition tube might affect the absorbance. It was found in experiments without the decomposition step that the addition of perchloric acid greatly enhanced the absorbance. Addition of 10 μl of 70% perchloric acid gave a constant and maximum absorbance value. Therefore the walls and the stirring rod were rinsed with the equivalent of this amount of acid. The rinsing with perchloric acid greatly improved the reproducibility of the complete procedure. Another modification is the addition of 0·1 ml of acetone to the organic phase. This addition prevents turbidity in the coloured solution.

For the blanks a mean value of zero was expected, and was found in about half the experiments, but all the others gave high blanks. The results suggest that the equivalent of 4 (or 2) ng of chromium are introduced in part of the experiments, but not in others. Statistical conclusions based on the Gaussian curve may therefore be incorrect. The mean value of the blanks is 1·4 ng, the standard deviation is 1·6 ng. This value is higher than the value of 1·0 ng found for the standard deviation in the measurement of samples, but it should be noted here that duplicate or triplicate samples were always analysed within one day, whereas the blanks were taken over a period of more than a week. The recovery experiments show recoveries of 96%, 103%, 93% and 94% (mean 97%) if a blank of 1·4 ng is substracted.

A calibration curve for the range 0–100 ng of chromium, obtained without application of the decomposition mixture and procedure, was linear, obeying the equation $1000A = 2{\cdot}16x + 1{\cdot}7$, where A was the absorbance and x the number of ng of chromium. A similar curve prepared with 1 ml of a serum and 0–50 ng of chromium was also linear, with the equation $1000A = 2{\cdot}15x + 8{\cdot}4$. The standard deviation was 0·6 ng in both cases.

From the analytical point of view it is concluded that the procedure described allows the determination of chromium in serum or plasma at the 0–50 ng level with a standard deviation of about 1 ng, but that blanks and samples should preferably be run at the same time. Fourteen experiments a day are possible.

BIOCHEMICAL APPLICATION

A total of 27 heparinized plasma samples and 10 sera samples from healthy adults were analysed for chromium, with the results given in Table I. Youden's formulae[12] were used to determine the standard deviations.

TABLE I.—CHROMIUM CONTENT OF THE SERUM OF 37 HEALTHY PERSONS FROM UTRECHT AND ROTTERDAM, AND RESULTS OF 14 BLANKS.

Cr found, *ng/ml*	Number of persons*	Number of blanks†
<1	3	7
1	10	1
2	16	2
3	2	0
4	1	4
5	4	0
6	1	0

* Most results were mean values for two (or three) analyses.

† Single experiments.

Most of the values given for the samples in the table are mean values of duplicate experiments; in a few cases only a single determination was made, in a few others triplicate experiments were run. From each set of duplicate or triplicate experiments (made on the same day) the standard deviation was calculated and a pooled value of 1·0 ng/ml was found with 31 degrees of freedom. No differences due to age or sex could be detected in the results. No difference was found between serum and plasma. Twelve blanks (including decomposition) and four recovery experiments were also run. Results for the blanks are given in Table I. Recovery experiments with 10 or 20 ng of Cr(VI) and 20 or 40 ng of Cr(III) gave measured values of 11, 22, 20 and 39 ng respectively. From a biochemical point of view we have to conclude that results for healthy persons are lower than might be expected from the review of Mertz[1] and the work of Niedermeier *et al.*[2] The best conclusion seems that practically all values are below 5 ng/ml and that 80 or 90% are below even 2 or 3 ng/ml. Clinical chemists are left to draw further conclusions but before they do so, repetition of the experiments may be useful. Two points are mentioned here, however. One is that Mertz[1] reports large geographical variation in the chromium content of several human organs. Another is that the loss of considerable amounts of chromium during storing of the sample, before the analysis, seems improbable, no decrease in chromium content being found during experiments on the same deep-frozen serum analysed at intervals during a fortnight.

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Zusammenfassung—Ein Verfahren zur Bestimmung von Chrom in 1 ml menschlichem Blutserum oder -plasma wird beschrieben. Es beruht auf nasser Zersetzung und spektrophotometrischer Bestimmung mit Diphenylcarbazid nach Extraktion mit Isobutylmethylketon. Eine 40 mm-Küvette mit weniger als 1 ml Probenvolumen wird beschrieben.

Die Ergebnisse sind niedriger als nach Literaturangaben erwartet; die meisten Proben von gesunden Personen enthalten weniger als 2 oder 3 ng/ml. Die Reproduzierbarkeit beträgt etwa 1 ng/ml.

Résumé—On décrit une méthode pour le dosage du chrome dans 1 ml de sérum ou de plasma humain. Elle est basée sur une décomposition par voie humide et un dosage spectrophotométrique avec le diphénylcarbazide après extraction en méthylisobutylcétone. On décrit une cuvette de 40 mm avec un volume d'échantillon moindre que 1 ml. Les résultats sont plus faibles que ceux attendus de la littérature, la plupart des échantillons de personnes en bonne santé contenant moins de 2 ou 3 ng/ml. La reproductibilité est d'environ 1 ng/mil.

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