A REINVESTIGATION OF METHYLGUANIDINE CONCENTRATIONS IN SERA FROM NORMAL AND URAEMIC SUBJECTS

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SUMMARY

1. A cation-exchange-chromatographic method for the determination of methylguanidine in serum is described.

2. In ten normal subjects, the mean serum methylguanidine concentration was 0·055 (SE 0·019) mg/100 ml and in ten uraemic patients it was 0·175 (SE 0·038) mg/100 ml. This difference is significant (P<0·02).

3. Recent claims that methylguanidine is present in uraemic serum in much higher concentrations are shown to be due to artifactual conversion of creatinine when methods involving charcoal chromatography are employed.

4. The results of intoxication experiments, in which blood concentrations of methylguanidine similar to those found by charcoal-chromatographic methods have been reproduced, must be interpreted with caution.

It has long been accepted that accumulation of toxic metabolites is responsible for many of the systemic effects of impaired renal function. However, there is no agreement about the relative importance of the many substances cited.

In recent years attention has turned to the possible role of methylguanidine in the genesis of the 'uraemic syndrome'. It has been claimed (Yatzidis, Oreopoulos, Tsaparas, Voudiclari, Stavroulaki & Zestanakis, 1966; Giovanetti, Biagini & Cioni, 1968a) that this substance is present in the serum of uraemic subjects in much higher concentrations than in normal subjects. Methylguanidine is known to have high acute toxicity (Mason, Resnik, Minot, Rainey, Pilcher & Harrison, 1937) and its chronic subcutaneous administration to normal dogs in amounts sufficient to produce methylguanidine concentrations similar to those reported in humans with severe renal failure (Yatzidis et al., 1966; Giovanetti et al., 1968a), induces a hypercatabolic state, impaired erythrocyte production and haemolysis, a decrease in platelet count, neuropathy, anorexia, vomiting, diarrhoea, gastrointestinal ulceration and haemorrhages, pulmonary oedema, tachycardia and arrhythmias (Giovanetti, Biagini, Balestri, Navalesi, Giagnoni, de Matteis, Ferro-Milone & Perfetti, 1969) as well as impairment.

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in intestinal calcium transport and hypertriglyceridaemia (Balestri, Biagini, Rindi & Giovanetti, 1970). In such concentrations methylguanidine in vitro produces as much haemolysis as is produced by creatinine in concentrations typical of severe renal failure (Giovanetti, Cioni, Balestri & Biagini, 1968b).

A specific method for the measurement of methylguanidine, based on cation-exchange chromatography, has been devised and amounts of this substance in uraemic serum have been found to be lower than those reported by others. Results from further experiments have enabled us to account for this discrepancy.

**MATERIALS AND METHODS**

The following chemicals and reagents were used: Dowex 50 (X 12; 100–200 dry mesh; H+ form) (Sigma Chemical Co.); phosphate buffer, pH 11.3, prepared by mixing 1 vol. of 0.1 M-disodium hydrogen phosphate (May & Baker Ltd), with 1 vol. of 0.05 M-sodium hydroxide; Sakaguchi reagent No. 1, prepared by mixing 9 vol. of 5% (w/v) urea with 1 vol. of 0.25% (w/v) α-naphthol (AnalaR; BDH Chemicals Ltd); Sakaguchi reagent No. 2, prepared by the addition of 0.7 ml of bromine to 100 ml of 5% (w/v) sodium hydroxide; dry activated powdered charcoal (BDH Chemicals Ltd) washed with dilute hydrochloric acid and treated with cyanide as described by Schramm & Primosigh (1943); creatinine and creatine (BDH Chemicals Ltd), L-arginine and 4-guanidinobutyric acid (Sigma Chemical Co.) methylguanidine sulphate (Kodak Ltd, London), guanidinoacetic acid (K and K Labs. Inc.), guanidinosuccinic acid (Mann Research Labs.).

**Method A (present authors)**

Serum (10 ml) was deproteinized by the addition of 2.8 ml of 20% (w/v) trichloroacetic acid. After centrifugation at 3000 rev./min for 30 min the supernatant was diluted to 50 ml with deionized water to decrease ionic strength and added to glass columns (12.5 cm × 1 cm) fitted with taps and containing 1 g of Dowex 50. Elution was carried out with 50 ml of phosphate buffer. Guanidinoacetic acid, guanidinosuccinic acid and guanidinobutyric acid, creatine, creatinine and arginine added to the system were eluted quantitatively. Methylguanidine, which at this stage was completely retained on the resin, was eluted quantitatively with 50 ml of 4 M-NaOH.

The Sakaguchi reaction was carried out by adding to a 5 ml sample of this eluate, 1 ml of Sakaguchi reagent No. 1 followed 1 min later by 1 ml of Sakaguchi reagent No. 2. Samples were read in a spectrophotometer (Unicam SP.500) at 500 nm against a 4 M-NaOH blank set at zero extinction. A standard curve was prepared with fresh solutions of methylguanidine sulphate in 4 M-NaOH (1.67 mg of methylguanidine sulphate in aqueous solution corresponds to 1 mg of methylguanidine base). The concentration of methylguanidine in the serum was then equal to 5 times the concentration in the eluate.

**Method B (after Yatzidis et al., 1966)**

To 2 ml of serum was added 16 ml of 0.415 M-sulphuric acid followed by 2 ml of 10% sodium tungstate. A 10 ml sample of supernatant was then shaken with 500 mg of activated charcoal in the presence of 2.5 ml of 4 M-sodium hydroxide and the mixture was filtered. The filter paper was placed in a flask containing a mixture of 25 ml of 95% (v/v) ethanol plus
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1 ml of concentrated hydrochloric acid and allowed to stand overnight. The supernatant was removed by filtration and the residual charcoal washed with acid–alcohol for about 30 min. The washings were removed by filtration and added to the earlier filtrate. The filtrate plus washings was evaporated to dryness in a rotary evaporator at 55°.

The residue was taken up in 50 ml of deionized water, the aqueous extract was subjected to Dowex 50 chromatography as described above, and the methylguanidine concentration in the eluate was measured.

Method C (after Giovanetti et al., 1968a)

To 10 ml of serum was added 10 ml of 20% (w/v) trichloroacetic acid and the precipitates were removed by filtration. Portions (10 ml) of the filtrates were shaken with 2 g amounts of activated charcoal in the presence of 1·5 ml of 40% (w/v) NaOH. The charcoal was removed by filtration and washed. The filter paper containing the charcoal was dried in an oven at 85° for 30 min, shaken with 50 ml of 95% (v/v) ethanol containing 1 ml of cone. HCl in which it was boiled for a few minutes. The samples were allowed to stand overnight when the alcoholic solution was filtered. A second equal volume of acid–ethanol was added to the charcoal and again removed by filtration. The filtrates were combined.

The ethanolic extract was evaporated to dryness as previously described, the residue was taken up in 50 ml of deionized water and Dowex 50 column chromatography performed in the manner described above.

RESULTS

In twenty experiments, involving elution from the Dowex 50 column only, recoveries of methylguanidine from aqueous solutions at concentrations ranging from 0·5 to 5·0 mg/100 ml were quantitative. The mean recovery of methylguanidine added to normal (twelve

\[ \text{Methylguanidine (mg/100 ml)} \]

\[ \text{Creatinine (mg/100 ml)} \]

Fig. 1. Apparent increase in methylguanidine concentration of uraemic serum to which creatinine has been added in amounts such as to raise the serum concentration by the values shown on the abscissa. The method of Yatzidis et al. (1966) was used for the determinations. The open circle represents the mean value obtained with two aqueous solutions of creatinine.
estimations) and uraemic (twenty estimations) sera in concentrations ranging from 0·5 to 4·0 mg/100 ml was 85%. The range was greater (53–127%) at a concentration of 0·5 mg/100 ml than it was at or above 2 mg/100 ml (92–105%).

The mean serum methylguanidine concentration in ten normal patients was 0·055 mg/100 ml (SE 0·019). In ten uraemic patients (seven men, three women) with mean blood urea 270 (120–430) mg/100 ml the mean serum methylguanidine was 0·175 mg/100 ml (SE 0·038). This difference is significant (0·01 < P < 0·02).

To examine the relative specificities of the three methods, creatinine was added to uraemic serum (serum creatinine 13·7 mg/100 ml) to raise the concentration by amounts ranging from 2·5 to 40 mg/100 ml. Creatine, L-arginine, 4-guanidinobutyric acid, guanidinoacetic acid and guanidinosuccinic acid were similarly added to give concentrations of 20 mg/100 ml. Samples of serum were then divided into three and a sample analysed by each of the three methods.

![Fig. 2. Results of a similar experiment to that described in Fig. 1, but employing the method of Giovanetti et al. (1968a) for the assay.](image)

When creatinine was added to serum, Sakaguchi-positive material, which behaved identically with methylguanidine during cation-exchange chromatography, was generated during the procedures described by Yatzidis et al. (1966) (Method B) (Fig. 1) and by Giovanetti et al. (1968a) (Method C) (Fig. 2). By contrast, the addition of creatinine to serum did not lead to an apparent increase in the concentration of methylguanidine in that serum if measurements were made by the cation-exchange procedure (Method A). Other compounds that were not converted into methylguanidine under any of the procedures used include creatine, L-arginine, guanidinoacetic acid, guanidinobutyric acid and guanidinosuccinic acid.

To study the mechanism of formation of methylguanidine from creatinine, protein-free filtrates of serum to which creatinine compounds had been added were treated with sodium hydroxide or boiled with acid–alcohol, or subjected to both treatments (as in the methods of Yatzidis et al., 1966, and Giovanetti et al., 1968a) in all cases without activated charcoal.
Methylguanidine was then determined by using column chromatography. In no case was the formation of methylguanidine observed. Aqueous solutions of creatinine or creatine, treated similarly, did not generate methylguanidine.

Prior treatment of the charcoal with cyanide (Schramm & Primosigh, 1943) did not alter the extent to which conversion of creatinine into methylguanidine occurred under the catalytic influence of the charcoal; when aqueous solutions of creatinine at a concentration of 20 mg/100 ml were subjected to assay, the mean apparent methylguanidine concentration was 2·5 mg/100 ml when cyanide-treated charcoal was employed and 2·6 mg/100 ml when untreated charcoal was used. These values agree well with those obtained when creatinine was added to uraemic serum (Fig. 1).

**DISCUSSION**

By using a method based on ion-exchange chromatography we have obtained lower methylguanidine concentrations in uraemic sera than those obtained by procedures involving adsorption on to charcoal in alkaline solution and elution with acidic ethanol (Yatzidis et al., 1966). Our ion-exchange method gave a mean value of 0·175 mg/100 ml of uraemic serum, whereas values as high as 4·2 mg/100 ml were obtained by the activated-charcoal procedure.

The original method with charcoal was not claimed to be specific for methylguanidine but was proposed as a method for the measurement of total monosubstituted guanidine bases. However, when Giovanetti et al. (1968a) modified the Yatzidis et al. (1966) procedure by the incorporation of descending paper chromatography to isolate methylguanidine and reported this compound to comprise nearly 90% of the Sakaguchi-positive material in uraemic serum, it appeared that by far the greatest contribution to the values obtained by Yatzidis et al. (1966) was made by methylguanidine. Further, comparison of the chromatographic mobility of the material measured by the Giovanetti procedure in six solvent systems left no reasonable doubt as to its identity.

Methylguanidine concentrations ranging from 0·6 to 3·2 mg/100 ml were obtained in uraemic serum by this procedure (Giovanetti et al., 1968a). It is clear from our results that the higher concentrations of methylguanidine found when procedures involving adsorption on charcoal are employed result from partial conversion of creatinine into the monosubstituted guanidine during separation. It seemed possible that metal ions adsorbed on the charcoal might be responsible for this conversion, since heavy metal ions are known to catalyse the conversion of creatinine (or creatine) into methylguanidine (Ewins, 1916; Baumann & Ingvaldsen, 1918; Greenwald, 1919). However, charcoal that had been treated with cyanide to remove metal ions (Schramm & Primosigh, 1943) was equally effective in catalysing the reactions. Methods that involve charcoal chromatography are unreliable for the measurement of methylguanidine in the presence of creatinine.

Gas–liquid chromatographic procedures have also been employed to measure methylguanidine concentrations in normal and uraemic sera. Values were reported to be approximately the same for the two groups (0·015 mg/100 ml) although recoveries of methylguanidine in control experiments were only 34 ± 16% (Beyermann & Wisser, 1969).

After precipitation of the protein and arginine from normal human serum with Ba(OH)2–ZnSO4 the amount of methylguanidine present in a filtrate was found to correspond to less than 0·02 mg/100 ml of serum (Van Pilsum, Martin, Kitto & Hess, 1956). Corrections for the
adsorption of methylguanidine on the Ba(OH)\textsubscript{2}-ZnSO\textsubscript{4} precipitate which sometimes occurs (Carr & Schloerb, 1960) seem not to have been applied. By using procedures that appear to be relatively specific, Carr & Schloerb (1960) demonstrated that normal and nephrectomized dogs have concentrations of 0·022 and 0·212 mg of methylguanidine/100 ml of serum respectively.

An important role of methylguanidine in the genesis of many of the complications of renal failure has been suggested directly (Giovanetti et al., 1969) or by implication (Giovanetti et al., 1968b) by extrapolation from experiments in which much higher concentrations of methylguanidine have been employed than are now shown to exist. Valid conclusions about the role of methylguanidine in this connection cannot be drawn from such experiments and it seems likely that the importance of this compound had been overestimated.

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REFERENCES


