Journal of Chromatography, 181 (1980) 11—16
Biomedical Applications

CHROMBIO. 436

DETERMINATION OF METHENAMINE IN BIOLOGICAL SAMPLES BY GAS—LIQUID CHROMATOGRAPHY

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(Received June 11th, 1979)

SUMMARY

Methenamine (hexamethylenetetramine), a urinary disinfectant, was determined in human plasma and urine by gas—liquid chromatography with a short (10 m) open-bore glass capillary column (split ratio 1:20) and nitrogen-selective detector. An almost quantitative recovery (92.1%) was achieved by simple dilution of water-containing samples (0.5 ml) with acetone (4.5 ml). After centrifugation an aliquot (2 μl) of the supernatant was injected into the gas chromatograph. Selectivity and sensitivity of the nitrogen detector allowed the quantitation of unchanged methenamine in plasma and urine up to 24 h after a single therapeutic dose of 1 g.

Reproducibility of the method was 7.6 and 2.1% (C.V.) in serum and urine, respectively. The time required for the analysis of one sample was approx. 2 min. Due to the simple extraction and short analysis time it was possible to analyze the samples concurrently with sample taking. Absorption of standard tablets and an enterosoluble preparation of methenamine hippurate was compared.

INTRODUCTION

Methenamine (hexamethylenetetramine), whose structure is shown in Fig. 1, is a long-established urinary disinfectant which is mostly used as a salt of

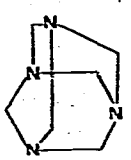

Fig. 1. The chemical structure of methenamine (hexamethylenetetramine).

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hippuric acid. It is especially suitable in the treatment of chronic urinary tract infections [1—4]. Its action is based on formaldehyde, which is liberated at the acidic pH of urine [5—7]. No formaldehyde has been found in tissues other than kidney and urinary tract [8, 9]. The most important advantages of methenamine salts as urinary disinfectants are (1) lack of serious side-effects, and (2) formaldehyde does not give rise to resistant bacterial strains [7, 10].

The determination of methenamine in biological samples has been performed mostly by spectrophotometry after acid hydrolysis by coupling the liberated formaldehyde with chromotropic acid [6], phenylhydrazine [11], or with 2-hydrazinobenzothiazole [12, 13]. Attempts to separate free formaldehyde from complexes with amino acids and proteins have not yielded satisfactory results [2].

In our hands the measurement of liberated formaldehyde was successful in buffered solutions but not in urine. This is possibly due to the reaction of formaldehyde with free amino groups in endogenous compounds in urine. Urinary recovery of formaldehyde was only a few per cent. The spectrophotometric methods lack sufficient sensitivity for measurement of methenamine concentrations in blood. Thus, they are not suitable in bioavailability studies. A new method was therefore developed.

EXPERIMENTAL

Subjects

Methenamine hippurate, as a 500-mg standard tablet or an enterosoluble preparation, was administered in cross-over design to six healthy volunteers (all females) mean age 35 years (range 26—48), mean weight 58 kg (range 47—70) and mean height 162 cm (range 154—170). Blood samples were collected from the cubital vein at 1-h intervals up to 10 h and at 12 and 24 h. Serum was separated as soon as possible. Urine samples, voided every 2 h, were also collected. After measurement of the volume, a 0.5-ml aliquot was separated. Extraction and analysis of the samples were performed immediately.

Apparatus

A Hewlett-Packard 5840A gas chromatograph and integrator terminal equipped with a dual flameless nitrogen-selective detector and open-bore glass capillary column (I.D. 0.3 mm, O.D. 0.9 mm) coated with a 0.23 μm thick film of OV-17, length 10 m, was used. The glass capillary column was prepared, filled and installed by Markku Reunanen and Mikko Murola in Åbo Academy, Turku, Finland. Make-up gas (nitrogen) with a flow-rate of 30 ml/min was passed to the detector. Hydrogen and air flow-rates were 3.0 ml/min and 50 ml/min, respectively. The split ratio for carrier gas (nitrogen) was 1:20. Carrier gas flow-rate through the capillary column was 1.0—1.2 ml/min. All gases were of standard purity and were purchased from AGA, Helsinki, Finland. Injector, oven and detector temperatures were 240°, 150° and 250°, respectively.

Quantitation of the integrated peaks was performed by internal standardization.

Reagents

Methenamine hippurate (synthesized by Leiras, Turku, Finland) and *p*-acetylbenzonitrile, used as internal standard (Aldrich-Europe, Janssen Pharmaceutica, Beerse, Belgium) were used without further purification. $NaHCO_3$ and acetone, both analytical grade, were obtained from Merck, Darmstadt, G.F.R.

Preparation of standard solutions

Standard solutions of methenamine were prepared by dissolving 100 mg of methenamine hippurate in acetone (100 ml). This solution was diluted with acetone to correspond to 0, 0.6, 2, 8 and 20 μg of methenamine hippurate per ml for serum samples, and 50, 100, 200 and 400 μg of methenamine hippurate per ml for urine samples. Internal standard was dissolved in acetone for use as extraction solutions at final concentrations of 5 and 100 μg/ml for serum and urine samples, respectively.

Analytical method

Serum, 0.5 ml, was shaken for 1 min with 4.5 ml of the extraction solution. After centrifugation about 500 μl were transferred to a clean test tube from which a 2-μl sample was injected into the gas chromatograph. Urine samples, 0.5 ml, were treated similarly except that the sample was made slightly alkaline by adding about 0.5 g of solid $NaHCO_3$ before extraction (to avoid hydrolysis of methenamine to formaldehyde at the acidic pH of the urine) and the extraction solution was used with the higher concentration of internal standard.

RESULTS

Precision of the analysis was studied by injecting identical samples fourteen times repeatedly into the gas chromatograph. The concentration of methenamine corresponded to 5 μg/ml (serum) and 250 μg/ml (urine). The concentrations of the internal standard were 2.5 and 500 μg/ml, respectively. The ratio of peak areas of sample to internal standard was calculated. The results, expressed as coefficient of variation (standard deviation/mean) × 100, are presented in Table I.

TABLE I

PRECISION, REPRODUCIBILITY AND RECOVERY OF METHENAMINE DETERMINATION IN SERUM AND URINE

For further details see text.

	Precision		Reproducibility		Recovery		
	C.V.	*n*	C.V.	*n*	$\bar{x}$ (%)	S.D.	*n*
Serum	4.2	14	7.6	9	93.1	5.3	12
Urine	1.1	14	2.1	9	91.2	1.8	12

Reproducibility was estimated by preparing and extracting nine identical samples from serum and urine. The concentrations were similar to the precision test. The results are presented in Table I. No day-to-day variation was calculated because the samples were analyzed immediately.

Recovery of the method was tested at concentrations of 10, 20 and 30 μg/ml in serum and at 100, 200 and 500 μg/ml in urine. The amounts of internal standard were 5 μg/ml (serum) and 250 μg/ml (urine). Samples without extraction (acetone solutions) were compared with extracted ones. Recovery per cent (mean 92.1) was similar in serum and urine, as shown in Table I, and was not dependent on methenamine concentration.

Chromatograms for serum and urine samples appear in Fig. 2A and B. Retention times of methenamine and internal standard were about 0.7 and 1.3 min, respectively. The standard curve was linear over the concentration range required in this study. Absorption curves of methenamine in one volunteer after a standard and an enterosoluble tablet following a single dose of 1 g are presented in Fig. 3. The urinary excretion of methenamine in the same volunteer after the same dose is illustrated in Table II.

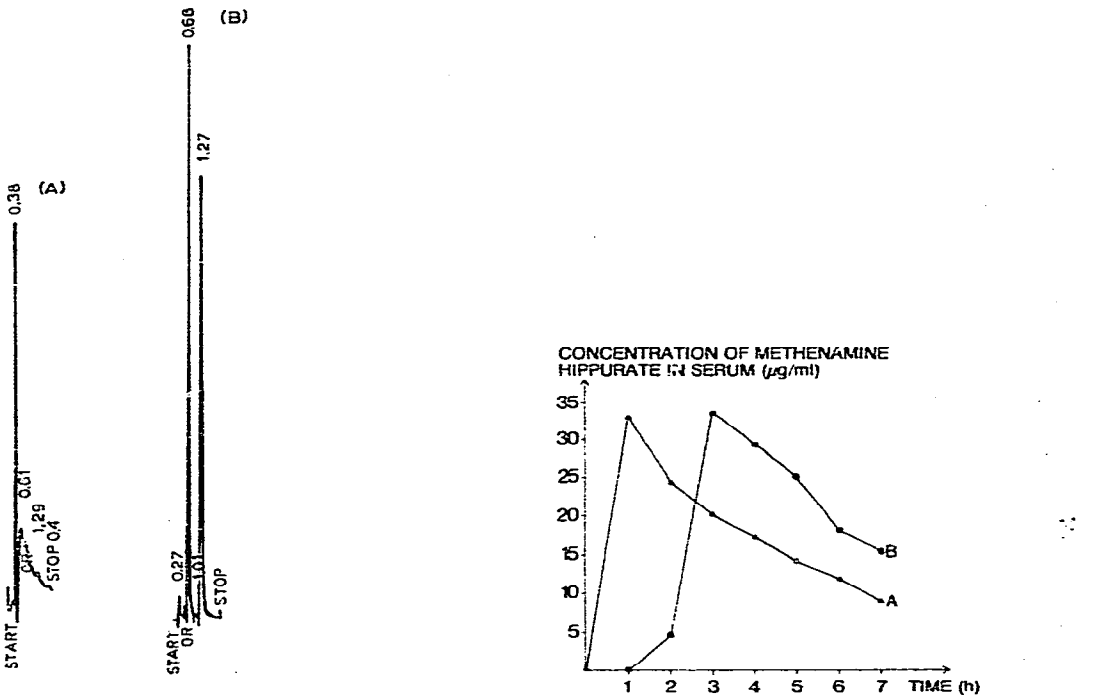

Fig. 2. Typical chromatograms of serum without methenamine and internal standard (A), and chromatogram of urine sample (B). Retention times of methenamine and internal standard were about 0.7 and 1.3 min, respectively.

Fig. 3. Typical absorption curve of methenamine for standard (A) and enterosoluble (B) tablets following a single dose of 1 g.

TABLE II

CUMULATIVE URINARY EXCRETION OF METHENAMINE IN ONE VOLUNTEER AFTER A SINGLE ORAL DOSE OF 1 g

Time of urine collection (h)	Amount excreted (mg)	
	Standard	Enterosoluble
0	0	0
0—2	289.0	65.0
2—4	477.5	266.0
4—6	594.8	546.7
6—8	654.7	706.5
8—10	707.2	841.1
10—12	740.8	892.2
12—14	767.7	945.2

DISCUSSION

Determination of methenamine in human samples has been a difficult problem. So far no reports of its concentrations in human serum have been reported. Even in the urine, where the concentrations are high, unchanged methenamine has not been measured. It has been cleaved by acid hydrolysis to formaldehyde, which has been quantitated by spectrophotometry after coupling with coloured complexes [6, 11—14]. The special difficulties of methenamine determination are (a) its lability in both acidic and alkaline conditions, (b) its hydrophilic nature, which seems to make its extraction from water-containing biological samples to water-insoluble organic solvents impossible, (c) rapid cleavage of the ring system if evaporation (concentration) is attempted, (d) its instability if an attempt is made to remove water from the samples (drying), and (e) the samples cannot be stored. For these reasons the extraction of methenamine cannot be made by the conventional method, that is extracting with organic solvent at a suitable pH, concentrating the sample by evaporation, drying (if necessary) and dissolving the sample in a small volume of solvent which is then analyzed by gas—liquid chromatography (GLC).

We failed in our attempts to concentrate the samples or to remove the traces of water by inorganic salts. The sample was therefore mixed with acetone and analyzed directly by GLC. The diluted sample contains 10% of water. This did not seem to disturb the function of either the glass capillary column or detector — possibly due to very small amounts of water entering the glass capillary column after the 1:20 split.

Methenamine is thermally stable in GLC analysis — at least it gives a single symmetrical peak in an OV-17 column as shown by Strom and Jun [15]. The method with a packed column and flame ionization detection was, however, used only for pharmaceutical purposes. Methenamine with four nitrogen atoms in the molecule (—NH groups) gives an intense response in the nitrogen-selective detector. It was therefore reasonable to use this detector in analyzing methenamine. The sensitivity of the detector proved to be sufficient for diluted serum samples stored for up to 24 h.

Most determinations of methenamine in human samples have been performed as urinary formaldehyde by spectrophotometry [6, 11—14]. Polar-

cgraphy [16], bioautographic visualization [17], refraction—extraction [18] and volumetric methods [19] have also been used. These methods are not suitable for the determination of methenamine in serum samples, because formaldehyde is liberated only in the urinary tract and not in serum.

The present method was applied to a bioavailability study of methenamine hippurate from various preparations marketed in Finland. A single dose of 1000 mg of methenamine hippurate was administered. Absorption curves of standard and enterosoluble tablets show that C_{max} is achieved later following administration of the enterosoluble preparation. The bioavailability seems to be the same for both preparations.

Our method does not measure the biologically active form of methenamine (= formaldehyde). Analysis of free formaldehyde is, however, as a whole a different problem and is not discussed in this paper. Measurement of total urinary formaldehyde after acid hydrolysis offers no advantages over the present method; on the contrary, in our hands the spectrophotometric methods suffered from poor sensitivity and poor recovery (10—20%), possibly due to reactions of formaldehyde with free amino groups in endogenous urinary compounds.

Details of the pharmacokinetics of methenamine in human serum and urine will be published elsewhere.

CONCLUSION

The present method allows the rapid and reliable measurement of methenamine in human serum and urine. It is very suitable in pharmacokinetic studies of methenamine.

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