Journal of Chromatography, 274 (1983) 27—35
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1619

HEAD-SPACE GAS—LIQUID CHROMATOGRAPHY FOR THE RAPID LABORATORY DIAGNOSIS OF URINARY TRACT INFECTIONS CAUSED BY ENTEROBACTERIA

N.J. HAYWARD

Bacteriology Laboratory, Alfred Hospital, Prahran 3181, Victoria (Australia)

(First received October 11th, 1982; revised manuscript received December 28th, 1982)

SUMMARY

Urine specimens were analysed in parallel in a hospital laboratory by routine methods which were regarded as the standard of correct diagnosis and by the rapid test already developed in a research laboratory. Technical modifications made to the rapid test ensured that its results agreed with those from routine methods and increased its rapidity so that a result was possible 4 h after receipt of the specimen. When 382 urine specimens were analysed by the modified test which is described in detail, there were neither false negatives nor false positives for infections with *Escherichia, Klebsiella, Citrobacter* or *Proteus* species.

INTRODUCTION

A test using head-space gas—liquid chromatography (HS-GLC) has been proposed for the rapid detection of bacteria in urine specimens [1]. The test depends on the production of ethanol from lactose or arabinose by *Escherichia coli* and some related species, and of methyl mercaptan from methionine by *Proteus* spp. Methyl mercaptan oxidizes spontaneously to dimethyl disulphide. Ethanol, methyl mercaptan and dimethyl disulphide appear early in growth and can be detected in one HS-GLC analysis. When the rapid test was applied in a research laboratory to urine specimens from patients, both *E. coli* and *Proteus* spp. were detected in specimens cultured in lactose methionine medium which was incubated and analysed after 3 and 5 h [2]. This was confirmed by Coloe [3] who used an arabinose methionine medium.

This paper reports technical modifications that were made to the rapid test when urine specimens were analysed in parallel by the rapid test and by routine methods in a hospital laboratory. Three trials were done in sequence, difficulties in the earlier trials being overcome in the final trial.

0378-4347/83/$03.00

EXPERIMENTAL

Gas chromatography and mass spectrometry

In HS-GLC the liquid to be analysed for volatile compounds is sealed in a container and the supernatant vapour (HS) is injected into the gas chromatograph. In the present investigation vapour pressures of volatile compounds in HS were increased by adding potassium carbonate to liquids for analysis (salting-out) and by heating the sealed containers to 60°C. Potassium carbonate salts out neutral and alkaline compounds into the HS. To prepare HS samples, 2 ml of each liquid for analysis were added to 3 g of potassium carbonate in a glass vial provided with the automatic head-space injector, sealed immediately, shaken on a Vortex mixer to disperse the salt in the liquid and held in the injector turntable at 60°C for injection.

The gas chromatograph was a Perkin-Elmer F45 with an automatic head-space injector, a flame ionization detector and a stainless-steel column, 2 m × 3 mm, packed with 0.4% Carbowax 1500 on graphite 60—80 mesh. The injector needle temperature was 150°C, the oven temperature 115°C and the injector and detector temperatures 140°C. Nitrogen carrier gas pressure was 180 kPa, hydrogen 400 kPa and air 380 kPa. The injection time was 3 sec and the analysis time 1.8 min.

Aqueous solutions of ethanol that had been redistilled over potassium hydroxide to constant boiling point, dimethyl disulphide (Eastman Organic Chemicals, New York, NY, U.S.A.), and S-methylthiourea which yielded methyl mercaptan were added to potassium carbonate in glass vials and analysed with each day's samples to check retention times for the identification of products from urine cultures.

The concentrations of ethanol, dimethyl disulphide and methyl mercaptan in urine cultures necessary for a positive response against the background of noise due to volatile compounds in the urine culture medium and urine specimens were 0.00022 *M*, 0.00048 *M* and 0.0024 *M*, respectively.

The mass spectrometer was a 30-cm radius 60° magnetic deflection instrument coupled to the gas chromatograph by a Watson—Bieman frit [4].

Specimens of urine

Specimens of urine (382) were refrigerated after voiding and later analysed by the HS-GLC rapid test in parallel with the routine laboratory test.

Method of HS-GLC rapid test

A basal methionine yeast-extract peptone medium was prepared by dissolving 37.5 g L-methionine (BDH), 25 g proteose peptone (Difco) and 7.5 g yeast extract (Oxoid) in 1 l 0.25 *M* sodium phosphate buffer, pH 7.2, dispensing in presterilized screw-capped bottles of 28-ml capacity, autoclaving at 115°C for 15 min and storing at 4°C. A 10% aqueous solution of L-arabinose was sterilized by membrane filtration and stored at 4°C. Within 24 h of use, medium for the HS-GLC rapid test was prepared by adding one part of arabinose solution to four parts of basal medium.

Urine cultures were inoculated in duplicate by mixing 1.1 ml of the urine specimen with 1.1 ml medium for the HS-GLC rapid test in screw-capped

bottles of 28-ml capacity. These volumes provided 2 ml of culture for HS-GLC analysis. The final concentrations of the ingredients were 1% (0.07 *M*) L-arabinose, 1.5% (0.1 *M*) L-methionine, 1% peptone, 0.3% yeast extract, 0.1 *M* phosphate buffer and 50% urine. Cultures were incubated for 3.5 h. Two degrees of aeration were provided, lower aeration in an unshaken culture sloping at an angle of 12° from the horizontal, and higher aeration in a culture standing in a shaker operating at 200 horizontal 20-mm oscillations per min.

Medium for the HS-GLC rapid test, diluted with an equal volume of sterile water, was incubated and analysed with each day's urine cultures. This analysis was to detect volatile substances originally present in the medium (medium blank). A 1-ml aliquot of each urine specimen was diluted with 1 ml water and analysed without incubation. These analyses were to detect volatile substances originally present in the urine specimens (non-incubated urine).

Method of routine laboratory analysis

MacConkey agar without salt was prepared as directed by the manufacturer (Oxoid). Firm blood agar [5] contained 6% equine blood and 3% agar (Oxoid) in a nutrient broth base. For the routine laboratory analysis of urine, the number of colony-forming units (CFU) per ml urine was determined on MacConkey agar and on firm blood agar after incubation for 18—24 h at 37°C. If more than one colony type was distinguished, each was counted and recorded separately. If the count for an organism was significant, that is $\geqslant 10^5$ CFU per ml urine [6], it was isolated and identified by standard methods [7] taking at least another 18—24 h. Species of the genera *Escherichia*, *Klebsiella* and *Citrobacter*, all of which were detected by ethanol production from arabinose in the HS-GLC analysis, are grouped together for the purposes of this paper and called "coliforms".

RESULTS

Technique of HS-GLC analysis

For the final trial, modifications were made to the technique of the earlier trials. The analysis time was reduced and operator time was saved so that results could be reported earlier. The accuracy of analysis was improved and the volume of urine specimen required was reduced.

Analysis time. The column was shorter and contained a lower concentration of the stationary phase on a coarser support, and the oven temperature was higher than in previous investigations. Consequently retention times (t_R) were short, 0.275 min, 0.325 min and 1.7 min for methyl mercaptan, ethanol and dimethyl disulphide, respectively. The analysis time of 1.8 min, compared with 2.5 min in earlier trials, was a significant saving when 50 or more analyses were to be carried out in a hospital laboratory. The increased throughput of analyses enabled a larger number of urine specimens to be tested in a given time and the results of all of them to be reported earlier. No further peaks eluted after the analysis time of 1.8 min.

Operator time. Proportionately more potassium carbonate was used to

prepare HS compared with previous investigations, sufficient to saturate the solution. This ensured that the solutions were always at the same concentration without the time-consuming need for a high degree of accuracy in weighing potassium carbonate.

The automatic HS injector was important in saving operator time. After the turntable had been loaded with sample vials, chromatograms were interpreted by the operator while analyses were proceeding and results could be reported earlier than with manual injection in which the operator could not start interpreting chromatograms until the last sample had been injected.

When manual injection was used it was difficult to detect partial blockage of the syringe needle impeding the passage of vapour from HS to the barrel of the syringe. This led to errors and duplicate analyses were essential. By contrast, results from automatic injection were highly reproducible and only one analysis was done on each culture. This halved operator time in preparation of culture medium, inoculation of cultures, and analysis of cultures by HS-GLC.

Precision. Automatic recording of times of injection enabled the t_R of each volatile compound in a chromatogram to be measured with a high degree of precision. The identification of these volatile compounds was therefore made with more confidence than was possible with manual injections.

When a 1-m column was used in preliminary work before the start of the final trial, the t_R of a compound that was expected to be methyl mercaptan was noted to be 0.025 min longer than the t_R of the known sample of methyl mercaptan. This small difference would not have been detected with manual injection. Analysis by mass spectrometry (MS) showed that the 1-m column had failed to separate two compounds, methyl mercaptan and trimethylamine. The mass spectrum of trimethylamine has distinctive peaks, at *m/e* 58, 42, 59 and 30 in order of intensity, which were clearly distinguishable from methyl mercaptan in the mass spectrum of the composite peak. The two compounds were separated from each other and from ethanol with the 2-m column.

Ethanol, methyl mercaptan and dimethyl disulphide, the volatile products that indicated coliforms and *Proteus* spp., were previously identified by MS [1—3,8].

Volume of urine specimen. In the preparation of HS for each analysis, only a small volume of urine culture and therefore of urine specimen was required. This is an advantage because patients with urinary tract infections often suffer from frequency or oliguria and are unable to provide large specimens for diagnostic tests. A further halving of the volume of urine specimen required for each analysis was achieved because results were highly reproducible (see above) and only one analysis was required on each culture.

The volume of urine required for the HS-GLC rapid test was 3.2 ml, 1 ml for the analysis of nonincubated urine and 2.2 ml for the urine cultures.

Technique of culture of urine specimens

Modifications were made to improve agreement with routine methods which were regarded as the standard of correct diagnosis. Results from HS-GLC rapid tests of urine specimens in the final trial (Table I) were divided into Groups 1 to 4 on the basis of routine results. To arrive at the HS-GLC results, the chromatograms from urine cultures (viz. Figs. 1 and 2) were interpreted in

TABLE I

COMPARISON BETWEEN RESULTS OF ROUTINE LABORATORY AND HS-GLC RAPID TESTS OF URINE

Sig. = Significant, i.e. $\geqslant 10^5$ colony-forming units (CFU) per ml urine.

Group	Routine laboratory result	No. of specimens	HS-GLC rapid test				
			Aeration of culture	HS-GLC result			
				Sig. coliform	Sig. *Proteus*	Sig. coliform + *Proteus*	Not sig. coliform or *Proteus*
1	Sig. coliform	111	Unshaken	111	0	0	0
	10^4 CFU coliform per ml urine	14	Unshaken	0	0	0	14
2	Sig. *Proteus*	22	Shaken	0	22	0	0
3	Sig. coliform + *Proteus*	11	One unshaken and one shaken	0	4	7	0
4	Not sig. coliform or *Proteus*	224	One unshaken and one shaken	0	0	0	224

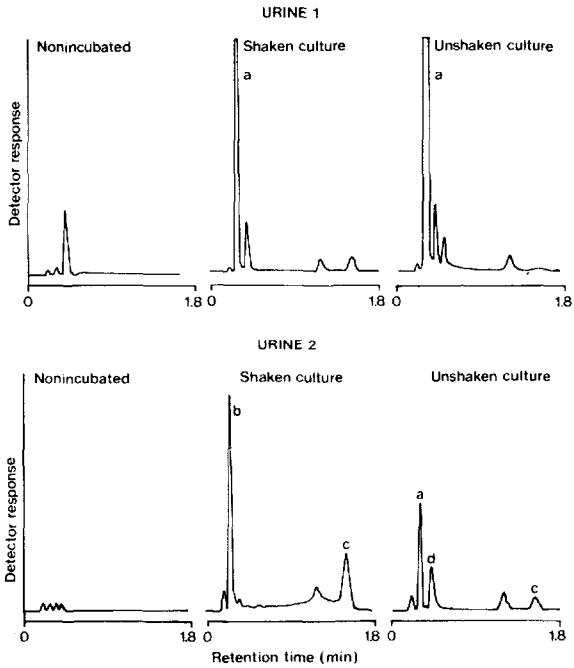

Fig. 1. Chromatograms of urine specimens diluted with an equal volume of water (nonincubated) or diluted with an equal volume of medium for the HS-GLC rapid test and incubated either shaken or unshaken for 3.5 h. Urine 1 contained $>10^5$ CFU *E. coli* per ml. Urine 2 contained $>10^5$ CFU *P. mirabilis* per ml. Peaks: a = ethanol, b = methyl mercaptan, c = dimethyl disulphide, d = trimethylamine.

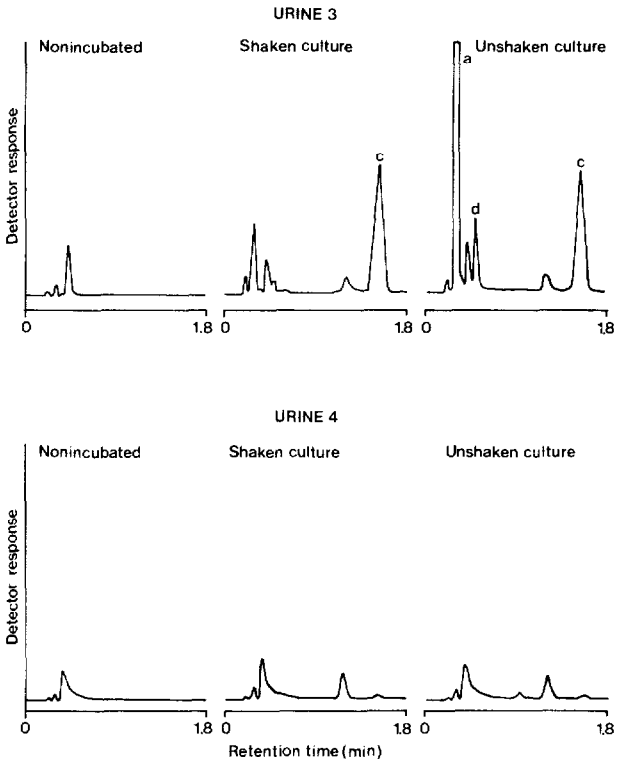

Fig. 2. Chromatograms of urine specimens diluted with an equal volume of water (nonincubated) or diluted with an equal volume of medium for the HS-GLC rapid test and incubated either shaken or unshaken for 3.5 h. Urine 3 contained $>10^5$ CFU *E. coli*, $>10^5$ CFU *P. vulgaris* and $>10^5$ CFU *P. morganii* per ml. Urine 4 did not contain $>10^5$ CFU per ml of any microorganism. Peaks: a = ethanol, c = dimethyl disulphide, d = trimethylamine.

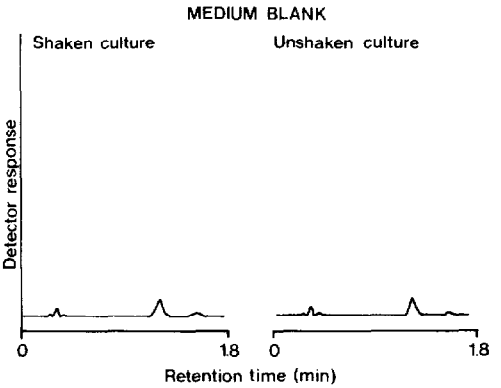

Fig. 3. Chromatograms of medium for the HS-GLC rapid test diluted with an equal volume of water and incubated either shaken or unshaken for 3.5 h.

the light of the chromatograms of the medium blank (viz. Fig. 3) and of non-incubated urine specimens (viz. Figs. 1 and 2).

Effect of period of incubation of urine cultures on detection of significant numbers of coliforms (Group 1). The incubation period set the quantitative

parameter of the HS-GLC test. In an early trial it was 3 h. Ethanol was detected by HS-GLC only in unshaken cultures of urine specimens with higher counts and not in cultures of specimens with counts at or near 10^5 CFU coliforms per ml urine. When the incubation period was increased to 4 h, ethanol was detected in unshaken cultures of all urine specimens containing 10^5 CFU coliforms per ml urine but also in cultures of specimens containing 10^4 CFU coliforms per ml urine, a count that is not generally regarded as significant.

In the final trial the incubation period was 3.5 h. Ethanol was detected in unshaken cultures of all 111 urine specimens containing $\geqslant 10^5$ CFU coliforms per ml urine (viz. Urine 1, Fig. 1). The 111 coliforms included 84 *E. coli*, 7 A—D group, 10 *K. aerogenes*, 2 *K. oxytoca*, 5 *C. freundii* and 3 *C. koseri*. Ethanol was not detected in cultures of 14 urine specimens containing 10^4 CFU coliforms per ml urine.

The incubation period of 3.5 h was suitable for the detection of *Proteus* spp. (Group 2).

Effect of aeration of urine cultures on detection of significant numbers of Proteus spp. (Group 2). Oxygen promoted the production of dimethyl disulphide by *Proteus* spp. After incubation for 3.5 h, dimethyl disulphide and sometimes also methyl mercaptan, was detected by HS-GLC in shaken cultures of all 22 urine specimens containing $\geqslant 10^5$ CFU *Proteus* spp. per ml (viz. Urine 2, Fig. 1). The 22 *Proteus* spp. included 17 *P. mirabilis*, 1 *P. vulgaris*, 2 *P. morganii* and 2 *P. stuartii*. The yield of dimethyl disulphide was usually small in the unshaken culture, despite being incubated at a slope to increase its surface area and consequently its aeration. By contrast, coliforms produced higher yields of ethanol in unshaken culture (viz. Urine 1, Fig. 1). It was concluded that two cultures of each urine specimen were necessary, one shaken and the other unshaken.

The detection of trimethylamine in most unshaken cultures of urine specimens containing significant numbers of *Proteus* spp. was a new observation. It was usually associated with a small ethanol peak.

Detection of significant numbers of both coliform and Proteus spp. occurring together in urine specimens (Group 3). After incubation for 3.5 h, ethanol was detected by HS-GLC in unshaken cultures and dimethyl disulphide in shaken cultures of 7 out of 11 urine specimens containing $\geqslant 10^5$ CFU coliform and *Proteus* spp. per ml urine (viz. Urine 3, Fig. 2). Out of the 7 urine specimens, 4 contained *E. coli* and *P. mirabilis*, 2 contained *K. aerogenes* and either *P. mirabilis* or *P. morganii*, and 1 contained *E. coli*, *P. vulgaris* and *P. morganii*. Ethanol was not detected in unshaken cultures but dimethyl disulphide was detected in shaken cultures of the remaining 4 polymicrobic specimens, 2 containing *E. coli* and *P. mirabilis*, 1 *K. oxytoca* and *P. mirabilis*, and 1 *E. coli* and *P. vulgaris*.

Results of rapid test on urine specimens that did not contain significant numbers of either a coliform or a Proteus sp. (Group 4). Neither ethanol nor dimethyl disulphide was detected by HS-GLC in 188 cultures of urine specimens that did not contain significant numbers of any organism (viz. Urine 4, Fig. 2) nor in 36 cultures of urine specimens containing significant numbers of an organism not detected by the HS-GLC rapid test. The organisms included *Staphylococcus epidermidis* (9), *Streptococcus faecalis* (7), *Pseudomonas*

aeruginosa (11), *Candida* sp. (3), *S. aureus* (1), *Serratia marcescens* (2), *P. aeruginosa* and *S. faecalis* (2), and *S. epidermidis* and *S. faecalis* (1).

Summary of results

The HS-GLC rapid test gave results that agreed with those from routine methods in the hospital laboratory. All urine specimens containing significant numbers of either a coliform or a *Proteus* sp. were detected. All polymicrobic urine specimens containing both a coliform and a *Proteus* sp. were detected as infections, both organisms being found in the majority of them. Thus the test did not yield any false negative result for urine specimens containing a coliform or a *Proteus* sp.

All urine specimens containing no organism in significant numbers or an organism that was neither a coliform nor a *Proteus* sp. were negative. Thus the test did not yield any false positive results.

DISCUSSION

The laboratory diagnosis of urinary tract infections requires both a quantitative and a qualitative microbiological analysis of urine specimens. Almost all urine specimens contain contaminating microorganisms added during voiding in addition to pathogenic microorganisms from the bladder. The contaminants and the pathogens are generally the same species and the distinction between them is quantitative. Contaminants should not exceed 10^4 CFU per ml urine were thought to be significant in a particular patient, for example a patient receiving antibiotic therapy, this lower concentration of bacteria could be detected by incubating the urine culture longer than 3.5 h.
urine cultures for the HS-GLC test. This was long enough to detect *Escherichia*, *Klebsiella*, *Citrobacter* and *Proteus* spp. in significant numbers. If $<10^5$ CFU per ml urine were thought to be significant in a particular patient, for example a patient receiving antibiotic therapy, this lower concentration of bacteria could be detected by incubating the urine culture longer than 3.5 h.

Analysis of urine cultures by HS-GLC separated specific chemical markers for the commonest causes of urinary tract infections. In this way the HS-GLC test was qualitative and pointed to the identity of the infecting bacterium on the day the specimen was received in the laboratory instead of the next day, the time required for routine laboratory results.

Several factors contributed to the rapidity of the HS-GLC test. Chemical analysis by GLC is very sensitive and when used to detect bacteria by analysis for their metabolic products it was possible to detect them in the earliest stages of growth of cultures of urine specimens. The advantage of this short period of incubation was reinforced by the rapid preparation of urine cultures. Coloe [3] used the centrifuged sediment from 60 ml of urine for each culture. This step causes a delay of about 20 min. In the present investigation whole urine was simply mixed with culture medium, a step that took less than 1 min before incubation of the cultures could commence. The use of HS (in preference to other kinds of sample such as solvent extracts) for GLC analysis of the culture after incubation further ensured the rapidity of the whole test. The preparation of HS samples was simple and compounds in HS were few and highly

volatile so that the GLC analysis time was short. Consequently bacteria could be detected by HS-GLC in a given urine specimen within 4 h. Urine specimens are among the most numerous processed in diagnostic microbiological laboratories. A gas chromatograph with an automatic head-space injector was invaluable for the early reporting of large numbers of analyses of urine cultures done in sequence on a single gas chromatograph.

It has appeared possible that a sloping unshaken culture might provide a level of aeration that would permit the detection of both coliforms and *Proteus* spp. in a single culture and obviate the need for two cultures from each urine specimen. However the detection of *Proteus* spp. was often uncertain in the unshaken culture. A shaken culture is necessary if methyl mercaptan production from methionine is the marker for *Proteus* spp. The observation that trimethylamine was produced by *Proteus* spp. in unshaken cultures merits further investigation because this marker might allow the detection of both coliforms and *Proteus* spp. in a single unshaken culture.

In this hospital laboratory trial of the HS-GLC rapid test the theoretical concept on which it is based [1] was fully supported. With technical modications it was rapid and reliable for the diagnosis of urinary tract infections caused by enterobacteria.

ACKNOWLEDGEMENTS

My sincere thanks are due to Dr. Valerie Asche, Queen Victoria Medical Centre and Mr. Reginald Butcher, Austin Hospital for providing urine specimens from their patients, to Dr. A.J.C. Nicholson, C.S.I.R.O. Division of Chemical Physics for mass spectral analyses, to the Alfred Hospital Wholetime Medical Specialists' Trust Fund for buying the Perkin-Elmer gas chromatograph with automatic head-space injector, and to the Alfred Hospital Medical Research Fund for a personal grant.

REFERENCES

1 N.J. Hayward, T.H. Jeavons, A.J.C. Nicholson and A.G. Thornton, J. Clin. Microbiol., 6 (1977) 195.
2 N.J. Hayward and T.H. Jeavons, J. Clin. Microbiol., 6 (1977) 202.
3 P.J. Coloe, J. Clin. Pathol., 31 (1978) 365.
4 W. Stark, J.F. Smith and D.A. Forss, J. Dairy Res., 34 (1967) 123.
5 N.J. Hayward, G.M. Incledon and J.E. Spragg, J. Med. Microbiol., 11 (1978) 155.
6 E.H. Kass, Trans. Assoc. Amer. Physicians, 69 (1956) 56.
7 S.T. Cowan, Cowan and Steel's Manual for the identification of medical bacteria, Cambridge University Press, London, 2nd ed., 1974.
8 N.J. Hayward, T.H. Jeavons, A.J.C. Nicholson and A.G. Thornton, J. Clin. Microbiol., 6 (1977) 187.