Gas liquid chromatography for detection of bacteriuria: examination for volatile acidic and neutral compounds

ELAINE BARRETT, G. LYNAM, AND SHEILA TRUSTEY

From the Division of Hospital Infection, Clinical Research Centre and Northwick Park Hospital, Harrow, Middlesex, HA1 3UJ, UK

SUMMARY Direct injection of clinically infected urines on porous-polymer columns was investigated to determine which microbial metabolites were consistently detectable, and whether their presence could be used as a reliable index of infection. Chromosorb 101 was found to be the most suitable porous polymer for the detection of microbial metabolites; greater sensitivity of detection was achieved by partial purification of the urine before injection. Acetic acid was the only compound found consistently and it enabled 10^6 microorganisms per ml to be detected in urine. However, as urinary tract infection is diagnosed by the presence of 10^5 organisms or more per ml, our method is insufficiently sensitive for the detection of bacteriuria. *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella* spp, *Proteus* spp, *Staphylococcus albus*, and *Streptococcus faecalis* were detectable by our method but *Pseudomonas aeruginosa* and *Candida albicans* were not.

Screening of urine for the presence of significant numbers of microorganisms contributes considerably to the workload of a department of diagnostic microbiology. A diagnosis of urinary tract infection is indicated by a simple viable count revealing the presence of $>10^5$ microorganisms per ml of urine, and a report is sent after further tests for identification and antibiotic sensitivity of the causative agent. Because many specimens are received containing insignificant numbers of microorganisms, the viable count is time-consuming and expensive of materials and technician time. It would be valuable if a rapid screening procedure could be devised to select the few specimens requiring further work. A suitable method should be easily automated and sufficiently sensitive to detect 10^4 microorganisms per ml, thus ensuring reliable selection of all positive specimens.

Our approach was to use gas-liquid chromatography (GLC) to detect microbial metabolites in urine. The potential use of GLC as a diagnostic aid in clinical microbiology has been indicated by other workers, in particular, Mitruka *et al.* (1972), and it has also been demonstrated that the technique can be readily automated for the detection of microbial metabolites (Brooks *et al.*, 1973). We considered

that volatile acidic and neutral compounds of low molecular weight, such as the lower fatty acids and alcohols, would be among the main detectable substances produced in urine by the microorganisms responsible for urinary tract infection; these substances can be easily detected in culture media by injection of an aqueous solution on columns packed with porous polymers (Cecchini and O'Brien, 1968; Rogosa and Love, 1968; Yoshioka *et al.*, 1969; Henkel, 1971).

Material and methods

GAS-LIQUID CHROMATOGRAPHY

A Pye series 104 gas chromatograph with heated dual flame-ionisation detectors was used. The instrument was operated isothermally as a single-channel instrument with glass columns of 1.5 m length and 4 mm internal diameter.

Column packings

Chromosorb 105; Chromosorb 101; Poropak P; Poropak Q; Poropak N; Poropak T. All were 80-100 mesh (Phase Separations Ltd, Clwyd).

Gases

The carrier gas was nitrogen, at a flow rate of 50 ml per minute. Hydrogen and compressed air were operated with optimised flow rates.

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Improvement of chromatographic technique

This was applied in all but the preliminary experiments. Ghosting was reduced to a minimal level, enabling reproducible results to be obtained by:

- i 1 μ l on-column injection and rigorous syringe cleaning;
- ii treatment of column glass with dilute hydrochloric acid after cleaning;
- iii use of Teflon wool for column plugs;
- iv thorough washing of porous polymers with acetone, methanol, and water before packing.

We found the last procedure to be the most significant in limiting ghosting. Other workers have advocated the incorporation of formic acid vapour in the carrier gas for this purpose (Geddes and Gilmour, 1970; Ackman, 1972; Van Eenaeme *et al.*, 1974). However, at the concentration required to limit ghosting, the use of fractionated formic acid to generate formic acid vapour so increased the noise and standing current on the detector that the use of the gas chromatograph was restricted to unacceptably high levels of attenuation.

MICROORGANISMS

All the microorganisms were isolated from patients with urinary tract infection. *Candida albicans*, *Escherichia coli*, *Klebsiella* spp, *Pseudomonas aeruginosa*, *Proteus* spp, *Staphylococcus albus*, and *Streptococcus faecalis* were obtained from the Department of Microbiology at Northwick Park Hospital, and *Enterobacter cloacae* from the Salmonella and Shigella Reference Laboratory, Colindale, London.

URINE

Clinical urine specimens were obtained from the Department of Microbiology at Northwick Park Hospital. They were considered to be infected when 10^5 microorganisms per ml were present, as estimated by a standard-loop technique. Normal urine specimens were obtained from healthy laboratory staff.

'Cleaning up' of urine

Five millilitres of urine was treated before injection by removal of:

- i cells and microorganisms by pressure filtration through a membrane filter, pore size 0.22 μ m (Millipore Corporation);
- ii high-molecular-weight compounds such as proteins by pressure filtration through an ultrafiltration membrane (Millipore Corporation);
- iii cations by shaking with cation-exchange resin AG 50W-X4 in the hydrogen form, 200-400 mesh (Bio-Rad Laboratories). This treatment also ensured the liberation of the acids as free acids at pH 1.

Concentration of urine

Urines 'cleaned up', as described above, were concentrated 20-fold by slow evaporation at 60°C under a stream of nitrogen. The acid components were preserved by prior conversion to their sodium salts with 10 M sodium hydroxide, and before injection the concentrates were acidified with 8 M hydrochloric acid to liberate the free acids. Volatile neutral compounds would, however, have been driven off.

VIALE COUNTS

Viable counts were performed by a modification of the method of Miles *et al.* (1938), in which 0.01-ml volumes were delivered from an automatic pipette.

Results**INJECTION OF UNTREATED URINE**

Our first objective was to discover whether the simple procedure of injection, on a porous-polymer column, of urine infected with the commonest urinary tract pathogen, *E. coli*, could reveal the presence of enough metabolites to serve as an indicator of significant bacteriuria.

Preliminary comparison of chromatograms of infected and uninfected urines

Eighty-six clinical urines infected with *E. coli* were chromatographed by injection of 3 μ l on a column of Chromosorb 105. The only peak obtained consistently had a retention time (R_t) of 7.5 minutes and a mean height of 29.3 mm. The height of this peak above which a urine could be designated as 'infected' was determined by study of a control group of 37 uninfected clinical urines. These were chromatographed in the same way as the infected urines and gave a mean peak height of 3.4 mm with standard deviation of 2.3 mm. The threshold value for the peak height was set, 2.5 standard deviations above the mean at 11 mm.

A comparison of the peak heights of the uninfected and infected urines is shown in Fig. 1; with the threshold value for 'infection' set at 11 mm, only 3% of uninfected urines were recorded as false positive but 36% of infected urines were recorded as false negative.

Relationship of height of 7.5-minute peak to viable count of E. coli

The viable count was correlated with the height of the 7.5 R_t peak for 29 clinical urines infected with *E. coli* and 35 experimentally infected urines, as shown in Table 1. No urine containing less than 10^7 *E. coli* per ml produced a significantly raised peak; but some urines with high viable counts failed to produce significantly raised peaks.

Fig. 1 Height of 7.5 R_t peak from 37 uninfected clinical urines and 86 clinical urines infected with *E. coli* $> 10^5$ per ml. Column packing—Chromosorb 105; oven temperature 190°C ; detector-oven temperature 250°C ; injection-port heater temperature 220°C ; injection volume $3\ \mu\text{l}$; attenuation 2×10^{-10} .

Table 1 Relationship of 7.5 R_t peak height to *E. coli* count in urine

Viable count (cells per ml)	Number of urines giving peak height > 11 mm/number tested	
	Clinical	Experimentally infected normal
10^4	0/2	NT
10^5	0/2	0/2
10^6	0/6	0/10
10^7	9/12	9/16
10^8	5/7	7/7

NT = not tested

Relationship of height of 7.5-minute peak to concentration of urine

Osmolality was used as a relative measure of concentration. For 19 clinical urines infected with $> 10^7$ *E. coli* per ml, the osmolality was correlated with the height of the 7.5 R_t peak produced, as shown in Table 2. All the concentrated urines produced significantly raised peaks whereas more than half of the dilute urines failed to do so.

TESTING OF 'CLEANED UP' URINE

Although the technical procedure was essentially simple, problems were encountered: the phenomenon of 'ghosting'—the appearance of a peak related to a previous injection rather than the current injection—gave rise to spurious results; and charred deposits rapidly accumulated at the injection site, causing column deterioration. We thought that treatment

Table 2 Relationship of 7.5 R_t peak height produced by urine infected with 10^7 *E. coli* per ml to dilution of urine; osmolality was used as a relative measure of dilution

Osmolality (mosm/kg)	Number of urines giving peak height ≥ 11 mm/number tested
100-199	1/2
200-299	1/2
300-399	0/2
400-499	4/5
500-599	3/3
600-699	2/2
700 and over	3/3

of the urine to remove non-metabolites before injection and general improvement of chromatographic technique would improve column sensitivity and might enable us to detect metabolites other than that associated with the 7.5 R_t peak. This was done as described in Methods.

Porous polymers other than Chromosorb 105 were also tested, as described below, and other microorganisms responsible for urinary tract infection were investigated in addition to *E. coli*, for example, *C. albicans*, *Enterobacter cloacae*, *Klebsiella* spp, *Ps. aeruginosa*, *Proteus* spp, *Staph. albus*, and *Str. faecalis*.

Choice of most suitable porous polymer for detection of bacterial metabolites in urine

A culture in the same normal urine was prepared for each of the main species of microorganisms responsible for urinary tract infection. After treatment, as described above, each culture and a control uninoculated urine were injected on columns of Poropak Q, N, T, and Chromosorb 101 and 105. The chromatographic profiles produced by the cultures were compared with those produced by the control. Differences between culture profiles and the control profile were observed only when either Chromosorb 101 or Poropak P was used. When the injections on Chromosorb 101 and Poropak P were repeated with optimum operating conditions for each polymer, the results for each polymer were the same; the culture profiles showed an increase in peak height over the control profile for one or more of three peaks. Chromosorb 101 was selected for further study.

Consistency of metabolite production in different urines

Two cultures of *E. coli* in two different normal urines were prepared in addition to the urine previously used, and the same procedure was used to produce two more sets of chromatographic profiles from Chromosorb 101. Only one of the three raised

Fig. 2 Chromatograms from 'purified' preparations of two normal urines before and after incubation with *E. coli*. Arrow indicates peak showing a large rise after culture of each urine. Column packing—Chromosorb 101; oven temperature 185°C; detector-oven temperature 230°C; on-column injection volume 0.5 µl; attenuation 1×10^{-10} .

peaks that had appeared in the culture profile of the original urine showed a large consistent rise for all three urines; we referred to this as the 'infection peak'. The culture profiles obtained from two urines before and after culture are shown in Figure 2.

The same result was obtained for cultures of *Enterobacter cloacae*, *Klebsiella* spp, *Proteus* spp, *Staph. albus*, and *Str. faecalis*, but there was no consistent rise in any peak from *C. albicans* or *Ps. aeruginosa*.

Ability of Chromosorb 101 to detect likely microbial metabolites

A series of volatile acidic and neutral compounds likely to be produced as metabolites by the types of organisms responsible for urinary tract infection was prepared in urine, and the minimum quantities detectable by injection on Chromosorb 101, after the treatment procedure described above, were measured. The results are shown in Table 3. n-Pentanol was then selected for use as an internal standard, and the relative retention times of the compounds were subsequently determined in relation to it.

Identity of infection peak

The internal standard, n-pentanol, was added to the urines that had been cultured with *E. coli*, and they were re-chromatographed to determine the relative retention times of the infection peak. The value obtained was 0.333. Reference to Table 3 shows that, of the compounds tested, the only likely microbial metabolite with the same relative retention time was acetic acid.

The metabolite responsible for the infection peak was identified with a Varian Model MAT112 Mass Spectrometer. It gave the same ratio of the same fragmentations as acetic acid.

Comparison of areas of acetic-acid peaks in chromatograms from clinically infected and uninfected urines

The relative areas of the acetic-acid peaks from 43 clinical urines infected with one or more species of the microorganisms responsible for urinary tract infection after chromatography on Chromosorb 101 were compared with those produced by a control group of 51 uninfected clinical urines.

After addition of 326 ng of the n-pentanol internal standard to 5 ml of urine, the chromatograms were obtained as described above for cultures of normal urine, and the areas of the acetic-acid peaks were expressed as a percentage of the area of the internal standard. The mean relative peak area of the control group was 1.66% with standard deviation 1.30%. As previously where the heights of the 7.5 R_t peak produced by urines infected with *E. coli* were considered, the threshold value above which a urine could be designated as 'infected' was set 2.5 standard deviations above the mean. This gave a threshold for relative peak area of 4.9% of the n-pentanol standard.

The comparison of the relative peak areas for the infected and uninfected groups is illustrated in Figure 3. The results are similar to those for peak height (Fig. 1) in that only 2% of uninfected urines produced relative areas above the threshold value,

Table 3 Amounts of likely microbial metabolites required to produce 10% full-scale deflection (FSD) when injected in urinary solution on Chromosorb 101, and relative retention time

Compound	Quantity required to produce 10% FSD (ng)	Retention time relative to n-pentanol
Acetic acid (ethanoic acid)	48.154	0.329
Propionic acid (propanoic acid)	53.793	0.553
Butyric acid (butan-1-oic acid)	74.524	1.000
Iso-butyric acid (butan-2-oic acid)	36.706	0.794
Valeric acid (pentan-1-oic acid)	121.429	1.713
Caproic acid (hexan-1-oic acid)	194.118	3.102
Ethanol	7.647	0.195
Propanol (propan-1-ol)	9.630	0.313
Isopropanol (propan-2-ol)	7.647	0.228
Butanol (butan-1-ol)	31.200	0.542
n-Pentanol (pentan-1-ol)	138.356	1.000
Pentan-3-one	22.429	0.668
Acetoin (3-hydroxybutan-2-one)	127.632	0.863
Propane-1,3-diol	102.459	1.520
Butan-2,3-diol	51.148	1.249

IUPAC nomenclature in parentheses

Fig. 3 Relative area of acetic-acid peak from 51 uninfected clinical urines and 43 clinical urines infected with $> 10^5$ per ml of one or more of eight species of microorganism mentioned in text. Chromatographic conditions as indicated for Figure 1.

but 37% of infected urines produced relative peak areas below this value.

Relationship between area of acetic-acid peak and viable count

The 43 clinically infected urines for which the

relative peak area of the acetic-acid peak had been determined, as described above, were considered in four groups: *E. coli* infection, *Ps. aeruginosa* infection, *Proteus* infections, and mixed infections. For each group the viable count was correlated with the relative area of the acetic-acid peak, as shown in Table 4. All the urines infected with *Ps. aeruginosa* produced relative peak areas below the threshold for 'infection' (4.9%), and urines infected with other organisms required the presence of at least 10^6 microorganisms per ml to produce relative peak areas above the threshold.

TESTING OF CONCENTRATED URINE

It seemed possible that acidic and neutral compounds other than acetic acid were not demonstrable in infected urine because their concentration was lower than the limit of detection on Chromosorb 101. Concentration of the urine might therefore enable them to be detected.

Detection of microbial metabolites in concentrated urine by Chromosorb 101

The loss of acetic acid during 20-fold concentration by the procedure described in Methods was determined for a standard solution and was found to be insignificant.

Table 4 Relationship of acetic-acid peak area to number of microorganisms in urine

	<i>E. coli</i> (clinical)	<i>E. coli</i> (experimentally infected)	<i>Ps. aeruginosa</i> (clinical)	<i>Proteus spp</i> (clinical)	Mixed (clinical)
10^4	0/1	0/2	NT	NT	NT
10^5	0/4	0/4	0/1	NT	0/5
10^6	5/6	2/4	NT	1/1	6/6
10^7	11/11	2/3	0/2	2/3	3/3

NT = not tested

Comparison of the chromatographic profiles of concentrates of *E. coli* culture with those of the controls revealed no metabolites other than the acetic acid detectable in unconcentrated urine. The profiles obtained for one urinary concentrate are presented in Figure 4. The same finding was obtained for concentrated cultures of the other microorganisms responsible for urinary tract infection. Volatile acid metabolites other than acetic acid were not produced in detectable amounts.

Fig. 4 Chromatograms from concentrated preparations of normal urine before and after incubation with *E. coli*. Arrow indicates peak corresponding to acetic acid and showing a large rise after culture of each urine. Chromatographic conditions as indicated for Figure 2.

Discussion

The simple procedure of injecting untreated urine on a porous polymer column, Chromosorb 105, provided a rapid method of detecting infection in some urines by recording a raised acetic-acid peak. Too many false negatives occurred, however, for the method to be useful as a screening test for bacteriuria. The insensitivity of the method—detection of only 10^7 organisms per ml whereas 10^5 organisms per ml is the accepted criterion of significant bacteriuria—and recording of false negatives in dilute urine with high bacterial counts together with charring of the crude material introduced into the gas chromatograph contributed to the failure of the procedure.

Detection of low urinary concentrations of likely volatile acidic and neutral microbial metabolites was achieved by selection of a more suitable porous polymer, Chromosorb 101, improved chromato-

graphic procedure, and the use of 'cleaned-up' urine; but when it was used to analyse urines infected with each of the principal microorganisms responsible for urinary tract infection, the only microbial metabolite consistently detected was acetic acid. This was detectable only when at least 10^6 microorganisms per ml were present, and was not produced by all the species tested, for example, *Pseudomonas* and *Candida*. Concentration of the urine by evaporation did not reveal the presence of metabolites other than acetic acid.

We have found that the use of solvent extraction to obtain and concentrate acidic and neutral metabolites, although simple, was less effective than the direct injection of aqueous solutions, because of the poor partition characteristics of the volatile acids in organic solvents.

It must be concluded that the microorganisms responsible for urinary tract infection liberate insufficient quantities of volatile small acidic and neutral molecules into the urine to serve as a reliable index of infection. Basic or non-volatile compounds may be more suitable indices, and work on their detection is now in progress.

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- Requests for reprints to: G. Lynam, Division of Hospital Infection, Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ, UK.