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Short communication

New method for determination of fecal sterols in urine using non-chlorinated solvents

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Abstract

A new method has been developed to determine a number of sterols in urine using non-chlorinated solvents, namely methyl *tert*.-butyl ether and methanol (the MTB method). The method involves liquid–liquid extraction, saponification, reextraction, silylation and final identification and quantification by GC–FID. The sterols determined were coprostanol, epicoprostanol, cholesterol and dihydrocholesterol. 5α-Cholestane was used as internal standard. The limit of detection for the sterols in this experiment was 2 μg l^{-1} urine. Recovery of coprostanol over the range 5–100 μg l^{-1} urine by this method was between 80 and 92%. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Urine has traditionally been used in many countries as a source of nutrients for agriculture. A waste recycling research program in Sweden aims to improve sustainable handling of human wastes by 'at the source' separation of human urine from feces. This division of waste products improves many of the problems associated with centralised sewage treatment and disposal caused by having large quantities of liquids (urine and water) mixed with solid wastes. The strategy of urine separation will enable urine to be more widely utilised as a source of nutrients, and contribute to sustainable waste handling by complimenting existing sewage treatment systems.

However, there is a risk of transmitting infectious pathogens when the urine is stored, transported and applied to the soil as a nutrient. The risk appears to occur when the urine and/or the inlet pipes are inadvertently contaminated with feces. Therefore it is necessary to use a suitable tracer to indicate whether contamination has occurred. The traditional method to determine fecal contamination is to evaluate coliform bacteria concentrations. However, studies of fecal bacteria in urine [1] showed that the survival time of these in stored urine differs very much between organisms.

Sterols are widely used as biomarkers for fecal contamination in sediments and water [2–4]. Their efficiency as indicator of feces in urine was, therefore, of great interest. The aim of this study was to develop a method for determining sterols in urine. To minimise the environmental effects of our work,

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it was desirable to use non-chlorinated solvents for the extraction. We report here a method to analyse sterols, including the fecal sterol coprostanol, and compare this with corresponding analyses using chlorinated solvents [5].

2. Experimental

2.1. Sample collection

Collection of source-separated human urine was performed by the authors in the eco-villages Understenshöjden, Stockholm and Åkesta, Västerås, Sweden. Samples were taken from the collection tank with a 50-ml syringe fixed to a long metal pole. Altogether 300 ml were collected in a glass bottle for each sample and refrigerated. The sample was then stored frozen at −20°C until analysis.

2.2. Reagents

The following standards were used for calibration (trivial names in italics): 5α-cholestane (internal standard, I.S.), 5β-cholestan-3β-ol (98%) coprostanol, 5β-cholestan-3α-ol (95%) epicoprostanol, 5α-cholestan-3β-ol (95%) dihydrocholesterol and cholest-5-en-3β-ol (99%) cholesterol. HPLC-grade methanol, heptane and methyl *tert*.-butyl ether (MTB) were used for extraction and clean-up. Dimethylformamide (HPLC-grade), hexamethyldisilane (puriss) and trimethylchlorosilane (puriss) were used for the silylation. All chemicals were from Lab Kemi (Stockholm, Sweden).

2.3. Calibration

For long-term storage, a stock solution of 5α-cholestane diluted in MTB ether was prepared at a concentration of 1000 μg ml^{-1}. A solution containing 5α-cholestane (100 μg ml^{-1}) was prepared as a working internal standard. Similar solutions were prepared for all the above sterols.

2.4. Sterol extraction, saponification and silylation

A 100-ml volume of urine was transferred into a separation funnel together with 2.5–100 μg of the internal standard. A 10-ml volume of MTB ether and 20 ml of methanol were added; the mixture was monophasic. The sample was shaken for 30 min and then left overnight. A further 20 ml of MTB ether were added to separate the two phases and the sample was shaken for another 30 min. The lower urine phase was saved in a glass bottle and the upper organic phase was poured into a 50-ml glass tube. The funnel was rinsed with 5 ml of MTB ether which was added to the organic phase. The organic phase was centrifuged for 5 min at 924 *g* and the upper layer was transferred into a 100-ml pear-shaped flask. The remaining urine phase in the tube was transferred into the separating funnel together with the saved portion of urine and another 20 ml of MTB ether. The extraction procedure was repeated once (without standing overnight) and the pooled ether-phase was evaporated under vacuum. The sample residue was dissolved with 3×0.8 ml of 50% MTB ether in methanol and evaporated in a small tube to dryness under a stream of nitrogen.

The sample was saponified with 2 ml of 1 *M* potassium hydroxide in 90% ethanol at 80°C for 70 min. After cooling to room temperature, 1.6 ml of water was added and the sterols were extracted twice with 1 ml of heptane. The upper heptane layers were transferred to 2-ml sample tubes and evaporated under a stream of nitrogen. The tube walls were washed with 100 μl of heptane–methanol (2:3, v/v) which was evaporated. This was repeated twice.

Sterols were converted to their corresponding trimethylsilyl (TMSi) ethers by treatment with 100 μl of CHOL-TMS containing dimethylformamide, hexamethyldisilane and trimethylchlorosilane (4:4:0.1, v/v) according to Miettinen et al. [6]. The silylation was carried out at 60°C for 60 min. Samples were then evaporated under nitrogen to dryness and were re-dissolved in 50–500 μl of heptane prior to analysis.

2.5. Experimental conditions

GC analyses were performed with a Chrompak 9000 GC, equipped with a 50 m×0.25 mm I.D. (0.12 μm film thickness) fused-silica capillary column (Chrompack CP-sil 8 CB for pesticides), a flame ionisation detector (FID) and a split/splitless injector, all from Chrompack Sverige (Stockholm,

Sweden). Samples were injected (0.8 μl out of typically 50–500 μl of sample) in the split mode (split flow 30 ml min^{-1}) at 170°C and after 2 min the oven temperature was raised to 260°C at 20°C min^{-1} and then after 55 min to 300°C at 20°C min^{-1}. Helium (N47 grade, 99.997%) was used as a carrier gas and the inlet pressure was 130 kPa. Peak areas were obtained from the chromatograms generated by the data-handling program PCI (Chrompack Sverige). Component identification was based on comparison of retention times with those of the standards. The relative standard deviation for replicate injections was 5%. The FID response was found to be linear in the practical concentration range (2–100 ng of individual component injected). Verification of compound identification was performed by GC–MS analysis of samples on a HP 5890 GC and mass-selective detector fitted with a direct capillary inlet and a split/splitless injector. Data were acquired and processed on a HP 59970C Workstation operated in scan acquisition mode. Operating conditions are described in detail elsewhere [7,8].

3. Results and discussion

3.1. Gas chromatography–mass spectroscopy (GC–MS)

Representative chromatograms illustrating sterol profiles for a urine sample with added sterols, analysed with the MTB method and from laboratory standards are shown in Fig. 1.

The retention times and fragmentation patterns in GC–MS measurement for the TMS ethers of the sterols under study are shown in Table 1.

3.2. Limit of detection, limit of quantification and linearity

The limit of detection was determined from repeated analyses of sterols at low concentrations. The concentration at which the mean exceeded the baseline noise by more than 3 S.D. was taken as the limit of detection.

The limit of detection in this study was 2 μg l^{-1} urine of individual sterols. This corresponds to a contamination rate of approximately 0.5 mg feces per litre urine [9].

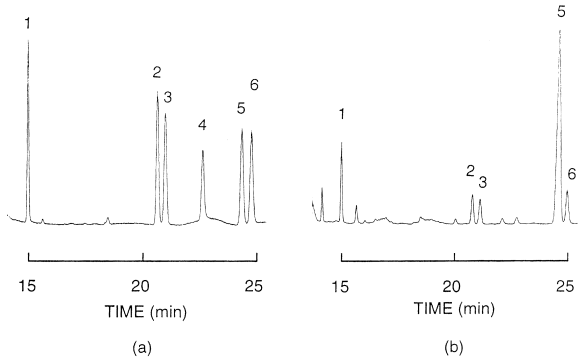

Fig. 1. Partial total ion chromatograms of sterols from (a) a standard mix and (b) a urine sample with added sterols. Key to sterols: (1) 5α-cholestane (internal standard, I.S.); (2) coprostanol; (3) epicoprostanol; (4) coprostanone; (5) cholesterol; (6) dihydrocholesterol. One μl is injected in split mode.

The limit of quantification in this study was 7 μg l^{-1}. It was calculated as 10 times the standard deviation of the baseline noise for the blanks.

A linearity test was made using standard sterols added to fresh urine. The linearity was good over the range 5–100 μg/l for coprostanol ($r=0.9572$, $P<0.0001$), epicoprostanol ($r=0.9274$, $P<0.0001$) and dihydrocholesterol ($r=0.8505$, $P<0.0005$).

The linear regression line and the correlation coefficient of the standard curves were: 5α-cholestane, $y=0.6366+9.0798\times10^{-3}$, $r=0.9990$; coprostanol, $y=0.2655+8.0925\times10^{-3}$, $r=0.9996$; epicoprostanol, $y=1.4758+7.8675\times10^{-3}$, $r=0.9991$; cholesterol, $y=0.6343+8.1919\times10^{-3}$, $r=0.9989$; and dihydrocholesterol, $y=0.1668+8.1479\times10^{-3}$, $r=0.9985$ in the given concentration range.

3.3. Accuracy and precision

Recovery data for the internal standard and four sterols are presented in Table 2. The sterols were added to fresh urine and extracted by the method described in this paper. The mean recovery was found to be between 87 and 100% and the variation was less than 9.5% for all sterols.

In order to check the precision of the method, a urine sample spiked with raw sewage sludge was prepared and analysed. Five parallel samples were

Table 1
Retention times and fragmentation patterns of 5α-cholestane and other sterols in urine

Compound	Retention time (min)	M	M−15	M−90	Other
5α-Cholestane (I.S.)	15.33	372 (38)	357 (47)	—	217 (100)
Coprostanol	21.09	460 (1)	—	370 (100)	355 (40)
Epicoprostanol	21.43	460 (0.3)	—	370 (100)	215 (59)
Cholesterol	24.75	458 (43)	—	368 (78)	129 (88)
Dihydrocholesterol	25.18	460 (56)	445 (77)	—	75 (89)

M, molecular ion; M−15, molecular ion−15 mass units; M−90, molecular ion−90 mass units. Figures in brackets give size as percentage of base peak (100).

Table 2
Percentage recovery of added[a] sterols using the new MTB[b] method

Sample no.	5α-Cholestane (I.S.)	Coprostanol	Epicoprostanol	Cholesterol	Dihydrocholesterol
1	89	90	103	90	100
2	88	85	106	88	98
3	93	92	104	97	104
4	89	80	90	77	88
Mean	90	87	101	88	97
C.V.	2.4	6.2	7.3	9.5	7.1

[a]37.5 μg of each sterol added to all samples.
[b]Methyl *tert*.-butyl ether.

spiked and analysed at the Department of Microbiology, Sweden, and four at the CSIRO Division of Oceanography, Tasmania. The quantitative results obtained for cholesterol and coprostanol as the major components of free sterols are given in Table 3.

The data in Tables 2 and 3 indicate the good repeatability and reproducibility of the outlined method. Besides the errors resulting from repeated injection, further inaccuracies might be caused by inhomogeneity of the sample, but also by the addition of internal standard and by possible insufficiency of the derivatisation. Comparison of the data in Tables 2 and 3 shows that the contribution of these errors to the total standard deviation is small.

3.4. Method development considerations

During our efforts to find a useful method to measure sterols in urine, we analysed the particulate and dissolved fractions of feces contaminated urine after filtering according to Nichols et al. [3]. We found that the sterols were distributed between the

Table 3
Comparison of the analysis of coprostanol and cholesterol in urine added with sewage sludge

Sample no.	Tasmania		Sweden	
	Coprostanol (μg l^{-1})	Cholesterol (μg l^{-1})	Coprostanol (μg l^{-1})	Cholesterol (μg l^{-1})
1	83	351	59	884
2	84	387	53	854
3	83	389	49	835
4	88	405	61	770
5	—	—	55	865
Mean	84	383	55	842
C.V.	2.9	5.9	8.5	5.2

The analyses were made of two separate batches and by two different laboratories.

Table 4
Comparison of the new MTB[a] method and Bligh and Dyer extractions

Sample no.	MTB		Bligh and Dyer	
	Coprostanol ($\mu g\ l^{-1}$)	Cholesterol ($\mu g\ l^{-1}$)	Coprostanol ($\mu g\ l^{-1}$)	Cholesterol ($\mu g\ l^{-1}$)
1	83	351	82	381
2	84	387	84	398
3	83	389	85	395
4	88	405	82	381
Mean	84	383	83	385
C.V.	2.9	5.9	1.7	3.3

[a]Methyl *tert*.-butyl ether.

dissolved fraction and the particles, a fact that made us look for a method to extract the sample by a direct liquid–liquid extraction procedure. In this experiment solvent phase extraction would discriminate sterols adsorbed by particles trapped on the filter and would therefore be inaccurate.

3.5. Environmental benefits

The classical way to extract lipids from water and wet samples is to use methanol and chloroform; i.e. the method of Bligh and Dyer [5]. However, this method uses 250 ml of methanol and 250 ml of chloroform for each 100-ml urine sample. Chloroform is a designated a cancer hazard and requires an exemption certificate for use in Sweden. The methanol/MTB method uses only 20 ml of methanol and 55 ml of MTB per sample. This is not only a more environmentally friendly method but also less expensive. To compare the efficiency of these two methods on a real sample matrix, we added 100 ml of raw sewage to 900 ml of fresh urine. This was divided into 50-ml portions; four were analysed by the Bligh and Dyer method [5] and four by the MTB method. The coprostanol and cholesterol concentrations determined in the samples are presented in Table 4. The two methods showed extremely good agreement and the variance was very small. This indicates that the MTB method using non-chlorinated solvents has a similar extraction efficiency to that of the Bligh and Dyer method, at least in this matrix, and that the method is an environmentally more friendly way of analysing fecal sterols in urine.

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