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Short Communication

Sources of plant sterol contaminants encountered in low level steroid analysis

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ABSTRACT

During development of an analytical method to characterize ligands to new members of the steroid hormone receptor superfamily, a persistant contaminant profile was observed during gas chromatographic analysis of reagent blanks. Mass spectrometric analysis identified three of the contaminant peaks as cholesterol and the plant sterols stigmasterol and sitosterol. Laboratory articles made of natural rubber, *i.e.* pipette fillers and latex gloves, were found to be the source of these and other compounds in the reagent blank profile.

INTRODUCTION

An analytical method for the characterization of endogenous ligands to new members of the steroid hormone receptor family (orphan receptors) has been developed [1]. The ligand binding domain (LBD) of a candidate orphan receptor can be used as a probe to isolate its ligand from the appropriate biological extract. Using a hydrophobic gel, the protein-specific ligand complex is separated from the non-specifically bound and unbound ligands. The specific ligand is then identified by gas chromatography–mass spectrometry (GC–MS). The strength of this approach is dependent on the enrichment of the specific ligand in the final fraction for GC–MS analysis. A reduction in levels of all other endogenous compounds and the avoidance of exogenous contaminants in sample preparation is thus essential.

During the early development of the above method, it became apparent that high levels (nanograms to micrograms) of contaminants accompanied the specific ligand in the final fraction. Addition of such contaminants occurred after incubation of the LBD with the biological extract. A systematic study of the sample extraction, clean-up and derivatization procedure (including five different chro-

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matographic columns) by GC analysis of reagent blanks revealed an intermittent yet specific contamination profile that was resilient to all clean-up attempts, *e.g.* use of all-glass apparatus, redistillation of solvents and thorough washing of gels. GC–MS of the post-incubation reagent blank identified three of the contaminants as cholesterol and the plant sterols, stigmasterol and sitosterol.

Preparation of reagent blanks initiated at various stages in the post-incubation procedure failed to identify the source of the plant sterol contaminants amongst materials in direct contact with the sample. This was mainly due to the irregular occurrence of the contaminants in these systematic studies. It was thus considered possible that the source of contaminants might be laboratory articles coming indirectly in contact with the sample. To investigate this, O-methyloxime trimethylsilyl (MO-TMS) ether derivatives of hexane extracts of ten laboratory articles were prepared and the retention indices of the major peaks compared with those of the post-incubation contamination profile.

EXPERIMENTAL

Glassware and solvents

After normal washing, all glassware was rinsed in chloroform–methanol (1:1, v/v) followed by ethanol. All solvents were of reagent grade and were redistilled twice before use. Water was obtained from a Milli-Q reagent-grade water system (Millipore, Milford, MA, USA).

Extraction and derivatization

The following items were selected at random and do not exhaust the list of articles in the laboratory that are potentially in indirect contact with the post-incubation sample.

Adhesive tape, white, used to label glassware (Garco Industrier, Åstorp, Sweden).
Detergent (Decon 90, Decon Labs., Hove, UK).
Dust collected in the laboratory.
Glove, latex disposable (Everguard, Pro Benlee Industrial, Taipei, Taiwan).
Hand cream (Käsivoide C arom normal, Farmos Consumer Goods, Turku, Finland).
Paper towel, household (Pappersgruppen, Stockholm, Sweden).
Pasteur pipette bulb, white rubber (Nollapo, Torekov, Sweden).
Pen for labelling glass sample tubes (Pilot super colour markers, Pilot Pen Company, Tokyo, Japan).
Pipette filler (Peléus Standard, Wertheim Glaswerk, Wertheim, Germany).
PTFE tape used to create tight seal between column and tap (Kartell, Milan, Italy).

Milligram amounts of each of the above items were extracted in hexane (4 ml). After vortex-mixing and 2-min ultrasonication, the sample was centrifuged at 865 *g* for 10 min in a bench-top instrument, and 3 ml of supernatant were transferred

to a clean glass test-tube. These hexane extracts were evaporated to dryness under nitrogen, then MO-TMS derivatives were prepared and purified as described previously [1]. The purified derivatives were reconstituted in a volume of hexane which, on GC injection of 1 μl, produced an on-scale profile (volumes ranged from 10 to 1200 μl).

GC analysis was carried out using a Carlo Erba Strumentazione high-resolution gas chromatograph (Farmitalia Carlo Erba, Milan, Italy), housing a fused-silica capillary column (methyl silicone, 25 m × 0.32 mm I.D., 0.25 μm film thickness, Quadrex, New Haven, CT, USA). The samples were introduced by on-column injection, and detection was by flame ionization. The oven temperature programme was 60–220°C at a maximal rate (55°C/min) and then 220–280°C at 1°C/min. Helium was used as the carrier gas with an inlet pressure of 70 kPa, giving a flow-rate of *ca.* 1 ml/min.

Laboratory article extract profiles were compared with the profiles of a blank hexane extract (4 ml of hexane evaporated to dryness, derivatized and purified as above) and a post-incubation reagent blank prepared as described by Banner *et al.* [1]. Retention indices [2] were calculated from retention times of a series of C_{28}–C_{36} *n*-alkane standards.

RESULTS AND DISCUSSION

Table I shows the retention indices of major peaks in the derivatized extracts of each laboratory article, listed in order of decreasing peak area. For extracts with complex GC profiles, only the five largest peaks are listed.

A qualitative comparison of the various profiles with the post-incubation reagent blank profile should indicate the source of contamination in the procedure. Quantitative comparisons of compounds between extracts were not made.

The extracts of detergent, dust and paper produced GC profiles similar to the blank hexane extract and appear therefore to contribute no contaminating peaks to the steroid elution window. Of the seven profiles with substantially more contaminants than the control, all except that from the marker pen contained one (adhesive tape, hand cream and PTFE tape) or more peaks, with retention indices similar to those of peaks in the post-incubation reagent blank profile. Each article made from natural rubber, *i.e.* the glove, the Pasteur pipette bulb, and the pipette filler, contained compounds with retention indices identical with those of cholesterol, stigmasterol and sitosterol. The peak-area ratio of stigmasterol to sitosterol was *ca.* 1:5 in both the rubber article extracts and the post-incubation reagent blank profiles.

The GC profile that was most compatible with the post-incubation reagent blank was that of the Pasteur pipette rubber bulb extract, indicating that this article is the main source of contamination. The process by which indirect contact is made between the bulb and the sample is unclear. Contamination may be mediated by solvent vapour or by fragments of material falling into the pipette.

TABLE I

RETENTION INDICES OF MAJOR PEAKS IN MO-TMS-DERIVATIZED EXTRACTS OF LABORATORY ITEMS

Item	Retention index	Item	Retention index
Reagent blank	3347[a]	Paper	—[d]
	3357		
	2899	Pasteur Pipette bulb	3347[a]
	2839		3154[b]
	3154[b]		3357
	3295[c]		2899
			3295[c]
Adhesive tape	3251		
	3146	Pen	2914
	3218		3030
			2920
Detergent	—[d]		3246
			2854
Dust	—[d]		
		Pipette filler	3159[b]
Glove	3148[b]		3355[a]
	3344[a]		3368
	3354		3305[c]
	2968		3290
	2994		
	3292[c]	PTFE tape	3426
			3252
Hand cream	2800		3075
	2893		2891
	2992		
	3186		
	3081		

[a] Sitosterol.
[b] Cholesterol.
[c] Stigmasterol.
[d] Blank hexane extract profile observed.

Variations in both the batch and age of the Pasteur pipette rubber bulbs used may therefore explain the intermittent occurrence of plant sterol contamination. This also obscured the fact that most of the contamination occurred at the final stage of the analysis, since cholesterol and the plant sterols should have been removed by the chromatographic step preceding derivatization.

Further analyses of reagent blanks prepared in an all-glass and PTFE system, avoiding the use of natural rubber products, produced GC profiles free of plant

sterols and associated contaminants. Alternatives to the Pasteur pipette rubber bulb are available, *e.g.* an all-glass syringe connected via an air-tight PTFE adaptor to a Pasteur pipette or an all-glass syringe pipette. These results emphasize the importance in low-level steroid analysis of ensuring not only that the sample has no direct contact with anything except glass and PTFE, but also that there is no indirect contact with organic materials of natural origin.

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