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Rapid and accurate high-performance liquid chromatographic method for the determination of 3-methylindole (skatole) in faeces of various species

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ABSTRACT

A rapid method for the determination of skatole (3-methylindole) in faeces by reversed-phase high-performance liquid chromatography is described. Samples of 0.5 g were extracted with 2 ml of methanol. The extract was purified on Amberlite XAD-8. The lower limit of detection was 2.5 ng per injection (0.2 μg/g faeces). The mean recovery of skatole was 95%, and the mean coefficients of variation were 7.0% (intra-assay) and 11.8% (inter-assay). Skatole concentrations were clearly lower in faeces from ruminants (average 2.6 μg/g for goat, sheep and cattle) than in those from monogastrics. Mean concentrations in human samples were 15.5 μg/g, and 10 μg/g in mature domestic pigs. An effect of the anabolic status on skatole concentrations in faeces of pigs is likely.

INTRODUCTION

Skatole (3-methylindole) is a volatile substance assumed to result from microbial degradation of tryptophan in the intestines of several species [1–3]. Its odour contributes to the characteristic smell of both faeces and liquid manure [4–7]. A fraction of synthesized skatole is resorbed from the intestine. Owing to its toxic properties it may lead to diseases of the respiratory tract, as described for ruminants [8,9]. In the pig, skatole accumulates in lean meat and adipose tissue, leading to a severe reduction of the carcass quality [10].

Several methods have been developed for determination of skatole, mainly in adipose tissue of pigs and mainly by gas chromatography (GC). Sample preparation was based either on volatility [11,12], or on extraction with organic solvents followed by purification prior to GC [13,14]. A method for the determination of skatole in tissue by high-performance liquid chromatography (HPLC) has been reported [15], but this also requires tedious concentration and purification steps.

For the measurement of skatole in faeces only GC methods have been reported so far. The sample preparation was performed by time-consuming distillation steps or by extraction using large volumes of organic solvent [5,6,16,17]. The

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performance features of these methods were only partly reported, and systematic measurements were carried out only for human and porcine faeces.

Therefore, it was the aim of our study to set up a rapid and accurate HPLC method for the measurement of skatole in faeces, and to demonstrate its performance characteristics. This method was also applied to determine the range of skatole concentrations found in faeces of several species.

EXPERIMENTAL

Equipment

An LKB (Bromma, Sweden) HPLC system was used, consisting of an HPLC pump (Model 2150), a solvent conditioner (Model 2156), a column oven (Model 2155), a variable-wavelength UV detector (Model 2151), and an autosampler (Model 2157) equipped with a Rheodyne injector (Model 7000). Separation of the analytes was performed on a 125 mm × 4.6 mm I.D. LiChrosper RP-18 column (particle size 5 μm, Bischoff Analysentechnik, Leonberg, Germany). An identically packed pre-column (15 mm × 4.6 mm I.D.) was used to protect the analytical column. Peaks were recorded and integrated using a Perkin Elmer Nelson Systems interface (Cupertino, CA, U.S.A.), linked to a personal computer.

Materials

All chemicals were analytical grade, and solvents were LiChrosolv quality (Merck, Darmstadt, Germany). Standard substances (skatole, 2-methylindole, indole-3-acetic acid, indole-3-acetic acid ethyl ester, indole and L-tryptophan) were reagent grade, and were supplied by Serva (Heidelberg, Germany). Fresh stock solutions of all standards were prepared at monthly intervals at a concentration of 0.1 mg/ml in methanol.

Amberlite XAD-8 resin (Merck) was used to prepare solid-phase extraction columns (27 mm × 5 mm I.D.). The columns were filled with 0.5 ml of Amberlite XAD-8, which had been activated with methanol. They were washed with 2 ml of water and equilibrated with 3 ml of 0.05 *M* Tris buffer in 0.05 *M* NaCl (pH 8.3) prior to use.

Origin of samples

Fresh faeces were collected from mature cattle, goats, sheep and wild and domestic pigs. Samples from growing pigs (age three months) were also obtained. All animals were kept indoors and fed species-specific standardized diets *ad libitum*. Human faecal samples were provided by adults on non-standardized diets. All samples were stored at −20°C until assayed.

Preparation of samples and HPLC analysis

Aliquot portions of 0.5 g of faeces were transferred to 10-ml reagent tubes in duplicate. 2-Methylindole (2.5 μg) was added as an internal standard to each

tube. This substance was chosen because it is chemically similar to 3-methylindole but does not occur naturally in faeces. The samples were suspended in 2 ml of methanol, and the mixture was vortexed for 30 s. After centrifugation (15 min at 1200 *g*), the supernatant was transferred to a second 10-ml reagent tube. After the addition of 4 ml of Tris buffer, the mixture was placed on top of the solid-phase extraction column. The column was washed with 4 ml of Tris buffer, and the analytes were eluted with 2 ml of acetonitrile. Aliquot portions of 50 μl were analysed by HPLC.

The mobile phase was 0.02 *M* acetic acid–2-propanol–acetonitrile (60:15:25, v/v). The flow-rate was 1.0 ml/min. The column was operated at 40°C. The detection wavelength was 280 nm, and the absorbance range was 0.08 a.u.f.s. at 100 mV full scale.

Quantification

The calibration curves were prepared by adding various amounts of skatole (0.5–10 μg for determination at 280 nm) and a constant amount of 2.5 μg of 2-methylindole either to 6 ml of Tris buffer–methanol (66:34, v/v) or to faeces samples taken from a bulk amount of faeces that had previously been shown to contain no detectable amount of endogenous skatole. All calibration samples were treated identically with the biological samples, when added both to buffer and to faeces. The peak areas of the calibration samples and the internal standard were determined, and the ratio was used to evaluate the biological samples.

Determination of the quality of the method

Skatole has an absorption maximum at 220 nm, and an increased absorption at 280 nm. Therefore the range of linearity of the calibration curve was determined separately at 220 and at 280 nm. The lower limit of sensitivity for both wavelengths was taken as the concentration of skatole at which the peak height of skatole exceeded the maximal noise of the baseline at least two-fold.

The accuracy of the measurements was determined by adding known amounts of skatole (0.5, 2.5 and 5.0 μg) to 0.5-g samples of faeces. These samples had been pooled from material previously shown to contain no detectable amounts of skatole.

The reproducibility of the method was determined by repeated measurement of the same samples either on the same day (intra-assay variation, $n = 5$) or on consecutive days (inter-assay variation, $n = 10$). The specificity was evaluated by determining the retention times of other indoles (L-tryptophan, indole-3-acetic acid, and indole), after injecting them dissolved in the mobile phase.

Practicability is given as the mean number of samples that can be handled by one person in five days.

RESULTS

Quality criteria

The specificity of the method used was apparent from the clear differences in retention times observed between both skatole and 2-methylindole (internal standard), and other indoles that occur in faeces. The retention times for the standards were 2.28 min for indole-3-acetic acid, 4.14 min for L-tryptophan, 5.03 min for indole, 6.30 min for indole-3-acetic acid ethyl ester, 7.00 min for 2-methylindole and 7.98 min for skatole. As shown in Fig. 1, the assay of biological samples also demonstrated a clear separation of skatole and 2-methylindole (internal standard) from indole and other residual endogenous substances from faeces.

When evaluated at 220 nm, the lower limit of detection was 0.6 ng per injection (50 μl), corresponding to 0.048 μg/g of faeces. The sensitivity was identical whether it was determined in buffer or faeces. The upper limit of linearity was 300 ng per injection (24 μg/g of faeces). The calibration curves for skatole led to slightly higher peak ratios when the substance was added to faeces than when it was added to buffer ($r = 0.997$). The detection limit was 2.5 ng per injection (50 μl) at 280 nm, which corresponds to a lower sensitivity limit of 0.2 μg/g of faeces. The upper limit of linearity was 937 ng per injection (75 μg/g of faeces). The calibration curves in buffer and faeces were identical ($r = 0.999$).

Results defining the accuracy of the method (evaluation at 280 nm) are given in Table I. Depending on the concentrations, 90–98% of skatole added to faeces was measured (mean 95.3%). The mean intra-assay coefficient of variation (C.V.)

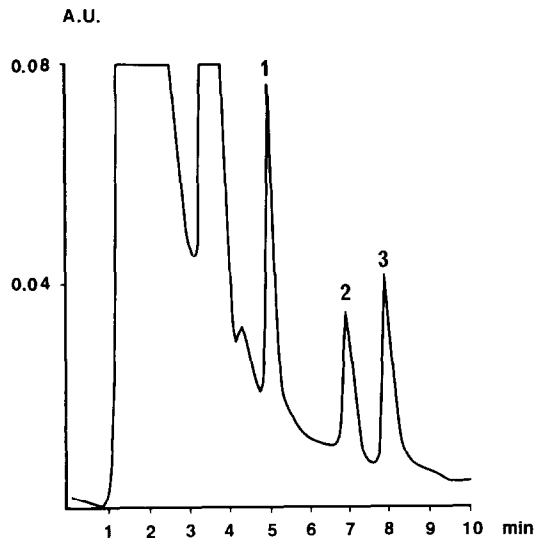

Fig. 1. Chromatogram of a pig faecal extract. Peaks: 1 = indole; 2 = 2-methylindole; 3 = skatole (see text for conditions).

TABLE I

ACCURACY AND INTRA- AND INTER-ASSAY COEFFICIENTS OF VARIATION OF SKATOLE DETERMINATIONS

Known amounts of skatole were added to skatole-free faeces and were measured by HPLC after extraction on Amberlite XAD-8 column.

Concentration (μg/g)		Coefficient of variation (%)	
Added	Measured (mean ± S.D., n = 5)	Intra-assay (n = 5)	Inter-assay (n = 10)
1.0	0.98 ± 0.12	16.4	24.9
5.0	4.88 ± 0.12	2.1	5.0
10.0	9.04 ± 0.25	2.5	5.6

was 7.0% (range 2.1–16.4%, depending on the concentration). The inter-assay C.V. was 11.8% (5.0–24.9%).

One person can process *ca.* 80 samples per week in duplicate, including sample weighing and evaluation of the chromatograms. The capacity of the HPLC instrument is not limiting, and would easily allow the four-fold analysis of samples.

Determination in biological samples

The concentrations of skatole in faeces of several species are shown in Table II. Compared with those in monogastric species, the concentrations in the faeces of ruminants are generally lower. The mean concentrations varied between 0.5 μg/g

TABLE II

CONCENTRATIONS OF SKATOLE IN FAECES OF SEVERAL SPECIES

N.D. = not detectable.

Species	Sex	n	Concentration (μg/g wet weight)	
			Mean ± S.D.	Range
Sheep	Female	10	5.1 ± 3.3	N.D.–11.4
Goat	Female	10	1.8 ± 0.8	0.5–3.2
Cattle	Female	10	0.5 ± 1.4	N.D.–4.5
	Male	10	3.2 ± 2.3	N.D.–6.6
Wild pig	Female	8	3.2 ± 3.1	N.D.–7.5
	Male	4	3.5 ± 3.3	N.D.–6.5
Domestic pig	Immature	10	26.6 ± 4.4	16.0–35.9
	Female	15	9.9 ± 8.6	N.D.–27.1
	Male	15	10.0 ± 5.4	1.5–23.1
Human	Female	6	15.1 ± 14.9	2.8–43.9
	Male	9	15.9 ± 11.6	1.2–35.4

of faeces (cows) and 5.1 μg/g of faeces (ewes). In cows, nine out of the ten samples were below the detection limit, the remaining sample revealed a concentration of 4.5 μg/g, explaining the low average and the high standard deviation.

The concentrations of skatole in the faeces of mature wild pigs are lower ($p \leq 0.05$) than in mature domestic pigs. The mean concentrations did not vary with the sex of the animal in either wild or domestic pigs. Female domestic pigs, however, showed a greater variation than males. By far the highest skatole concentrations were measured in the faeces of young growing pigs.

Skatole concentrations in human samples were found to be in the same range as those in porcine samples, but revealed the highest variability, as indicated by both the large standard deviations for both sexes and the range of concentrations. No clear sex differences were found.

DISCUSSION

A rapid and accurate method for skatole determination in faeces was developed. Compared with other methods [1,5–7,16,17], the sample preparation was improved in that only small volumes of organic solvents are necessary. The evaporation of solvents is also avoided, thus improving both the precision and the practicability. Only one method has been previously reported for skatole determination using a colorimetric determination [18], which is more rapid, although the precision and accuracy are apparently inferior to that of GC [14].

The accuracy and precision of our method are comparable with other methods, which require a much more time-consuming sample preparation. The sensitivity is higher at 220 nm than at 280 nm. However, because of the high concentrations that occur naturally in faeces, the sensitivity is not crucial. We suggest routine determination at 280 nm, which also covers the range of high concentrations generally found in faeces. In addition, determination at 280 nm allows the use of a calibration curve in buffer and hence a simplified preparation, instead of addition to faeces.

The skatole concentrations in the faeces of several species clearly differ between ruminants and monogastrics. In ruminants it is assumed that the low amounts of skatole originate from ruminal fermentation [19,20], whereas a contribution of the intestinal tract has not been determined so far.

Skatole concentrations in the faeces of pigs were reported for immature growing animals [5,7,16], and were similar to those found in our study. In addition, measurements along the intestinal tract [1,17] revealed that the caecum and colon are the main sites of skatole formation. The concentrations in these compartments agree well with those in porcine faeces.

The measurements in samples of mature wild pigs and domestic pigs suggest a dependency on the anabolic status. Three-fold increases in the concentration in samples from domestic pigs compared with those from identically fed wild pigs are consistent with the three-fold difference in the endogenous level of growth

hormone reported earlier [21]. The higher variation of skatole concentrations in female pigs may be attributable to differences in the stage of the ovarian cycle. Such a dependency, however, needs further clarification.

In case of human faecal samples, the high variation may be explained by individual eating habits, which were not standardized in this screening study. Excessive protein consumption far beyond requirements probably led to the very high concentrations. An attempt [22] to relate skatole concentrations in human faecal samples to diseases of the colon revealed a range similar to that found in our study, but did not confirm the suggested relationship.

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