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QUALITATIVE AND QUANTITATIVE ANALYSIS OF SOME SYNTHETIC, CHEMICALLY ACTING LAXATIVES IN URINE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A method for the qualitative and quantitative simultaneous analysis of dioxyanthraquinone, desacetyl-Bisacodyl, phenolphthalein and Oxyphenisatin in human urine using gas chromatography—mass spectrometry (GC—MS) has been developed. The compounds were extracted from urine at pH 7.5 with diethyl ether using Extrelut extraction columns, followed by evaporation and trimethylsilylation.

The method used electron beam ionization GC—MS employing a computer-controlled multiple-ion detector (mass fragmentography). The recovery from urine for the various compounds was between 80% and 100%. The detection limit for these compounds was in the range 0.01—0.05 μg/ml of urine.

The method proved to be suitable for measuring urine concentrations for at least four days after administration of a single oral low therapeutic dose of the laxatives to sixteen healthy volunteers.

INTRODUCTION

It has been demonstrated, that chemically acting laxatives are more dangerous than was expected [1—3]; for example, Oxyphenisatin caused liver damage after use over a prolonged period [4—6] and the number of false diagnoses due to laxative abuse increased [7]. The need for reliable information on laxative abuse is evident in the light of these experiences. It seemed appropriate, therefore, to select dioxyanthraquinone, Bisacodyl, phenolphthalein and Oxyphenisatin as the most frequently used laxatives and investigate the possibilities for their qualitative and quantitative analysis in human urine. The chemical structures of these compounds are given in Fig. 1.

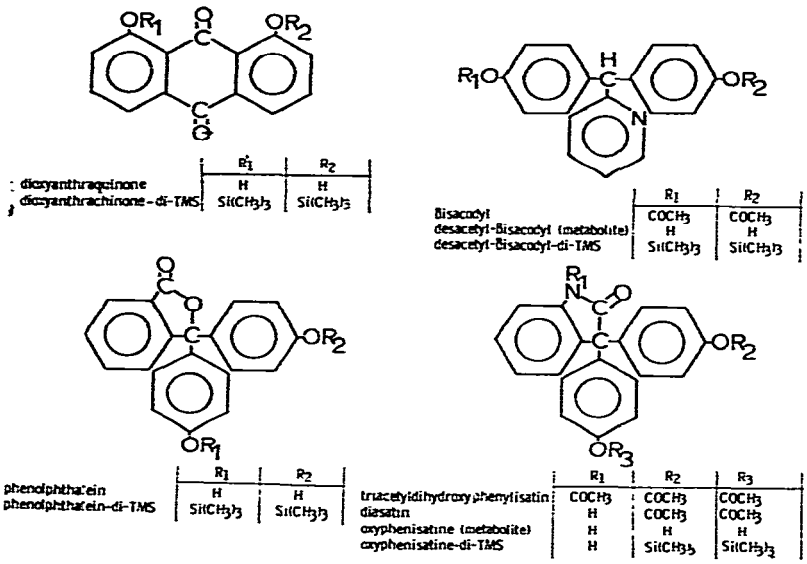

Fig. 1. Chemical structures of the laxatives, some metabolites and their TMS derivatives.

Only a limited amount of work has been done on the qualitative and quantitative analysis of laxatives in human urine. A method for the determination of underivatised dioxyanthraquinone in human urine based on gas chromatography (GC) with flame-ionization detection (FID) has been described [8]. However, dioxyanthraquinone showed unfavourable GC properties, while the other laxatives did not elute at all. This problem can be solved by trimethylsilylation. The method, however, requires extensive clean-up of samples because of many naturally occurring interfering substances.

Some methods for the analysis of laxatives using thin-layer chromatography (TLC) have been developed in various laboratories. A TLC method for the determination of dioxyanthraquinones based on fluorescence densitometry has been described [9]. The method is sensitive, but requires fluorescent laxatives and was not applied to the analysis of human urine. Some qualitative TLC methods [10, 11] based on visualisation of the spots in UV light or using various spray reagents have been developed, but only one method [12] has been used for qualitative analysis of Bisacodyl in human urine and faeces. When this method was used for the simultaneous qualitative analysis of the four selected laxatives in human urine, many problems were encountered with naturally occurring substances, due to lack of selectivity of the spray reagents. Most problems in analysis were caused by Bisacodyl and Oxyphenisatin, which are excreted in low concentrations in urine. In these cases it was necessary to repeat the TLC analysis with the isolated suspected spots from the first TLC separation, but the results were inadequate. Since desacetylation of Bisacodyl is a very important metabolic pathway [13, 14], TLC analysis of desacetyl-Bisacodyl in the urine was also performed, but only slightly better results were obtained.

The GC–mass spectrometric (MS) method reported here quantitates dioxyanthraquinone, desacetyl-Bisacodyl, phenolphthalein and Oxyphenisatin as

their trimethylsilyl derivatives. The sensitivity and specificity of the method fulfil the requirements for its use in investigations into abuse of the selected laxatives. The GC—MS method is able to quantitate 0.1 μg of each compound per milliliter of urine and has a detection limit for the different compounds of about 0.01—0.05 μl/ml.

The method was successfully tested for each investigated laxative on five healthy volunteers who each received a single oral low therapeutic dose. Their 24-h urines were collected over the next four consecutive days [15]. However, Oxyphenisatin was given to only one healthy volunteer because of its dangerous side-effects.

EXPERIMENTAL

Instrumentation and analysis conditions

All analyses were carried out on a Finnigan 3200 mass spectrometer connected by a glass jet-separator to a Finnigan 9500 gas chromatograph. Multiple-ion monitoring (mass fragmentography) was processed by the Finnigan 6000 data system. The mass spectrometric conditions were: electron energy, 70 eV; emission current, about 250 μA; ion source temperature, 80—100°C.

The GC column was a silanized U-shaped column, 180 cm × 3 mm I.D., packed with 3.8% SE-30 on Chromosorb W AW DMCS HP (80—100 mesh), operated isothermally at 280°C with a helium flow-rate of 20 ml/min. Injection port temperature was 280°C, and the glass jet-separator temperature was 270°C.

The derivatization conditions for the trimethylsilyl reagent were investigated using a Hewlett-Packard 5730A gas chromatograph equipped with a flame-ionization detector under the following conditions: injection port temperature, 300°C; detector temperature, 300°C. A silanized spiral-shaped glass column, 180 cm × 3 mm I.D., packed with 3.8% SE-30 on Chromosorb W AW DMCS HP (80—100 mesh), was operated with a temperature program from 230°C to 270°C at a rate of 8°C/min. Helium was used as carrier gas with a flow-rate of 35 ml/min.

Reagents and solvents

The extraction was carried out using Extrelut extraction columns (E. Merck, Darmstadt, G.F.R.) with diethyl ether (pro Analyse, Merck).

The trimethylsilyl reagent was prepared just before use by mixing trimethylchlorosilane, hexamethyldisilazane and pyridine (1:3:6, v/v; pro Analyse, (Merck).

Enzymatic hydrolysis of glucuronides was carried out using Ketodase (5000 Fischmann units of β-glucuronidase per milliliter; Warner Lambert Company, New Jersey, Ireland).

Chloroform (pro Analyse, Merck) was used for the preparation of standard solutions.

Reference compounds

The compounds used were dioxyanthraquinone and phenolphthalein (both from Brocades ACF, Maarssen, The Netherlands), Oxyphenisatin (Winthrop

Laboratories, Fawdon, Great Britain) and desacetyl-Bisacodyl, prepared in our laboratory by acid hydrolysis of Bisacodyl (Brocades ACF).

Standard curves

Known amounts of the pure laxatives were dissolved and diluted in chloroform to give the desired concentration range. For dioxyanthraquinone, desacetyl-Bisacodyl and Oxyphenisatin, standard curves of 25—500 μg/ml were used, covering urine concentrations of 0.17—3.33 μg/ml. For phenolpthalein a standard curve of 40—900 μg/ml was used, covering urine concentrations of 0.27—6.0 μg/ml.

A 100 μl volume of each standard solution was evaporated to dryness and derivatized with 100 μl of trimethylsilyl reagent prior to GC—MS analysis in the same series as the urine extracts.

Hydrolysis of glucuronides

To 15 ml of urine in a 25-ml conical flask was added 1 ml of acetic acid—sodium acetate buffer (pH 4.5, 1 *M*) and if necessary adjusted to pH 4.5. Then 0.5 ml of Ketodase preparation was added and the sample was placed in a thermostatted water-bath at 37°C and allowed to hydrolyse for 1 h.

Extraction

The hydrolysed urine sample was adjusted to pH 7.5 by adding 2 ml of phosphate buffer (pH 7.5, 1 *M*) and if necessary a few drops of 1 *N* NaOH. The sample was then poured into an Extrelut extraction column and allowed to absorb for 10 min. Then the laxatives were extracted by eluting the column first with 40 ml and then with 20 ml of diethyl ether. The combined eluents were dried over anhydrous Na_2SO_4 and collected in a 100-ml evaporating dish. The extract was concentrated on a steam-bath, quantitatively transferred to a 10-ml Teflon-stoppered glass tube and evaporated to dryness in a thermostatted water-bath of 37°C under a gentle stream of dried air.

Derivatization

The residue was dissolved in 100 μl of trimethylsilyl reagent and allowed to react for 15 min at room temperature in a Teflon-stoppered glass tube prior to GC—MS analysis.

Gas chromatography—mass spectrometry

Between 0.5 and 5.0 μl of derivatized urine extract was injected into the GC—MS interspersed with 5-μl injections of derivatized standard solutions to provide a standard curve. The multiple-ion detector measured the abundances of the following selected ions: *m/e* 297, 369 for di-trimethylsilyl-dioxyanthraquinone and *m/e* 343, 421 for di-trimethylsilyl-desacetyl-Bisacodyl during the first 4.5 min; *m/e* 253, 418 for di-trimethylsilyl-phenolphtahlein and *m/e* 268, 432 for di-trimethylsilyl-Oxyphenisatin during the next 9.0 min.

Calculations

For qualitative analysis the area ratio of the two selected ion profiles for each trimethylsilylated laxative as well as the GC retention time of the sub-

stance isolated from the urine sample were compared to the pure trimethylsilylated standard, which was injected in the same series.

For quantitative determination a direct calibration curve was used (ion-peak area vs. concentration). The standards were injected alternately with unknowns. The concentration of the unknowns were calculated from the peak area ratio of the selected ion profile of the unknown and the standard and afterwards corrected for recovery, the aliquot injected and dilutions incurred during the sample processing.

The ions *m/e* 369, 421, 418 and 432 were used for quantitation of dioxyanthraquinone-di-TMS, desacetyl-Bisacodyl-di-TMS, phenolphthalein-di-TMS and Oxyphenisatin-di-TMS, respectively.

Recovery

The overall recovery of dioxyanthraquinone, desacetyl-Bisacodyl, phenolphthalein and Oxyphenisatin was determined by adding known microgram amounts to blank urine, and analysing them by the procedure described above.

To obtain the right concentration of a laxative in urine, 1.0 ml of a standard solution in chloroform at the desired concentration was evaporated to dryness in a water-bath at 37°C under a gentle stream of dried air. The residue was dissolved in 100 μl of ethanol and then in 99.9 ml of urine. In this way two different urine concentrations were prepared for each laxative (exact concentrations are given in Table I). The standard urines were stored at −20°C and defrozen in a water-bath at 37°C prior to analysis.

Test of the method in healthy volunteers

The method was tested in sixteen healthy volunteers. Each time a low therapeutic oral dose was taken of one of the laxatives according to Table II. After intake of the laxative the urine was collected in 24-h volumes for four consecutive days. The 24-h urine samples were homogenized and an aliquot of 100 ml was stored at −20°C until analysis. The urine samples were defrozen at 37°C in a thermostatted water-bath and 15 ml were used for analysis.

RESULTS AND DISCUSSION

Experience in our laboratory over the last five years in the analysis of laxative abuse has indicated a lack in specificity of detection using TLC or GC combined with FID. False positives were readily obtained when a GC method was used without the benefit of a mass spectrometer. The mass spectrometer was used in the multiple-ion detector mode, which offers the required sensitivity to produce reliable quantitative data.

The combination of trimethylsilyl derivatisation with gas chromatography on SE-30 was chosen because of the excellent separation and peak shape characteristics obtained with this system (Fig. 2). The trimethylsilylation of the laxatives proceeded within 5 min at room temperature. Reaction times of up to 1 h at room temperature were investigated. Reaction times longer than 5 min did not increase the peak height of the trimethylsilyl derivatives. A disadvantage, however, was that the trimethylsilyl derivative of dioxyanthraquinone de-

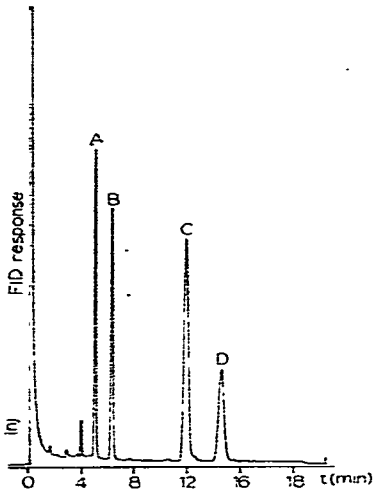

Fig. 2. Temperature-programmed gas chromatogram of the TMS derivatives of the laxatives with FID. (A) di-trimethylsilyl-dioxyanthraquinone; (B) di-trimethylsilyl-desacetyl-Bisacodyl; (C) di-trimethylsilyl-phenolphthalein; (D) di-trimethylsilyl-Oxyphenisatin.

composed slightly under these chromatographic conditions, but this never exceeded 5% (calculated from the peak area ratios of the decomposition product and dioxanthraquinone-di-TMS, with FID).

Mass spectra of the four trimethylsilylated laxatives are shown in Fig. 3.

Linear standard curves (six points), constructed by plotting the peak areas of the selected fragment ions against the concentration of the four trimethylsilylated laxatives, were obtained. Correlation coefficients in the concentration range 25—500 μg/ml of 0.997, 0.999 and 0.998 were found for dioxyanthraquinone-di-TMS, desacetyl-Bisacodyl-di-TMS and Oxyphenisatin-diTMS, respectively. The correlation coefficient for phenolphthalein-di-TMS in the concentration range 40—900 μg/ml was also 0.999.

Extraction of urine at pH 7.5 provided quantitative extraction of the four laxatives investigated and a relative minimum of endogenous substances were co-extracted. Extraction using Extrelut extraction columns instead of separation funnels was less time-consuming and more reproducible because no strong emulsions could form, and cleaner extracts were obtained. The overall recovery and reproducibility of the method for each laxative at two concentration levels in urine are summarized in Table I.

The detection limit was found to be 0.01 μg/ml of urine for dioxyanthraquinone and desacetyl-Bisacodyl, 0.03 μg/ml of urine for phenolphthalein, and 0.05 μg/ml of urine for Oxyphenisatin (signal-to-noise ratio ≥ 3).

The time required for enzymatic hydrolysis of the glucuronides of the phenolic laxatives was investigated for up to 15 h at 37°C. Incubation times longer than 1 h did not increase the yield of free phenolic laxatives.

The method was tested in sixteen healthy volunteers. Using the method it was possible to measure the administered laxative or its metabolite in the urine of every volunteer for at least four days. Representative mass fragmentograms of the urine extracts are shown in Fig. 4.

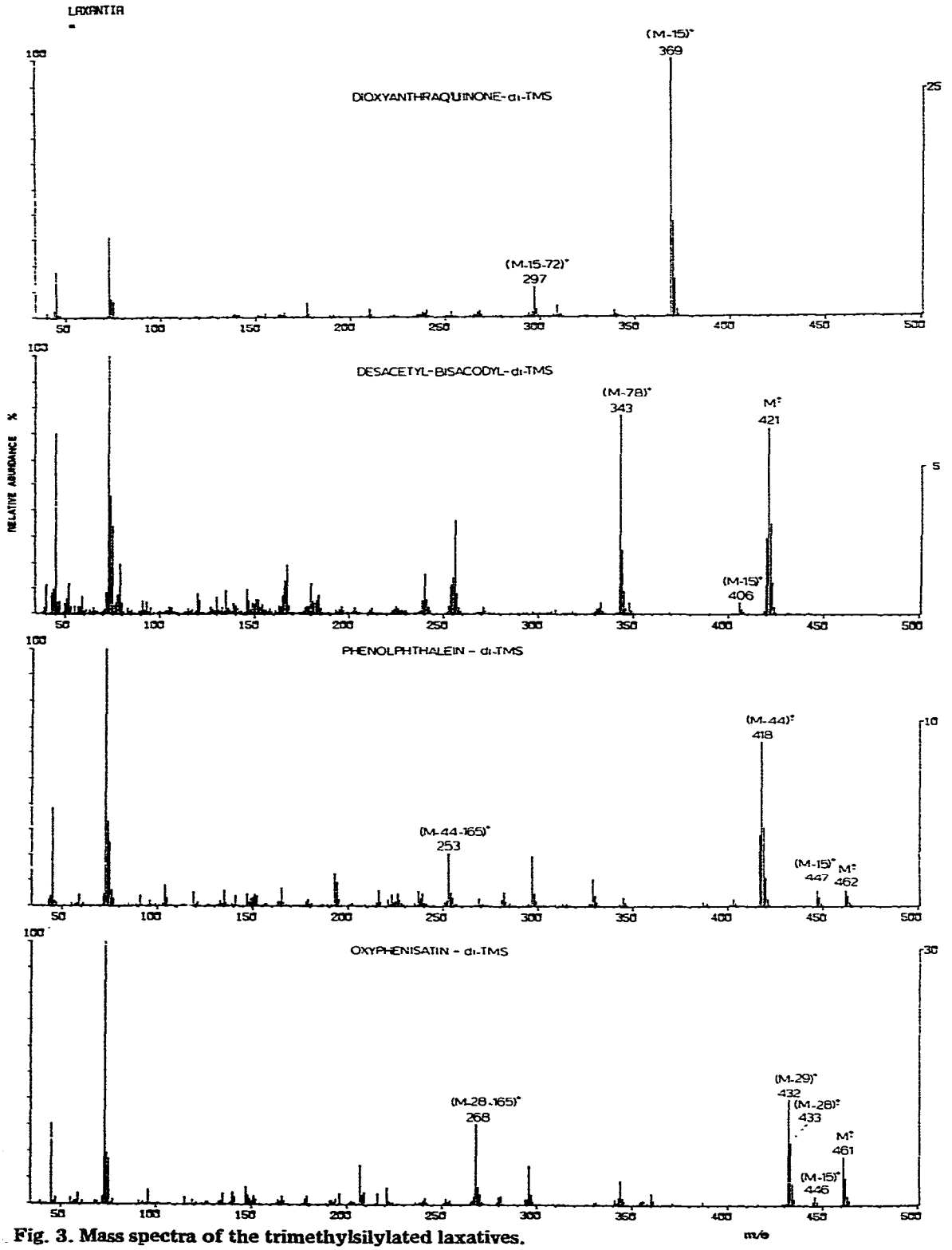

Fig. 3. Mass spectra of the trimethylsilylated laxatives.

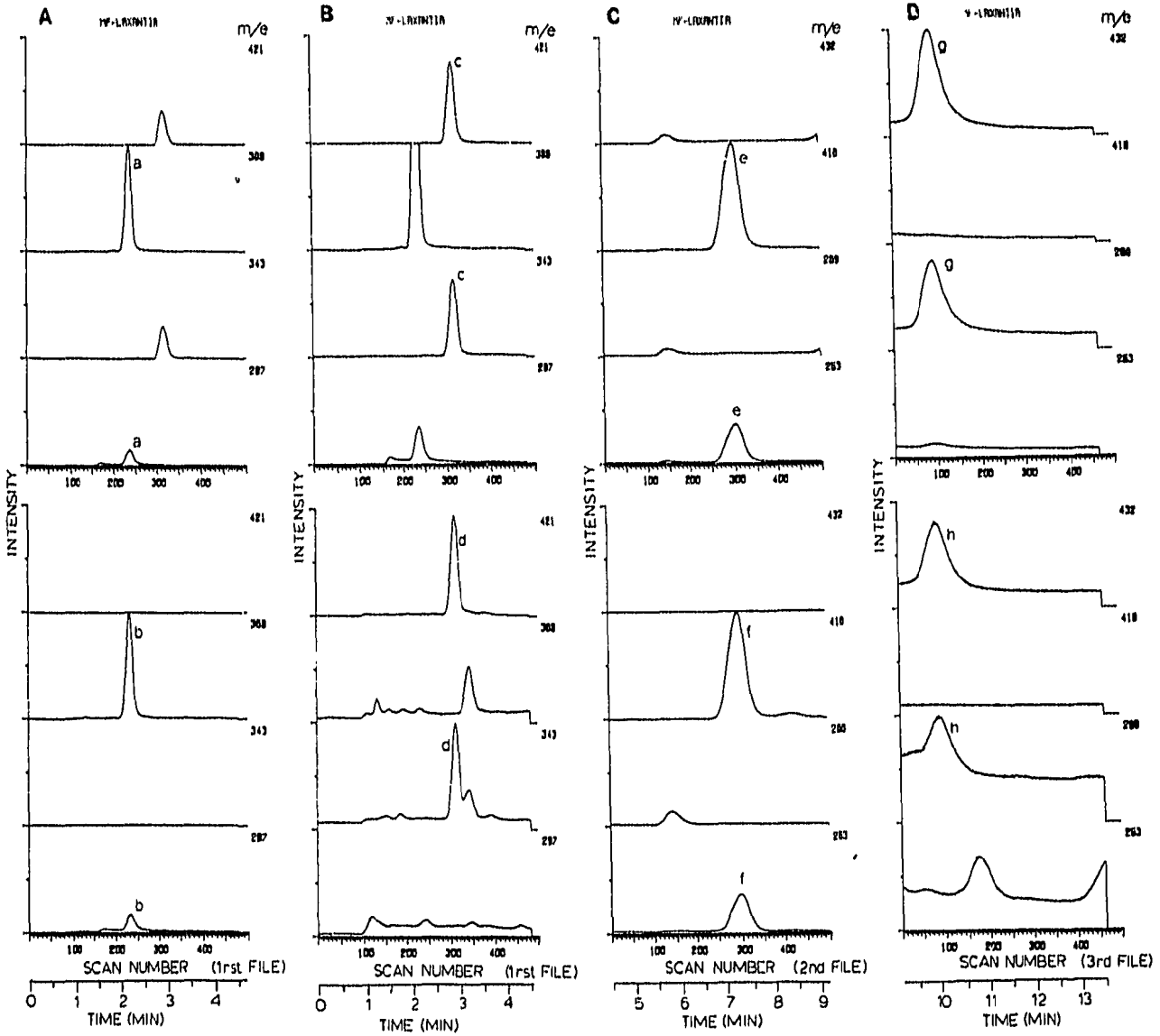
A
B
C
D
m/e
INTENSITY
SCAN NUMBER (1rst FILE)
SCAN NUMBER (2nd FILE)
SCAN NUMBER (3rd FILE)
TIME (MIN)

TABLE I

OVERALL RECOVERY AND REPRODUCIBILITY OF THE METHOD

	Concentration after addition to urine (mg/l)	No. of determinations	Mean concentration found in urine (mg/l)	Standard deviation (mg/l)	Mean recovery (%)
Dioxyanthraquinone	0.254	8	0.261	0.029	103
	2.54	9	2.10	0.092	83
Desacetyl-Bisacodyl	0.218	8	0.233	0.015	93
	2.18	9	2.20	0.129	101
Phenolphthalein	0.434	8	0.427	0.032	98
	4.34	9	3.95	0.214	91
Oxyphenisatin	0.250	8	0.248	0.029	99
	2.51	9	2.539	0.195	101

The concentration range found for each laxative or its metabolite in the urine is summarized in Table II. These calculations resulted in the following quantitative information about excretion rate. Dioxyanthraquinone is mostly excreted in the first 24 h after administration, and ranged from 7% to 23% of the dose in five volunteers, with a mean of 17%. In the following three consecutive days the excretion of dioxyanthraquinone was less than 1% of the dose in each volunteer.

TABLE II

CONCENTRATION RANGE FOR EACH LAXATIVE OR METABOLITE FOUND IN THE URINE AFTER ORAL ADMINISTRATION

No. of volunteers	Laxative tested	Formulation	Oral dose	No. of consecutive days over which analyses were performed	Found concentration range in urine (mg/l)
5	Dioxyanthraquinone	1 X ½ tablet Istizin	75 mg	4	0.5—30
5	Bisacodyl	1 X 1 drageee Dulcolax	5 mg	4	0.1— 1.4
5	Phenolphthalein	1 X 1 tablet Fructine	120 mg	4	1 —16
1	Oxyphenisatin	1 X 1 tablet Diasatine	5 mg	4	—

Bisacodyl was measured in the urine as its metabolite desacetyl-Bisacodyl. Among the five volunteers a large variation in excretion was observed. The total excretion over four days ranged from 8% to 46% of the dose with a mean of

Fig. 4. (A) Representative mass fragmentograms of dioxyanthraquinone-di-TMS analysis of (b) the first 24-h urine from a healthy volunteer after a single oral dose of 75 mg dioxyanthraquinone, and (a) the pure dioxyanthraquinone-di-TMS standard. (B) Representative mass fragmentograms from desacetyl-Bisacodyl-di-TMS analysis of (d) the first 24-h urine from a healthy volunteer after a single oral dose of 5 mg of Bisacodyl, and (c) the pure desacetyl-Bisacodyl-di-TMS standard. (C) Representative mass fragmentograms from phenolphthalein-di-TMS analysis of the (f) first 24-h urine from a healthy volunteer after a single oral dose of 125 mg of phenolphthalein, and (e) the pure phenolphthalein-di-TMS standard. (D) Representative mass fragmentograms from Oxyphenisatin-di-TMS analysis of (h) the first 24-h urine from a healthy volunteer after a single oral dose of 5 mg of Diasatin (diacetyl-Oxyphenisatin) and (g) the pure Oxyphenisatin-di-TMS standard.

23%, while the maximum excretion was reached on the second day. These quantities correlate with earlier published data on the excretion of desacetyl-Bisacodyl as its glucuronide after intake of 5 mg of Bisacodyl [16].

The phenolphthalein excretion over four days ranged from 11% to 31% with a mean of 22%, with maximum excretion in the first two days.

Oxyphenisatin can be administered in several forms, for example as the di- and tri-acetate. Analogous to Bisacodyl, di- and tri-acetyl-Oxyphenisatin are deacetylated in an alkaline reaction in the intestinal tract [17]. One healthy volunteer took 5 mg of Diasatin (diacetyl-Oxyphenisatin) and it was possible to measure Oxyphenisatin for three days, with maximum excretion on the second day. Since Oxyphenisatin was tested in only one volunteer, mean values for excretion are not available.

Finally, it may be concluded that the GC—MS method described had proved to be sufficiently specific and sensitive to analyse laxative abuse for the four investigated laxatives both qualitatively and quantitatively. The method may be helpful in investigating questions dealing with the extent of biotransformation, the dependence of the excretion rate on urinary pH [18] and laxative abuse.

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