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SCREENING PROCEDURE FOR STIMULANT LAXATIVES IN URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE ARRAY DETECTION

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SUMMARY

We describe a liquid chromatographic screening procedure for the detection of stimulant laxatives in urine. A 2-ml urine sample was incubated with 500 U of β-glucuronidase for 2 h at 60°C. The sample was acidified with sodium acetate (pH 5.0) and extracted with 5 ml of an isopropanol–chloroform (1:9) mixture. The organic layer was cleaned up further by washing with 5 ml disodium hydrogen-phosphate (pH 7.5) before being transferred to a conical tube and evaporated to dryness The residue was reconstituted in 100 μl mobile phase and 3 μl were injected onto a Hewlett-Packard Hypersil ODS (5 μm) column. The ultraviolet absorbance of the eluent was monitored at 225 nm. Rhein, bisacodyl diphenol, bisoxatin diphenol, phenolphthalein, bisacodyl, bisoxatin and danthron all eluted within 6 min. The screen was evaluated using urine specimens obtained from 19 patients who claimed they had taken one or more of the laxatives under consideration within the past 48 h. Only two patients who claimed to have taken Coloxyl with Danthron® showed negative results. Eighteen of twenty laxatives (90%) taken by the patients were detected and their identity verified by plotting post-run ultraviolet spectra. We therefore conclude that the screen is sufficiently reliable to be of help in the early detection of surreptitious abusers of stimulant laxatives.

INTRODUCTION

The deliberate administration of laxatives is one of the oldest forms of medical treatment known to man [1]. Unfortunately this tradition of colon cleanliness persists even in today's twentieth century lifestyle and many reports of laxative abuse have appeared in the literature in this decade alone [2–24]. The great majority of long-term and often surreptitious abusers of laxatives are usually unaware of the serious gastrointestinal and electrolyte disorders, among others, that may occur with constant purgation [25]. Patients who conceal their laxative taking may undergo prolonged and uncomfortable investigations which could lead

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to surgery if a positive diagnosis of laxative abuse is not made. Thus, when reliable information is not available from the patient, the screening of a urine sample for the presence of laxatives may help in the early diagnosis of laxative abuse.

The stimulant laxatives, composed of the diphenylmethanes (e.g. phenolphthalein, bisacodyl and bisoxatin) and the anthraquinones (e.g. senna, cascara, frangula, aloes and danthron), cause the greatest side-effects and are the most abused by the public [18,26]. Only four screening procedures for the detection of these laxatives in biological specimens have been reported [27–30]. Three of these methods used thin-layer chromatography (TLC) [27–29]. The method of Vyth and Kamp [27] only addressed the detection of anthraquinone glycosides by the identification of rhein (a metabolic end product) in urine. The diphenylmethane laxatives were not considered at all. De Wolff et al. [28] examined all the stimulant laxatives but required large samples volumes, needed to run two chromatographic plates, each taking 12 min, and were unable to verify spots with retention factors corresponding to a laxative. In a modification of the above procedure, Morton [29] demonstrated that only one chromatographic plate needed to be run if bisoxatin and oxyphenisatin were excluded from the screen. The inability to verify spots obtained by TLC, however, could lead to a high incidence of false positives, as has been reported [30]. Kok and Faber [30] used a gas chromatographic–mass spectrometric (GC–MS) screening procedure and could verify peaks by obtaining mass spectra. Their procedure, however, did not detect the naturally occurring anthraquinone laxatives senna, cascara, frangula and aloes.

This paper describes a high-performance liquid chromatographic (HPLC) procedure with ultraviolet spectrophotometric diode array detection to identify the covert use of laxatives in the stimulant class.

EXPERIMENTAL

Reagents

All reagents were of analytical or HPLC grade. Water was deionized and filtered (18 MΩ purity).

Laxative compounds used in the study, either directly or as a derivatized product thereof, were kindly provided by the manufacturers: bisacodyl and danthron, Fawns and McAllan (Croydon, Australia); phenolphthalein and cascara, Parke-Davis (Caringbah, Australia); senna and rhein, Reckitt and Colman (West Ryde, Australia); and bisoxatin, Boehringer Ingelheim (Artarmon, Australia). Stock solutions in ethanol containing 0.4 mg/ml of each drug/metabolite were stored at 4°C.

β-Glucuronidase used to hydrolyse drug glucuronide conjugates was purchased from Sigma (St. Louis, MO, U.S.A.). The β-glucuronidase was from *Escherichia coli* and had an activity of 1 375 000 Sigma units per gram.

Chromatography

A Hewlett-Packard 1090A liquid chromatograph equipped with a diode array detector was used for all screens. A Hewlett-Packard Hypersil ODS (5 μm) column, 100 mm×2 mm I.D., was used at 50°C to separate the laxatives studied.

The flow-rate of the mobile phase, acetonitrile in 0.01 mol/l phosphate buffer (pH 5.0), was maintained at 0.5 ml/min while a gradient was used to increase the acetonitrile content from 20% initially to 45% at 3.0 min. All seven peaks of interest eluted within 6.0 min. The diode array detector was set to monitor the signal to the integrator at 225 nm, however, spectral data between 210 and 400 nm were stored in computer memory (Hewlett-Packard 85B) and this could be plotted at the end of the run.

Synthesis of metabolites

Bisacodyl and bisoxatin are both metabolised in vivo via deacetylation to the active metabolites bisacodyl diphenol and bisoxatin diphenol, respectively, which in turn are glucuronidated prior to excretion in urine. These metabolites were prepared by alkaline hydrolysis of the parent drugs: to 5 ml of 0.4 mg/ml solutions of bisacodyl and bisoxatin in ethanol were added 25 μl of 6 mol/l sodium hydroxide. The tubes were heated at 60°C for 1 h, cooled and then neutralized with 25 μl of 6 mol/l hydrochloric acid. The solutions were stored at 4°C.

This procedure ensured 98% conversion of these drugs into the diphenol products with less than 2% of the respective monophenol products being obtained due to incomplete hydrolysis.

Urine extraction

As phenolphthalein, bisacodyl diphenol and bisoxatin diphenol are excreted in the urine only as the glucuronide conjugates, it was necessary to liberate the free drug by carrying out a β-glucuronidase incubation.

To 2-ml urine samples were added 100 μl of 3.6 mg/ml (5000 U/ml) β-glucuronidase in 0.1 mol/l Na_2HPO_4–KH_2PO_4 buffer (pH 6.8). Following incubation for 2 h at 60°C, 200 μl of 2.2 mol/l sodium acetate buffer (pH 5.0) and 5 ml of a 10% isopropanol in chloroform mixture were added. The tubes were vortex-mixed for 2 min and centrifuged at 1500 *g* for 5 min. The aqueous layer was then removed by aspiration and 5 ml of 0.1 mol/l Na_2HPO_4 (pH 7.5) were added to the remaining organic phase. The samples were again extracted for 2 min and centrifuged as above. The organic phase was transferred to a conical tube and evaporated to dryness at 37°C under a gentle stream of nitrogen. The residue was then reconstituted with 100 μl of 50% acetonitrile in 0.01 mol/l phosphate buffer (pH 5.0) of which 3 μl were injected into the chromatograph.

Recovery study

Urine samples were spiked to contain 5 μg/ml of each of the drugs/metabolites being studied and extracted as described. The chromatographic peak heights obtained were then expressed as a percentage of the respective peak heights of an aqueous solution containing 100 μg/ml of each drug injected directly into the chromatograph.

The extraction recovery in the absence of the wash step with 0.1 mol/l Na_2HPO_4 (pH 7.5) was also evaluated in order to determine the amount of drug being lost at this stage.

Sensitivity study

This study was carried out to determine the detection threshold for each drug included in the screening process.

The 0.4 mg/ml solutions were used to prepare a composite solution containing 20 μg/ml phenolphthalein, 40 μg/ml bisacodyl, bisacodyl diphenol, bisoxatin, bisoxatin diphenol and danthron and 80 μg/ml rhein. The composite solution was used to spike urine samples in decreasing concentrations. These samples were then extracted and the minimum detectable amount determined.

Patient study

This study was conducted in order to determine the ability of the screening procedure to successfully detect laxatives taken by patients as part of their normal medical treatment and to investigate the possibility of false positive results due to interferences from a range of other medications.

Urine samples were obtained from nineteen patients. The majority of these patients were attending a clinic at Mount Royal (geriatric) Hospital and were taking several prescribed medications. Urine samples were collected from patients who claimed they had taken at least one of the laxatives under consideration within the previous two days. These samples were chromatographed to assess the reliability of the screening procedure.

RESULTS

Chromatography

Fig. 1 shows a chromatogram of the extract of a spiked urine sample. The urine sample contained the following concentrations: 4 μg/ml rhein, 2 μg/ml bisacodyl diphenol, bisoxatin diphenol, bisacodyl, bisoxatin and danthron and 1 μg/ml phenolphthalein. The tiny peaks with retention times of 3.32 and 3.61 min are bisacodyl monophenol and bisoxatin monophenol, respectively, which are by-products formed during chemical synthesis of the metabolites of bisacodyl and bisoxatin.

Fig. 2 shows a trace of drug-free urine.

In countries where bisoxatin is unavailable and where interest lies only in the detection in urine of the surreptitious ingestion of bisacodyl, phenolphthalein, danthron and the naturally occurring anthraquinone glycosides it is possible to shorten the chromatographic run time from 6.0 to 4.5 min by increasing the gradient of acetonitrile in the mobile phase from the initial 20% to 65% at 3 min. Fig. 3 shows a trace of an extract of a spiked urine sample run under these conditions. The urine sample contained the following concentrations: 4 μg/ml rhein, 2 μg/ml bisacodyl diphenol and danthron and 1 μg/ml phenolphthalein.

The identity of laxative peaks detected in a patient's urine extract were verified by plotting the UV spectra of these peaks between 220 and 400 nm and comparing them to the UV spectra of known standards. A positive result was reported only if the UV spectrum of the suspected peak was identical to that of the laxative standard, when superimposed. In some cases the upslope, apex and downslope spectra of the suspected peak were all superimposed as a further check of peak

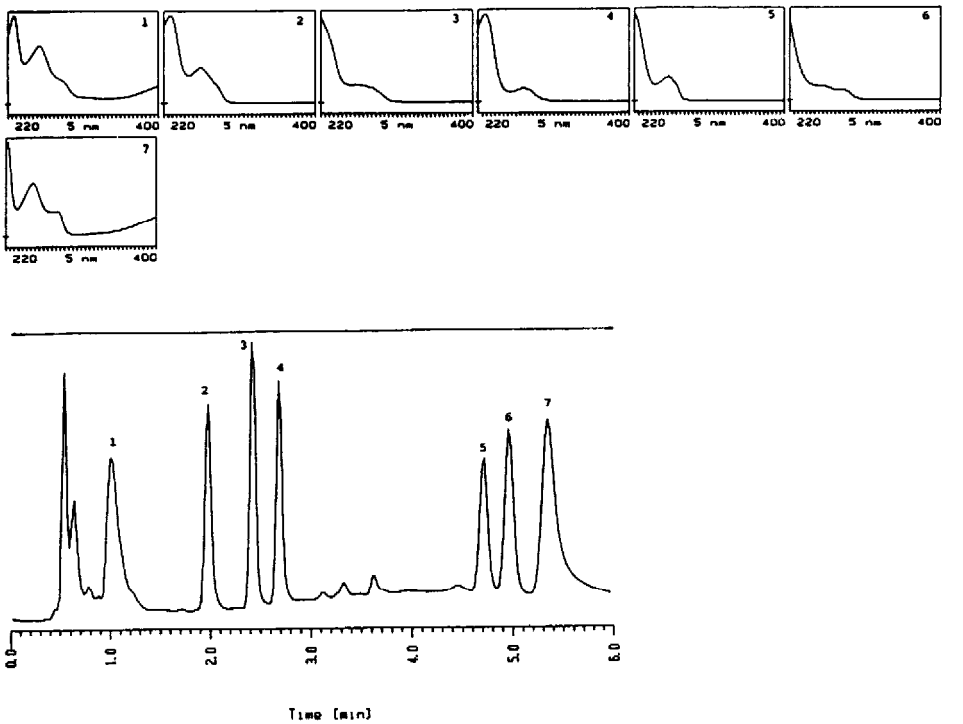

Fig. 1. Urine extract of seven laxatives/metabolites run at 0.1 a.u.f.s. (UV 225 nm). The UV spectrum of each peak is illustrated above the chromatogram. Peaks: 1 = rhein (4 μg/ml); 2 = bisacodyl diphenol (2 μg/ml); 3 = bisoxatin diphenol (2 μg/ml); 4 = phenolphthalein (1 μg/ml); 5 = bisacodyl (2 μg/ml); 6 = bisoxatin (2 μg/ml); 7 = danthron (2 μg/ml). The tiny peaks at 3.32 min and 3.61 min are bisacodyl monophenol and bisoxatin monophenol, respectively, being by-products from the chemical synthesis of bisacodyl and bisoxatin metabolites.

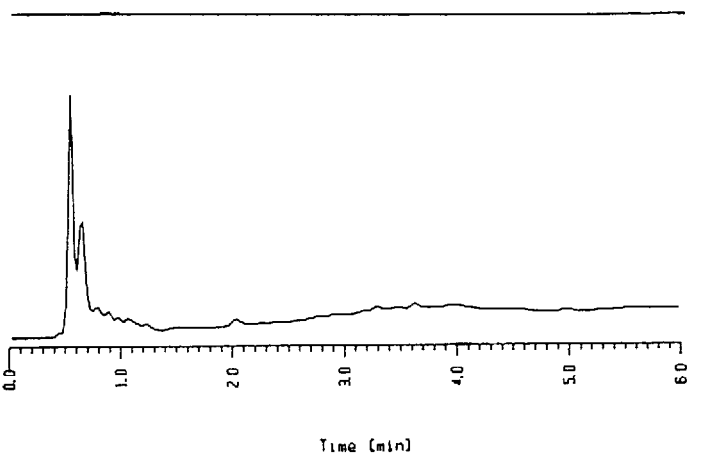

Fig. 2. Blank urine extract (at 225 nm).

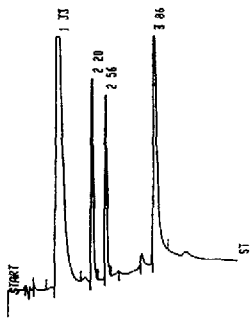

Fig 3 Urine extract of four laxatives/metabolites pertinent to Australia run at 0.128 a.u.f.s. (UV 225 nm). Peaks: 1.33 min = rhein (4 μg/ml); 2.20 min = bisacodyl diphenol (2 μg/ml); 2.56 min = phenolphthalein (1 μg/ml); 3.86 min = danthron (2 μg/ml).

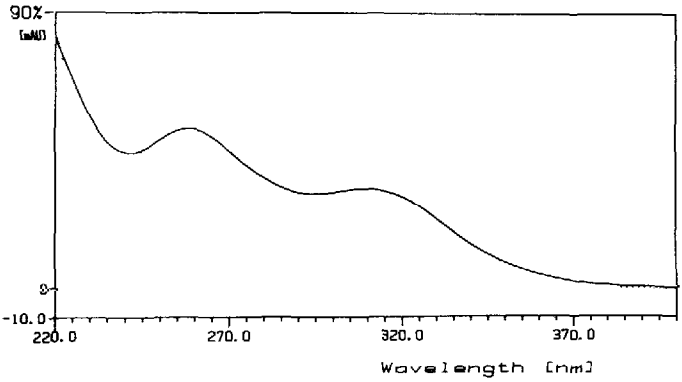

Fig. 4. Superimposed UV spectra of nitrazepam (—) and phenolphthalein (···) showing that they are easily distinguished despite their chromatographic retention times of 2.80 and 2.71 min, respectively.

purity. For example, nitrazepam (retention time 2.80 min) was found to co-elute with phenolphthalein (retention time 2.71 min), however, an examination of the UV spectra enabled these drugs to be easily distinguished (Fig. 4).

Recovery study

Recovery studies were based on extraction of 2-ml urine samples ($n=5$) containing 5 μg/ml of each drug. The percentages of each drug recovered in the extract are listed in Table I. The recovery figures ($n=3$) when the wash step with 0.1 mol/l Na_2HPO_4 (pH 7.5) was omitted from the extraction procedure have also been listed.

An examination of this table shows that, with the exception of rhein, the recovery figures are acceptable despite the wash step. The low recovery of rhein did not prove to be a problem in its detection as will be seen in the patient samples studied.

Sensitivity study

Chromatography was performed on extracts of 2-ml drug-free urine specimens ($n=5$) spiked to contain one quarter of the amount used in the reference stan-

TABLE I

EXTRACTION RECOVERY FROM 5 μg/ml SAMPLES

Drug	Recovery (mean ± S.D.) (%)	
	With wash step ($n=5$)	No wash step ($n=3$)
Rhein	13 ± 0.5	79 ± 1.5
Bisacodyl diphenol	84 ± 1.6	93 ± 2.9
Bisoxatin diphenol	68 ± 1.1	92 ± 2.1
Phenolphthalein	86 ± 1.1	92 ± 1.0
Bisacodyl	58 ± 1.5	62 ± 3.2
Bisoxatin	68 ± 1.9	68 ± 1.2
Danthron	71 ± 5.7	82 ± 3.1

TABLE II

SENSITIVITY GAUGE USING A 2-ml URINE SAMPLE ($n=5$)

Drug	Concentration (μg/ml)	Percent full scale deflection at 0.032 a.u.f.s. (mean ± S.D.)
Rhein	1.0	17 ± 1.3
Bisacodyl diphenol	0.5	66 ± 2.5
Bisoxatin diphenol	0.5	81 ± 3.3
Phenolphthalein	0.25	61 ± 2.8
Bisacodyl	0.5	25 ± 0.8
Bisoxatin	0.5	29 ± 1.1
Danthron	0.5	29 ± 1.5
Baseline noise	–	<1

dard, the final concentrations being 1.0 μg/ml rhein, 0.5 μg/ml bisacodyl diphenol, bisoxatin diphenol, bisacodyl, bisoxatin and danthron and 0.25 μg/ml phenolphthalein. At an integrator sensitivity setting of 0.032 a.u.f.s. all seven peaks were at least 17% of full scale deflection (Table II). Using 5% of full scale deflection as the detection limit (baseline noise <1%) the minimum detectable amounts of the laxative/laxative metabolites were: rhein 0.29 μg/ml, bisacodyl diphenol 0.04 μg/ml, bisoxatin diphenol 0.03 μg/ml, phenolphthalein 0.02 μg/ml, bisacodyl 0.10 μg/ml, bisoxatin 0.09 μg/ml and danthron 0.09 μg/ml.

Patient study

Of the nineteen patients who provided samples for screening (including one patient on two laxatives and two patients who provided samples on two separate occasions), ten had taken Coloxyl with Danthron®, three Agarol®, one Laxettes®, four Durolax® and two Senokot®.

The number of positive findings detected using the screening procedure, to-

gether with the determined concentrations, are shown in Table III. Only two patients who claimed to have taken Coloxyl with Danthron showed negative results. It is important to note that chromatograms of eight samples had peaks at retention times corresponding to a laxative other than that which the patient had taken. The post-run UV spectral analyses, however, revealed these peaks to be false positives in all cases.

TABLE III

RESULTS OF LAXATIVE SCREEN PATIENT STUDY

Patient	Age	Sex	Proprietary laxative	Approximate time since last dose (h)	Detectable ingredient	Urinary concentration (μg/ml)
AT	89	F	Coloxyl with danthron	48	Danthron	0.2
AT	89	F	Coloxyl with danthron	15	Danthron	1.0
EL	88	F	Coloxyl with danthron	17	Danthron	1.1
JB	88	F	Coloxyl with danthron	16	Danthron	1.3
FM	97	F	Coloxyl with danthron	24	Danthron	1.8
MC	76	F	Coloxyl with danthron	12	Danthron	3.1
LB	79	F	Coloxyl with danthron	15	Danthron	3.5
MB	81	F	Coloxyl with danthron	16	Danthron	4.0
WR	74	M	Coloxyl with danthron	14	Danthron	N.D *
SR	74	M	Coloxyl with danthron	16	Danthron	N.D.*
FM	88	F	Laxettes	36	Phenolphthalein	1.0
MH	82	F	Agarol	15	Phenolphthalein	1.9
ES	91	F	Agarol	16	Phenolphthalein	3.1
MO	79	F	Agarol	7	Phenolphthalein	8.2
MF	32	F	Durolax	10	Bisacodyl diphenol	0.3
UC	74	F	Durolax	36	Bisacodyl diphenol	0.7
HS	80	F	Durolax	15	Bisacodyl diphenol	0.7
MC	76	F	Durolax	12	Bisacodyl diphenol	2.5
RM	79	M	Senokot	15	Rhein	4.6
RM	79	M	Senokot	12	Rhein	6.8

*N.D. = not detected.

TABLE IV

LIST OF DRUGS PATIENTS WERE TAKING

Ascorbic acid	Frusemide	Paracetamol
Acetylsalicylic acid	Glyceryl trinitrate	Phenytoin
Amitriptyline	Hydrochlorothiazide	Prazosin
Amoxycillin	Ibuprofen	Prednisolone
Baclofen	Imipramine	Salbutamol
Benserazide	Isocal®	Sulindac*
Chloral hydrate	Levodopa	Sulphamethoxazole
Cimetidine	Methyldopa	Temazepam*
Codeine	Mianserin	Theophylline
Dienoestrol	Naproxen*	Tranylcypromine
Digoxin	Nitrazepam*	

*Drugs found to interfere with the screen.

All patients were taking their usual medications (Table IV) at the time of sample collection. An examination of the medication records of those eight patients whose samples produced a chromatographic false positive led to the drugs suspected of interference being injected directly into the HPLC system. Four drugs (asterisked in Table IV) were found to interfere with the chromatography and could only be distinguished by UV spectra analysis, as exemplified in Fig. 4. A separate comprehensive list of possible interfering substances was not prepared as all suspected laxative/laxative metabolite peaks were routinely checked by plotting and comparing UV spectra.

DISCUSSION

The HPLC system described here can be used to simply and reliably screen urine specimens/pharmaceutical preparations for seven laxatives or their metabolites. By slight alteration of chromatographic conditions the run time can be shortened to 4.5 min when screening urine samples only for laxatives available in Australia. Both procedures included detection of rhein which is a common metabolite of the naturally occurring anthraquinone glycoside laxatives (e.g. senna, cascara, frangula, aloes).

The sample preparation and chromatographic elution is rapid and compares favourably with other stimulant-laxative screening methods that utilize either TLC [27–29] or GC–MS [30]. The TLC methods do not have the ability to verify suspected spots and this could result in false positive findings being obtained as indeed has been reported by Kok and Faber [30] who have stated "Experience in our laboratory over the last five years in the analysis of laxative abuse has indicated a lack of specificity of detection using TLC or GC combined with flame ionization detection. False positives were readily obtained when a GC method was used without the benefit of a mass spectrometer". The GC–MS method described by Kok and Faber [30] had the ability to verify peaks by the collection of mass spectra but it did not allow detection of rhein and consequently the intake of the naturally occurring anthraquinone glycosides could not be established.

In the sample preparation described in the present study, a clean-up wash step with phosphate buffer pH 7.5 was necessary to obtain a clean extract for chromatography. This wash step caused the recovery of rhein to drop six-fold, from 79 to 13%. It was found, however, that the cleaner extract permitted the screen to be monitored at an eight-fold higher sensitivity setting than was previously possible and that the screening procedure was still capable of detecting rhein in urine down to levels of 0.3 μg/ml.

In the two patients who took senna, rhein was detected in levels (4.6 and 6.8 μg/ml) well above the detection limit (0.3 μg/ml) of the screen thus demonstrating that the reduction in extraction recovery from 79 to 13% was not deleterious. The only two negative results found in this study were both from patients who had claimed to have taken Coloxyl with Danthron. However, specimens from eight other patients who had taken the same laxative, 14 to 48 h prior to sample collection, showed positive results. This may mean a 20% false negative rate for the detection of danthron, but as danthron is readily detectable (average patient level 1.9 μg/ml and limit of detection 0.09 μg/ml) it may be that in the two

negative cases the dose was omitted or taken more than 48 h prior to sample collection.

A 42% false positive rate (eight of nineteen patients) would have occurred in the patient study if peak verification by obtaining UV spectra derived from the diode array detector had not been possible. This figure for false positives is in keeping with the findings of Kok and Faber [30] and exemplifies the absolute necessity for verification of peak identity. Finally, as eighteen of twenty laxatives (90%) taken by the patients, within 48 h of sample collection, were detected and identified using our screening procedure we conclude that the screen is sufficiently reliable to be of help in the early detection of surreptitious abusers of stimulant laxatives.

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