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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SCREENING FOR UNKNOWN SUNSET YELLOW FCF AND ORANGE GGN METABOLITES IN RAT FAECES USING A CONTINUOUS GRADIENT ELUTION SYSTEM

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SUMMARY

The water-soluble azo dyes, Sunset Yellow FCF and Orange GGN were administered in different doses to male Wistar rats by stomach intubation. The 24-h faeces were collected and analysed both for the parent dyes and those metabolic aromatic aminosulphonic acids which are also excreted in urine and are assumed to be potential metabolites in faeces: sulphanilic acid and N-acetylsulphanilic acid for FCF, metanilic acid and N-acetylmetanilic acid for GGN and the common metabolites 1-amino-2-naphthol-6-sulphonic acid (ANSA) and N-acetyl-ANSA.

Aqueous faeces suspensions were centrifuged and aliquots of the supernatants were injected directly into a high-performance liquid chromatographic (HPLC) system after filtration, and were also submitted to an ion-pair extraction procedure followed by HPLC analysis of the isolated purified metabolites.

Screening for several metabolic aromatic sulphonic acids of different polar characters could be performed in a single run by means of a reversed-phase ion-pair HPLC system with continuous linear gradient elution.

A method for differentiation between metabolite peaks and endogenous peaks has been worked out by comparing continuous gradient chromatograms of faeces extracts, recorded before and after administration of dye under similar conditions. These chromatograms clearly demonstrate that in addition to the metabolites mentioned, many new unknown dye metabolites are formed or excreted in rat faeces which are not present in urine.

By selecting the detector wavelength in the visible spectrum we also were able to prove that some of these new unknown metabolites obviously are coloured. This could be confirmed by a recently developed thin-layer chromatographic method.

INTRODUCTION

In order to obtain adequate toxicological data about dyes currently used in food or in cosmetics, a knowledge of their metabolic fate is required. An important step in metabolism research is the structural elucidation of unknown metabolites,

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which can be achieved only by spectroscopic techniques such as mass spectrometry and nuclear magnetic resonance spectrometry. The sample compounds, however, have to be relatively pure so that metabolites have to be isolated from their complex biological matrices, preceded or followed by purification steps. Before this preparative work can take place, another essential step must be undertaken in qualitative bioanalysis of metabolites, namely the screening for metabolites on an analytical level.

Daniel[1] obtained a few data on the fate of azo dyes in faeces by colorimetric methods. Radomski and Mellinger[2] described the absorption and excretion in rats of some water-soluble azo dyes, including Sunset Yellow FCF. The dye concentrations in the faeces were also determined colorimetrically. A paper chromatographic technique, however, was applied to urine and bile samples. Both groups showed that the reduction of the azo group in the dyes by azo-reductase in the liver is of minor significance. They also stated that the absorbed part of the administered dyes (2–4%) is rapidly excreted in the bile and then may be further reduced by the intestinal bacteria. Using antibiotics, these workers were able to show that the observed reductive fission of the azo group was due to the action of the gut microflora. In a review on the metabolism of azo compounds, Walker[3] stated that the generalization that highly sulphonated dyes are not absorbed to any great extent from the gastro-intestinal tract does not appear to apply equally to the metabolites produced by reduction of the azo function in the gut flora after oral dosing. The absorption of aminosulphonic acids may appear to conflict with the earlier concept that sulphonic acids are normally poorly absorbed. This anomaly is probably explicable in terms of the effect of the basic amino group. Indeed, sulphonated amines frequently appear in the urine or bile to a much greater extent than in faeces as found by Daniel[1] and Scheline and Longberg[4].

In a previous paper[5] we confirmed the presence in rat urine of different aromatic sulphonic acids as metabolites of Sunset Yellow FCF and Orange GGN (Fig. 1), using a combination of ion-pair extraction and reversed-phase ion-pair high-performance liquid chromatography (HPLC). This procedure offered the advantage of selective isolation and separation of the different aromatic sulphonic acids as possible urinary metabolites, so that free and N-acetylated derivatives such as sulphanilic acid and N-acetylsulphanilic acid for FCF, metanilic acid and N-acetylmetanilic acid for GGN and the common metabolite N-acetylANSA could be distinguished and identified.

In this paper, we describe a reversed-phase ion-pair HPLC system with continuous linear gradient elution as a general approach to the screening and separation

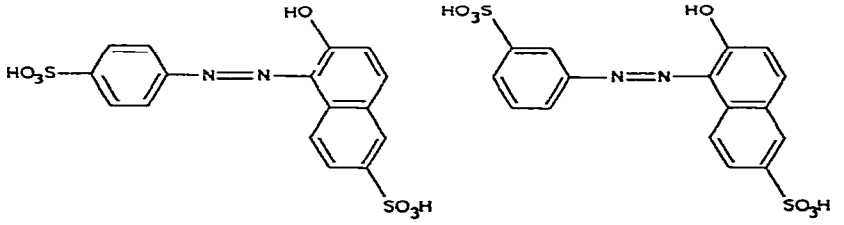

Fig. 1. Structures of Sunset Yellow FCF (I) and Orange GGN (II)

of various unknown Sunset Yellow FCF and Orange GGN metabolites in rat faeces. This technique is very suitable because we can screen in a single run a large number of aromatic sulphonic acids of low volatility having widely different polar characters.

EXPERIMENTAL

Instrumentation

The chromatographic separation was performed on an HPLC system consisting of two Altex Model 110A pumps, an Altex Model 421 solvent gradient programmer and a Hitachi Model 100-10 variable-wavelength UV–visible detector. The samples were injected with an Altex Model 210 injection valve, fitted with a 20-μl loop. In all experiments a reversed-phase 10-μm Hibar LiChrosorb RP-18 column (25 cm × 4 mm I.D.) (Merck, Darmstadt, G.F.R.) was used. The recorder was a single-pen Omniscribe Model 5117-2 (Houston Instruments). The mobile phase consisted of HPLC-grade water (solvent A) (Altech Assoc., Deerfield, IL, U.S.A.) and HPLC-grade methanol (solvent B) (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), mixed for each 1000 ml of solvent with the contents of one PIC Reagent A bottle (Waters Assoc., Milford, MA, U.S.A.) to form a 0.005 *M* solution of tetrabutylammonium phosphate (TBA^+ phosphate). Solvent A was filtered through a Millipore Type HA filter (0.45 μm) and solvent B through a Millipore Type H filter (0.5 μm). Before injection each extract solution was filtered through a Millipore Type FH filter (0.5 μm) by means of a 13-mm Stainless Swinny (Millipore, Bedford, MA, U.S.A.).

Reagents

Analytical-reagent grade chemicals were employed when available. Sulphanilic acid was supplied by Merck and metanilic acid (*m*-aminobenzenesulphonic acid) by ICN Pharmaceuticals (Plainview, NY, U.S.A.). Sunset Yellow FCF (E110, FD&C Yellow No. 6, C.I. Food Yellow 3) and Orange GGN (E111, C.I. Food Orange 2), obtained from Chroma-Gesellschaft Schmid (Stuttgart, Untertürkheim, G.F.R.) were used without further purifications. 1-Amino-2-naphthol-6-sulphonic acid (ANSA) was synthesized according to Larsen and Tarding[6]. N-Acetylsulphanilic acid, N-acetylmetanilic acid and N-acetyl-ANSA standards were obtained by derivatization for 1 h of the free sulphonic acids with acetic anhydride (Merck) in ethanol (Merck) at 60°C in a Silli-Vial system (Pierce, Rockford, IL, U.S.A.), followed by evaporation to dryness under a stream of nitrogen (L'Air Liquide Belge, Liège, Belgium). Tetrabutylammonium hydrogen sulphate (TBA^+ HSO_4^-) was obtained from Fluka (Buchs, Switzerland). The reagents and equipment used for the thin-layer chromatographic (TLC) procedure were the same as described by Van Peteghem and Bijl[7].

Faeces and urine samples

Male Wistar rats (*ca.* 200 g) from the animal house of the K.U. Leuven (Belgium) were kept individually in Makrolon metabolism cages (Hulskamp, Alkmaar, The Netherlands) and 24-h urine and faeces samples were collected free from cross-contamination. Food and water were provided *ad libitum*. Single doses of 100, 250 and 500 mg/kg of Sunset Yellow FCF and Orange GGN were administered in duplicate by stomach intubation.

TABLE I

CONTINUOUS LINEAR GRADIENT ELUTION PROGRAMME, CONTROLLED BY AN ALTEX MICROPROCESSOR, FOR HPLC ANALYSIS OF RAT FAECES EXTRACTS

Time (min)	*Function*	*Value*	*Duration (min)*
0.00	Flow-rate	1 ml/min	
0.00	% B	15%	
5.00	% B	100%	85
91.00	% B	15%	1
100.00	Alarm		0.2

Extraction and chromatographic procedures

For the extraction of the aromatic sulphonic acids from urine, we applied the previously described procedure[5] with slight modifications. Endogenous urinary organic acids were pre-extracted from 1.0 ml with 5.0 ml of diethyl ether and 5.0 ml of ethyl acetate. The final 3.0-ml dichloromethane phase, containing the extracted sulphonic acids as ion-pairs with the TBA^+ ion, was transferred into a clean test-tube and evaporated to dryness at 60°C under nitrogen. Before HPLC analysis, the recovered residue was dissolved in 1.0 ml of the same solvent mixture as the gradient elution programme started with, and filtered.

The collected 24-h faeces were weighed and mixed thoroughly with three times their weight of water, until a homogeneous suspension was obtained. This suspension was centrifuged for 15 min at 4000 rpm and decanted. Subsequently 1 ml of the supernatant was analysed exactly as the urine samples, with the same modifications. Simultaneously, part of the supernatants was filtered for direct HPLC analysis. Blank 24-h urine and faeces samples from three rats were run under identical conditions.

The HPLC analysis was performed at detection wavelengths of 240 nm (urine and faeces) and 360, 480 and 520 nm (faeces). The flow-rate was kept constant at 1 ml/min. Table I shows the eluent composition and the continuous linear gradient profile. During the first 5 min the eluent consisted of 15% solvent B in solvent A. Then at time 5.0 min, solvent B was added in a linear mode, increasing at 1% per minute up to 85 min, when the final eluent composition was 100% solvent B. The solvent gradient was subsequently reduced after 6 min to 15% solvent B in 1 min and allowed to equilibrate for 15 min between two injections. The faeces extracts were also analysed by TLC[7].

RESULTS AND DISCUSSION

Because it is the major excretory route for the compounds in question, faeces were chosen for further analysis. As we expected strong metabolic transformations of both dyes by the intestinal bacteria, yielding, in contrast to urine, many new unknown aromatic sulphonic acids with different polarities, we considered a gradient elution system starting with 85% water (0.005 *M* PIC A) and finishing with 100% methanol (0.005 *M* PIC A) to permit the elution of all metabolites and the parent dye from the column. This gradient profile offers the means of separating individual metabolites

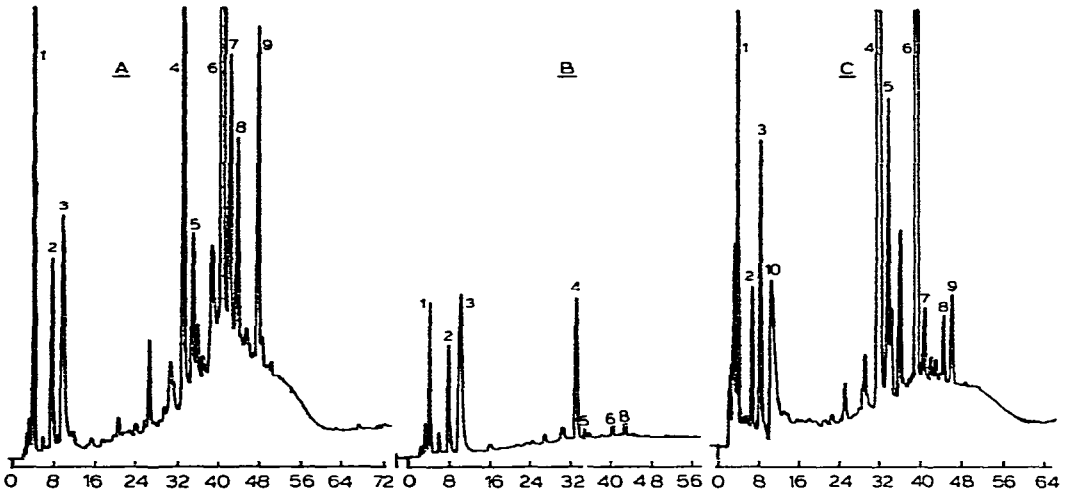

Fig. 2. Continuous gradient programmed HPLC traces, detected at 240 nm, (A) of the ion pair-extracted first 24-h rat faeces after administration of 500 mg/kg of Sunset Yellow FCF, (B) of the ion pair-extracted second 24-h faeces of the same rat and (C) of the direct aqueous macerate of the first 24-h faeces of the same rat. 1 = Sulphanilic acid; 2 = N-acetylsulphanilic acid; 3 = N-acetyl-ANSA; 6 = Sunset Yellow FCF; 10 = ANSA; 4 = orange-coloured metabolite; 5 = yellow-coloured metabolite; 7, 8 and 9 = red-coloured metabolites. Detector sensitivity: 0.5 a.u.f.s.

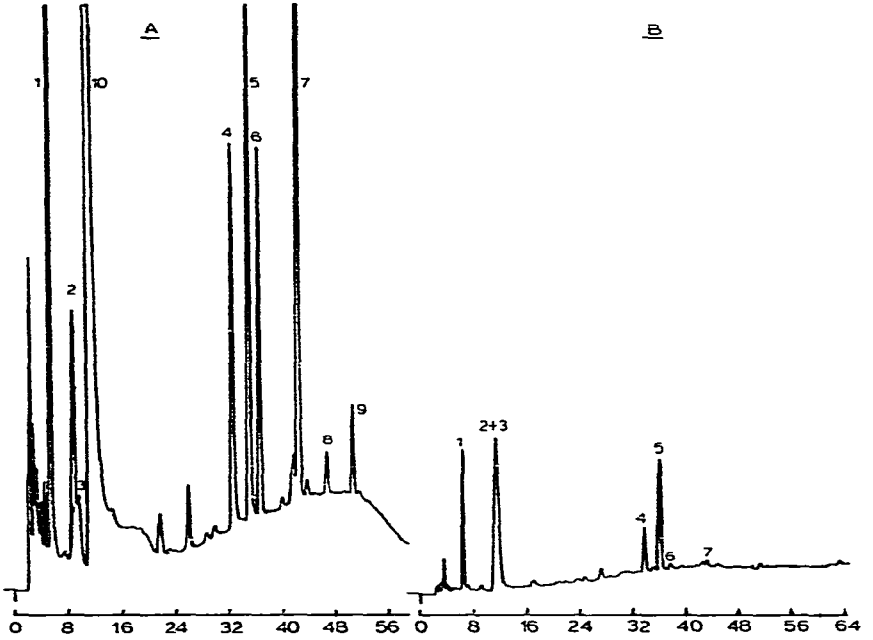

Fig. 3. Continuous gradient programmed HPLC traces, detected at 240 nm, (A) of the direct aqueous macerate of the first 24-h rat faeces after administration of 500 mg/kg of Orange GGN and (B) of the ion pair-extracted second 24-h faeces of the same rat. 1 = Metanilic acid; 2 = N-acetyl-ANSA; 3 = N-acetylmetanilic acid; 7 = Orange GGN; 10 = ANSA; 4 and 5 = orange-coloured metabolites; 6 = yellow-coloured metabolite; 8 and 9 = red-coloured metabolites. Detector sensitivity: 0.5 a.u.f.s.

with differing polarities from accompanying endogenous compounds and gives satisfactory results with respect to resolution, peak shape and reproducibility. An analogous approach has already been succesfully applied by Overzet *et al.*[8] for screening unknown Butoprozine metabolites in dog bile.

Preliminary work had revealed that a pre-extraction at acidic pH (1) with diethyl ether or ethyl acetate of aqueous solutions containing different aromatic aminosulphonic acids did not change their composition, even when the solutions were previously saturated with ammonium sulphate. Hence a first adequate clean-up step, eliminating many endogenous organic acids from urine and faeces extracts, could be achieved. To check the efficiency of the proposed ion-pair extraction method and to establish that no metabolite degradation occurs during the procedure, filtered aqueous faeces macerates were injected for direct analysis under identical HPLC conditions. Virtually identical peak patterns for both chromatograms indicated the reliability of the extraction procedure (Figs. 2 and 3). It became clear, however, that the very unstable metabolite ANSA, which is very sensitive to oxidation giving coloured *o*-quinoneimines, is poorly recovered by ion-pair extraction, but is actually present in freshly prepared aqueous faeces macerates as a true common Sunset Yellow FCF and Orange GGN metabolite.

As the administered doses were increased, higher metabolite peaks were recorded (Fig. 4), whereas analogous metabolite peaks appeared after administration of equal doses. Analysis of faeces samples collected during a second 24-h period revealed that the bulk of the parent dye and its metabolites are eliminated within the first 24-h faeces (Figs. 2 and 3). Unlike faeces, the first 24-h urine contains only low unchanged dye concentrations (Fig. 5).

The presence of the known metabolites in faeces was established by HPLC analysis of the corresponding standard compounds, running both the continuous

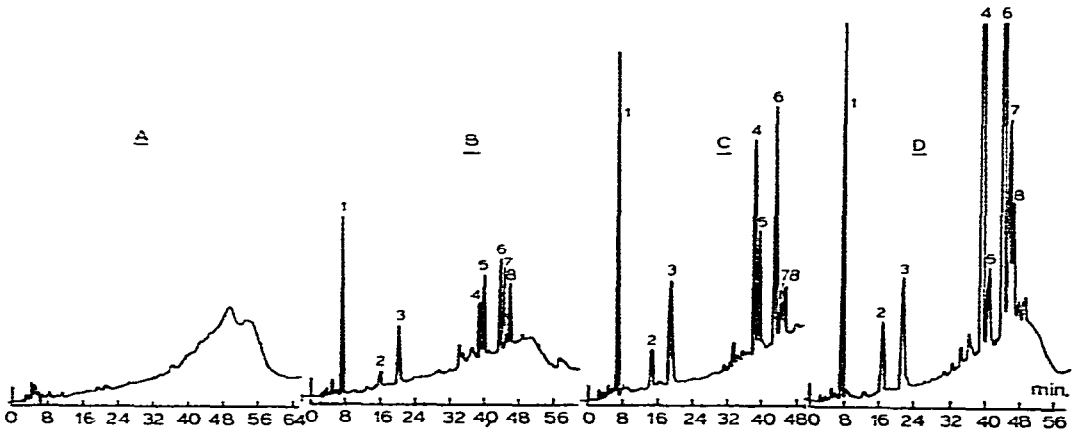

Fig. 4. Continuous gradient programmed HPLC traces, detected at 240 nm, (A) of ion pair-extracted 24-h blank rat faeces, (B) of the ion pair-extracted first 24-h rat faeces after administration of 100 mg/kg, (C) 250 mg/kg and (D) 500 mg/kg of Sunset Yellow FCF. Detector sensitivity: 0.5 a.u.f.s. For numbering see Fig. 2.

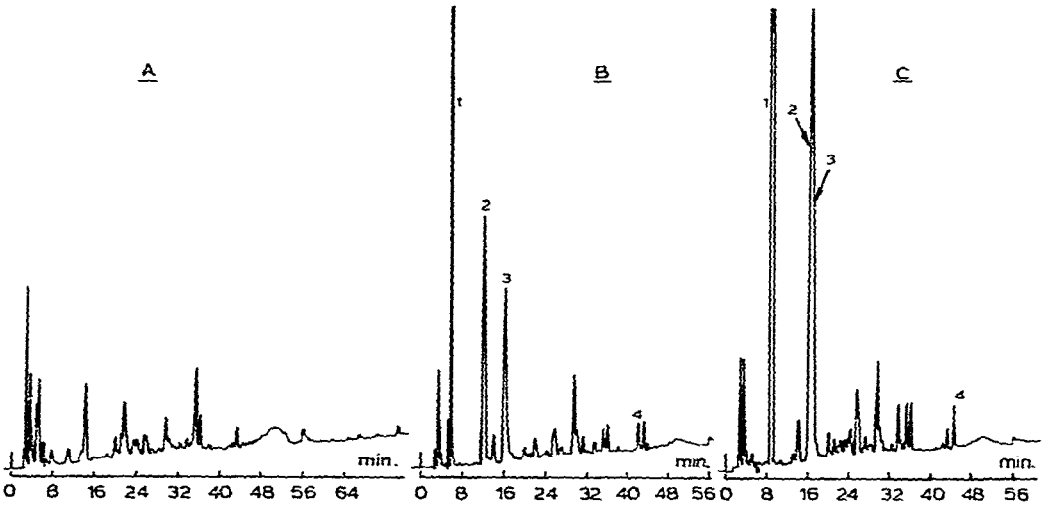

Fig. 5. Continuous gradient programmed HPLC traces, detected at 240 nm, (A) of ion pair-extracted 24-h blank rat urine, (B) of ion pair-extracted first 24-h rat urine after administration of 250 mg/kg of Sunset Yellow FCF (1 = sulphanilic acid; 2 = N-acetylsulphanilic acid; 3 = N-acetyl-ANSA; 4 = FCF) and (C) after administration of 250 mg/kg of Orange GGN (1 = metanilic acid; 2 = N-acetyl-ANSA; 3 = N-acetylmetanilic acid; 4 = GGN).

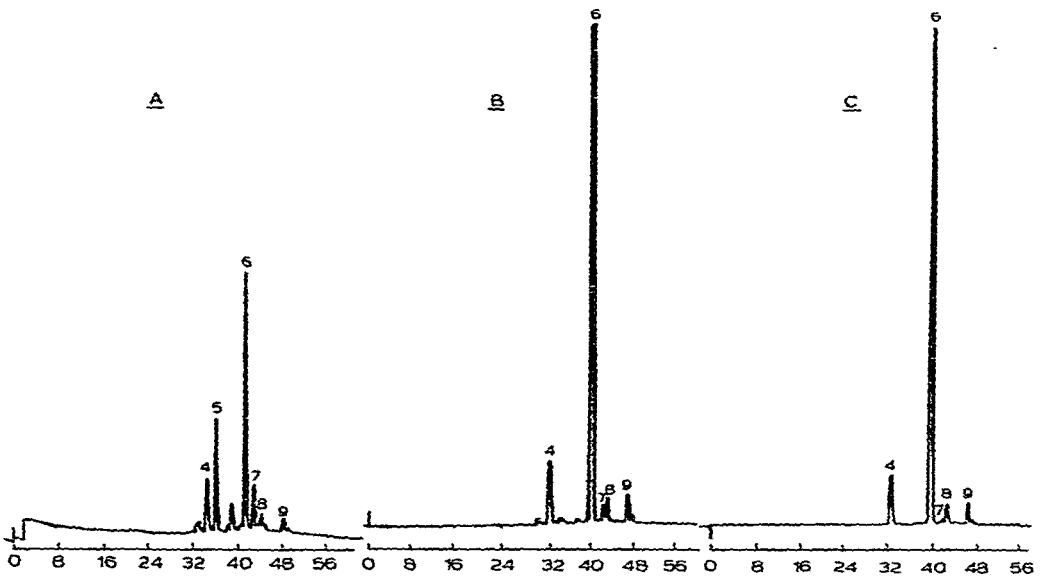

Fig. 6. Continuous gradient programmed HPLC traces of ion pair-extracted first 24-h rat faeces, after administration of 500 mg/kg of Sunset Yellow FCF, detected (A) at 360 nm, (B) at 480 nm and (C) at 520 nm. For numbering, see Fig. 2; peak 5 corresponds to a yellow FCF metabolite. Detector sensitivity: 0.5 a.u.f.s.

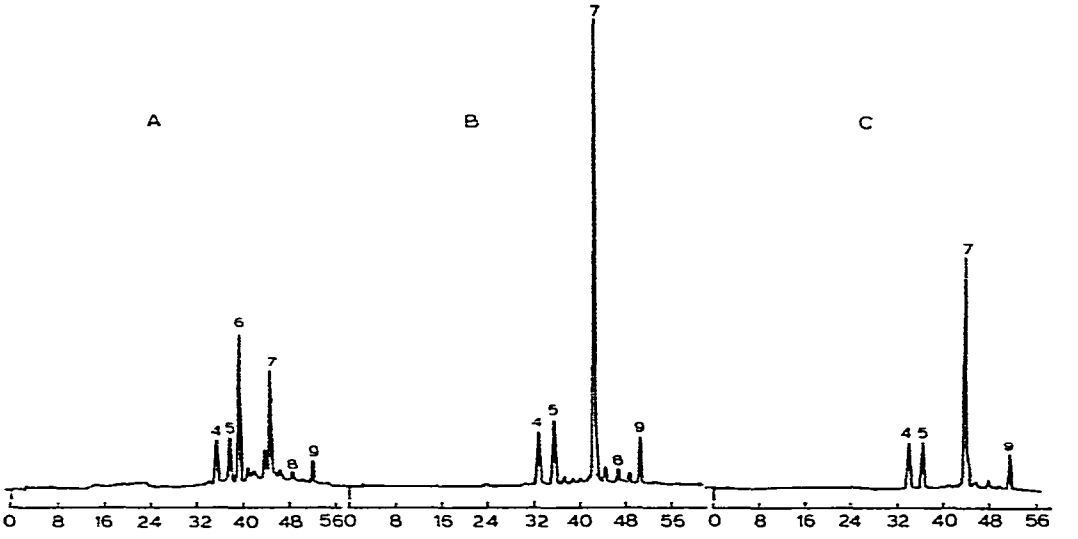

Fig. 7. As Fig. 6, but after administration of 500 mg/kg of Orange GGN. For numbering, see Fig. 3; peak 6 corresponds to a yellow GGN metabolite, which has a different retention time, however, to the yellow FCF metabolite (peak 5) in Fig. 6.

linear gradient system and the previously described[5] gradient and isocratic systems. In the present system ANSA elutes after N-acetyl-ANSA, whereas in the previously developed systems ANSA elutes before N-acetyl-ANSA. The acetic anhydride-derivatized ANSA always shows two peaks on the chromatograms, the first of which corresponds to N-acetyl-ANSA.

By selecting detection wavelengths in the visible spectrum we were able to extract from the faeces-extract HPLC traces, recorded at 240 nm, different coloured metabolites (yellow, orange and red) for both FCF and GGN (Figs. 6 and 7). Comparing the HPLC traces of the FCF faeces extracts recorded at 360, 480 and 520 nm with the corresponding chromatograms of the GGN faeces extracts, different peak patterns with different retention times for most coloured metabolites are observed, so that we can postulate that the non-common metabolites could be generated by modification of the entire dye molecules. Their presence was obviously confirmed by the TLC procedure on silica gel plates impregnated with cetyltrimethylammonium bromide, using the two recommended water-free mobile phases[7]. The different coloured faeces metabolites (yellow, orange and red) can be distinghuished visually, and are separated using both eluent systems, revealing lower R_F valves than the parent dyes FCF and GGN (R_F 0.32 for both in solvent A and 0.25 in solvent B).

CONCLUSION

The results clearly show the usefulness of the continuous linear gradient elution system in the analysis of dye metabolites in faeces and urine. By means of this procedure we could demonstrate that in the faeces of rats, that had received *per os* the water-soluble azo dyes Sunset Yellow FCF and Orange GGN, in addition to the same

aromatic aminosulphonic acids as are present in urine, many other unidentified coloured metabolites are present, probably formed by direct substitution of different functional groups on the unchanged dye molecules.

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