Journal of Chromatography, 237 (1982) 115–125
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,444

N-ACETYL-(1-AMINO-2-NAPHTHOL-6-SULPHONIC ACID), A COMMON METABOLITE OF SUNSET YELLOW FCF, ORANGE GGN AND 1-AMINO-2-NAPHTHOL-6-SULPHONIC ACID IN RAT URINE

JACQUES O. DE BEER*, PAUL J. DIERICKX and RÉNÉE I. GRIMÉE
Instituut voor Hygiëne en Epidemiologie, J. Wytsmanstraat 14, B-1050 Brussels (Belgium)
(Received September 17th, 1981)

SUMMARY

Sunset Yellow FCF, Orange GGN and 1-amino-2-naphthol-6-sulphonic acid (ANSA) were administered to male Wistar rats by stomach intubation. Aromatic sulphonic acids excreted in 24-h urines after enzymatic cleavage of the azo link in the dyes were isolated using a thoroughly elaborated ion-pair extraction method and further separated by means of a reversed-phase ion-pair liquid chromatographic system. Blank 24-h rat urines were extracted and run at the same time under identical analytical circumstances.

In addition to the peaks corresponding to sulphanilic and metanilic acid and their N-acetylated derivatives, which are metabolites arising from Sunset Yellow FCF and Orange GGN, respectively, an important common peak appeared on the chromatograms, which was absent from the blank urine extracts. After analysis of 24-h urine of rats that had received ANSA, a peak with the same retention time as this unknown common peak could be detected under the same liquid chromatographic conditions, the retention time of which was different from that of the ANSA standard.

However, after derivatization of the ANSA standard with acetic anhydride, followed by liquid chromatographic examination of the derivatised mixture, two peaks appeared on the chromatogram, the first of which had the same retention time and the same UV-spectrum as the unknown common peak in rat urine extracts. By means of semi-preparative liquid chromatographic separation and isolation, followed by further purification over a cation-exchange resin, the compound corresponding to the unknown common peak could be identified by FT-PMR spectroscopy as N-acetyl-ANSA.

INTRODUCTION

Water-soluble azo compounds are widely used as colour additives, *e.g.*, in foods, pharmaceuticals and cosmetics. Although they have been used for at least half a century, the toxicity and metabolism of these compounds have received attention only during the last two decades. The metabolism of azo compounds has been re-

0021-9673/82/0000–0000/$02.75

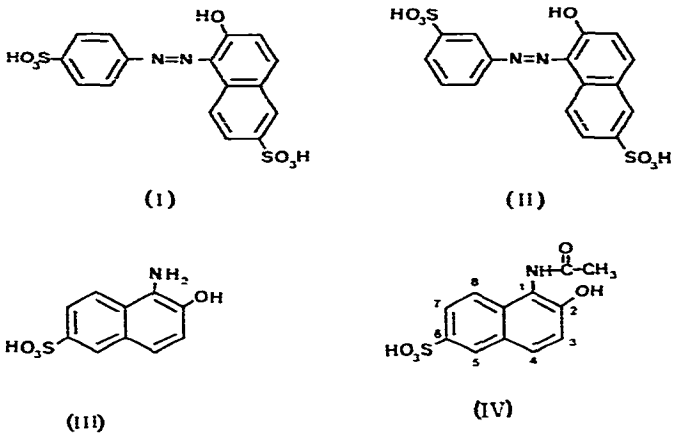

Fig. 1. Structures of Sunset Yellow FCF (I), Orange GGN (II) and 1-amino-2-naphthol-6-sulphonic acid (III).

viewed by Walker[1]. The most important metabolic reaction is cleavage of the azo link, primary amines being formed. This reaction is primarily performed by the gut flora. The mammalian hepatic azo-reductase is regarded as being of minor importance.

Acetylation of the generated primary amino groups is a common detoxification mechanism and the urinary excretion of acetylated amine metabolites has been described in many studies, including those by Bray *et al.*[2], Daniel[3], Jones *et al.*[4], Scheline and Longberg[5] and Stevenson *et al.*[6]. In some of these studies sulphanilic acid, its N-acetylated derivative and 1-amino-2-naphthol-6-sulphonic acid (ANSA) appeared to be metabolites in the urine of test animals after oral administration of Sunset Yellow FCF (I) (Fig. 1). The identification and quantification of these metabolites, however, were performed using colorimetric methods, with which the presence of the N-acetylated metabolites could not be proved directly. No results on the metabolism of Orange GGN (II) (Fig. 1) that demonstrate the formation of metanilic acid and its N-acetylated derivative have been published. For ANSA (III) (Fig. 1), its concentration in urine from test animals could only be determined colorimetrically, starting from freshly prepared standard solutions, which are very sensitive to oxidative changes, giving coloured *o*-quinoneimines. The urines had to be collected in receivers immersed in solid carbon dioxide. The presence of N-acetyl-ANSA as a urine metabolite has never been confirmed.

Larsen and Tarding[7] described the absorption and excretion of ^{35}S-labelled ANSA in rats and rabbits, measuring the radioactivity, without following the individual fates of possible different ANSA metabolites.

The combination of ion-pair extraction and reversed-phase ion-pair high-performance liquid chromatography (HPLC) offers the advantage of selective isolation and separation of the different aromatic sulphonic acids as possible urinary metabolites, so that free and N-acetylated derivatives can be distinguished and identified unambiguously. Semi-preparative HPLC isolation and purification of an unknown peak, not appearing in the blank urines, followed by spectroscopic identification can allow the detection of a new, undescribed metabolite.

EXPERIMENTAL

Instrumentation

The chromatographic separation was performed on a HPLC system consisting of two Altex Model 100A pumps, an Altex Model 421 solvent gradient programmer and a Hitachi Model 100-10 variable-wavelength UV–visible detector. Injections were made by means of an Altex Model 210 sample injection valve, supplied with loops of 20 μl and 2.0 ml. The isolation and collection of separated compounds eluting from the semi-preparative column were effected by means of an Altex automatic rotary valve (Altex, Berkeley, CA, U.S.A.).

The analytical column was a reversed-phase 10-μm Hibar LiChrosorb RP-18 25 cm × 4 mm I.D. column (Merck, Darmstadt, G.F.R.). The semi-preparative column was a reversed-phase 5-μm Ultrasphere ODS 25 cm × 10 mm I.D. column (Altex). The recorder was a single-pen Omniscribe Model 5117-2 (Houston Instruments). The UV spectra were recorded with a Beckman Model 25 UV-visible spectrophotometer, with digital reading and recorder (Beckman, Fullerton, CA, U.S.A.). Extracts were evaporated on a Büchi RE 120 Rotavapor (Büchi Laboratorium-Technik, Flawil, Switzerland).

The proton magnetic resonance (PMR) spectroscopic properties of the common metabolite were examined on a JEOL FX 100 nuclear magnetic resonance spectroscopic apparatus in the Fourier transform mode at a frequency of 99.55 MHz and a temperature of 26°C.

Reagents

All chemicals were of analytical-reagent grade unless specified otherwise.

Sulphanilic acid was supplied by Merck and metanilic acid (*m*-aminobenzenesulphonic acid, aniline-*m*-sulphonic 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) were obtained from Chroma-Gesellschaft Schmid (Stuttgart, Untertürkheim, G.F.R.). ANSA was synthesized according to Larsen and Tarding[8]. N-Acetylsulphanilic and N-acetylmetanilic acid standards were synthesized starting from the free sulphonic acids and acetic anhydride (Merck), and recrystallized according to Daniel[3]. Tetrabutylammoniumhydrogenium sulphate (TBA^+-HSO_4^-) and tetrapentylammonium bromide (TPA^+-Br^-) were obtained from Fluka (Buchs, Switzerland).

The pH 7.0 buffer was prepared by diluting a mixture of 50 ml 0.1 *M* potassium dihydrogen orthophosphate (Merck) and 29.1 ml of 0.1 *M* sodium hydroxide to 100 ml with doubly distilled water. The pH 10.0 buffer was prepared by dilution of a mixture of 50 ml of 0.05 *M* sodium hydrogen carbonate (Merck) and 10.7 ml 0.1 *M* sodium hydroxide (Merck) to 100 ml with doubly distilled water. HPLC-grade methanol was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

For the PMR spectroscopic examination, the purified residue of the common metabolite and the derivatized ANSA compound were disolved in 0.5 ml of ^2H_2O (Aldrich, Beerse, Belgium) containing 3-(trimethylsilyl)propionic acid-d_4 sodium salt (Uvasol, Merck) as reference.

Solvent A was a 0.01 *M* solution of sodium acetate and 0.005 *M* TPA^+–Br^- in distilled water, the pH being adjusted to 3.50 with orthophoshoric acid. Solvent B

contained the same amounts of salts, but with 50% (v/v) of methanol. Solvent A and the aqueous part of solvent B were filtered through a Millipore Type HA filter (0.45 μm). The extract solution injected for semi-preparative separation was filtered through a Millipore Type FH filter (0.5 μm) by means of a Millipore "Stainless Swinny" (13 mm) (Millipore, Bedford, MA, U.S.A.).

Dowex 50W-X8 (100–200 mesh) cation-exchange resin (Fluka), swollen for 24 h in doubly distilled water, was used to eliminate the quaternary ammonium ions from the isolated metabolite residue.

Urine samples

Male Wistar rats (*ca.* 200 g), obtained from the animal house of the KU Leuven, Belgium, were administered in duplicate by stomach intubation single doses of 20, 100, 250 and 500 mg/kg of Sunset Yellow FCF and Orange GGN and 150 and 300 mg/kg of ANSA, as an aqueous suspension. The 24-h urine samples, the volumes of which varied from 13.0 to 18.0 ml, were collected in plastic bottles without additives. They were centrifuged for 10 min at 4000 rpm and decanted.

Extraction and chromatographic procedures

For the extraction of the urinary aromatic sulphonic acid metabolites, prior to HPLC analysis on the analytical column, 1.0 ml of urine was mixed in a test-tube with 0.5 ml of 1.5 *M* orthophosphoric acid and 2.0 g of ammonium sulphate. This mixture was extracted twice with 5.0 ml of ethylacetate by vortexing for at least 2 min. After centrifugation (5 min at 3000 rpm) the ethyl acetate phases were discarded. The urine phase was neutralized to pH 7.0 adding 3–4 drops of 10 *N* sodium hydroxide solution and mixed with 1.0 ml of 0.1 *M* TBA^+–HSO_4^- in buffer solution of pH 7.0.

Some ammonium sulphate was added to maintain saturation. The urine phase was extracted further with 3.0 ml of dichloromethane by vortexing for at least 2 min and centrifuged for 10 min at 4000 rpm, the dichloromethane phase being transferred into a clean test-tube. Finally, 1.5 ml of this dichloromethane phase was extracted by vortexing for 2 min with 0.5 ml of 0.1 *M* sodium perchlorate in pH 10.0 buffer solution. After centrifugation (for 5 min at 1000 rpm, 20 μl of the aqueous layer were injected into the HPLC system.

TABLE I

HPLC GRADIENT PROGRAMME, CONTROLLED BY THE ALTEX MICROPROCESSOR, FOR THE RAT URINE EXTRACT ON THE ANALYTICAL COLUMN

Time	*Function*	*Value*	*Duration*
0.00	Flow-rate	1.25 ml/min	
0.00	%B	35%	
0.00	External flag*	1	
12.00	%B	75%	4 min
25.00	%B	100%	4 min
35.00	%B	35%	3 min
45.00	Alarm	–	0.1 min
50.00	External flag	1	0 min
50.00	Flow-rate	0 ml/min	0.2 min
50.2	End programme	–	

* External flag = apparatus that can be started by the microprocessor; *e.g.*, External flag 1 = recorder.

HPLC analysis on the analytical column was performed at two detection wavelengths (240 and 260 nm) by solvent gradient elution, controlled by the Altex solvent gradient programmer. The programme was started with 35% solvent B and increased by a two-step gradient programme to a final concentration of 100% B. This was accomplished over a 29-min period. The solvent gradient was reduced after 6 min to 35% B in 3 min and allowed to equilibrate for 15 min between two injections. The flow-rate was kept constant at 1.25 ml/min (Table I). Each extract was also submitted to an isocratic run, solvent A being 25% and solvent B being 75% at the same flow-rate.

The extraction procedure prior to the semi-preparative HPLC analysis was started with 5.0 ml of urine, the other reagents being added proportionally. The ion-pair extraction however, was performed twice with 5.0 ml of dichloromethane, which was ten evaporated at 60°C on the Büchi Rotavapor. The residue was dissolved in 2.0 ml of a mixture of solvents A and B (25:75, v/v), filtered and injected completely through the 2.0-ml loop on the semi-preparative column. This semi-preparative HPLC separation was performed by an isocratic run, the mobile phase being 25% A and 75% B, at a detector wavelength of 240 nm and a constant flow-rate of 2.25 ml/min. The column effluent, containing the probable common metabolite, was isolated and its UV spectrum was recorded. It was then evaporated to dryness at 70°C on the Büchi Rotavapor, the residue being dissolved in portions at 7–8 ml of dichloromethane which were collected and evaporated. By this means most of the dichloromethane-insoluble inorganic salts present in the column effluent were eliminated.

The salt-free residue was dissolved in 10 ml of doubly distilled water and freed from quaternary ammonium ions by passing the solution through a Dowex 50W-X8 cation-exchange column (3.0 × 1.5 cm I.D.) which was eluted with 20 ml of doubly distilled water. The aqueous effluent, free from quaternary ammonium ions, was evaporated at 70°C on the Büchi Rotavapor, the final purified extract obtained being submitted to PMR spectroscopic examination. Under identical analytical conditions, the same procedure was followed in order to isolate, purify and identify the compound with the same retention time and the same UV spectrum as the probable common metabolite obtained after injection of the concentrated mixture prepared by derivatization of 50 mg of ANSA with an excess of acetic anhydride in ethanol[3].

FT-PMR spectroscopic examination

On account of the small amount of residue of the purified metabolite, the spectra were recorded after accumulation of 1000 pulses, using the "double precision" programme. This programme improves the signal-to-noise ratio for peaks of weak intensity with respect to the intense signal of the solvent, which here is residual H_2O present in ^2H_2O. The spectra were recorded in a 1000-Hz width, using 8192 points in pulses of 45° (20 μsec) and a pulse repetition time of 5 sec. The chemical shifts are calculated in parts per million with respect to 3-(trimethylsilyl)propionic acid-d_4, sodium salt, as reference.

RESULTS

Some chromatograms of 24-h urine extracts separated on the analytical column by solvent gradient and by isocratic elution are shown in Figs. 2 and 3,

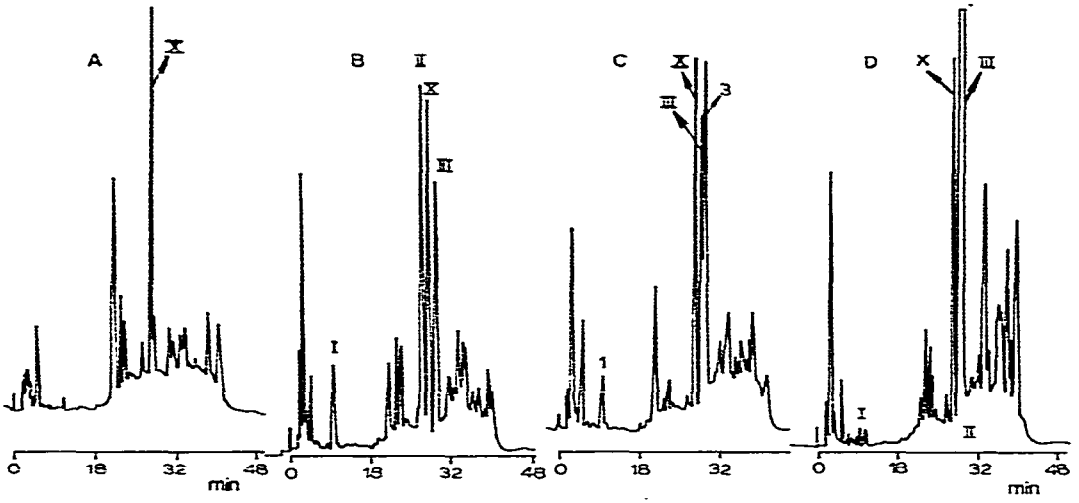

Fig. 2. Gradient-programmed HPLC chromatograms detected at 240 nm of a blank rat urine extract (A) and of 24-h rat urine extracts after administration of 100 mg/kg of Sunset Yellow FCF (B), 100 mg/kg of Orange GGN (C) and 150 mg/kg ANSA (D). I = Sulphanilic acid; II = N-acetylsulphanilic acid; III = common metabolite; 1 = metanilic acid, 3 = N-acetylmetanilic acid, X = endogenous rat urine component.

respectively. Chromatograms of a urine extract recorded at 240 and 260 nm are given in Fig. 4.

Some chromatograms of 24-h urine extracts separted on the semi-preparative column are shown in Fig. 5.

The UV spectrum of the unknown common metabolite, after administration of Sunset Yellow FCF, Orange GGN and ANSA, is given in Fig. 6.

The PMR spectrum of the purified common metabolite, which is the same as that of the derivatized ANSA compound having the same retention time and UV spectrum, is shown in Fig. 7.

DISCUSSION

Extraction procedure

In order to minimize sample work-up, direct injection of samples was attempted under various circumstances, but was unsuccessful as interfering peaks did not allow the resolution of the compounds of interest. Owing to the large difference in the physico-chemical properties between aromatic sulphonic acids, which are always ionized in water even at low pH, and most of the endogenous organic acids present in rat urine, pre-extraction of the urine with ethyl acetate at low pH seemed to be successful as a preliminary clean-up step. By adding high concentrations of ammonium sulphate, the endogenous organic acid extraction efficiency was enhanced, without the sulphonic acids leaving the aqueous phase.

The very polar aromatic sulphonic acids could be isolated from aqueous solutions by means of an ion-pair liquid–liquid extraction, this type of extraction being particularly efficient for polar and ionized compounds[9–11]. The pH, nature and concentration of the counter ion, ion strength and extraction solvent, however, must be

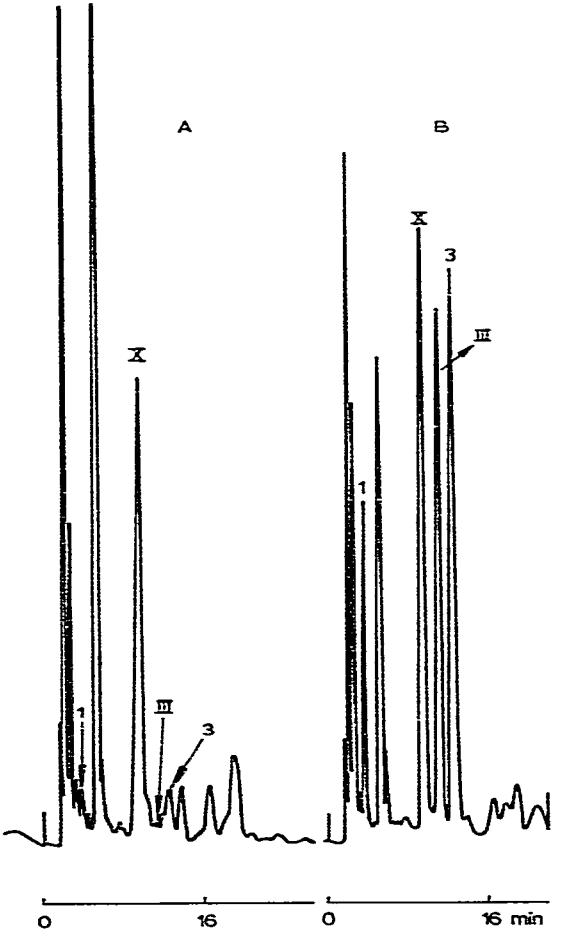

Fig. 3. Isocratic HPLC chromatogram detected at 240 nm of a 24-h blank rat urine extract (A) and a 24-h rat urine extract after administration of 100 mg/kg of Orange GGN. 1 = Metanilic acid; 3 = N-acetyl-metanilic acid; III = common metabolite; X = endogenous urine component.

optimized. A pH of 7.0–7.5 was found to be optimal in accomplishing complete extraction of Orange GGN, Sunset Yellow FCF, sulphanilic acid and metanilic acid together with their N-acetyl derivatives, TBA^+ being added as counter ion at an optimum concentration of 0.1 *M*. Lower counter-ion concentrations produced low and variable extraction efficiencies. The addition of high concentrations of ammonium sulphate to the system, producing a salting-out effect, was necessary to ensure a good extraction efficiency of sulphanilic acid, metanilic acid and their N-acetyl derivatives. Dichloromethane was preferred to chloroform as extraction solvent because also high sulphonic acid concentrations could be extracted efficiently as ion pairs. By vortexing the dichloromethane phase, containing the ion pairs, with 0.5 ml of 0.1 *M* sodium perchlorate in pH 10.0 buffer, the sulphonic acids returned to the aqueous phase, which was subsequently injected for HPLC analysis.

During the extraction procedure the probable common metabolite seemed to

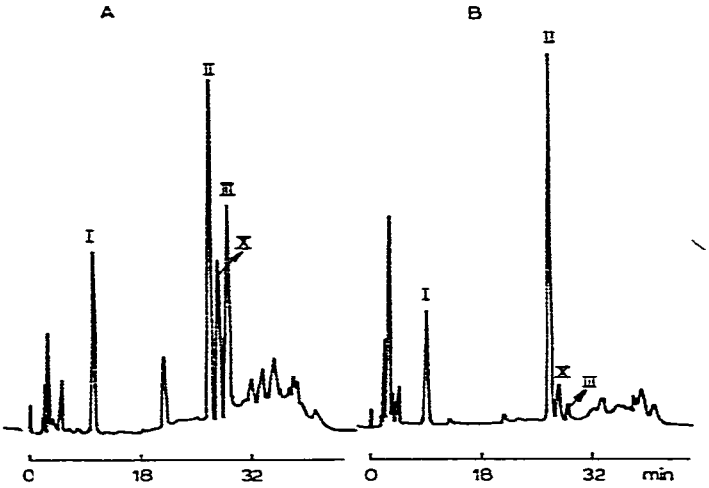

Fig. 4. Gradient-programmed HPLC chromatograms of a 24-h rat urine after administration of 100 mg/kg of Sunset Yellow FCF, detected at 240 nm (A) and 260 nm (B). I = Sulphanilic acid; II = N-acetyl-sulphanilic acid, III = common metabolite; X = endogenous urine component.

behave identically to the other aromatic sulphonic acids. In contrast, ANSA could not be recovered from spiked water and urine samples by means of this extraction method, perhaps because of its chemical instability. A partial recovery with poor reproducibility was obtained by adding ascorbic acid as an antioxidant to the sample before extraction. No peak corresponding to the ANSA standard (stable for many days in an aqueous solution with ascorbic acid as antioxidant), however, was found in real urine samples from rats given FCF, GGN or ANSA, not even when ascorbic acid was added in high concentrations before extraction.

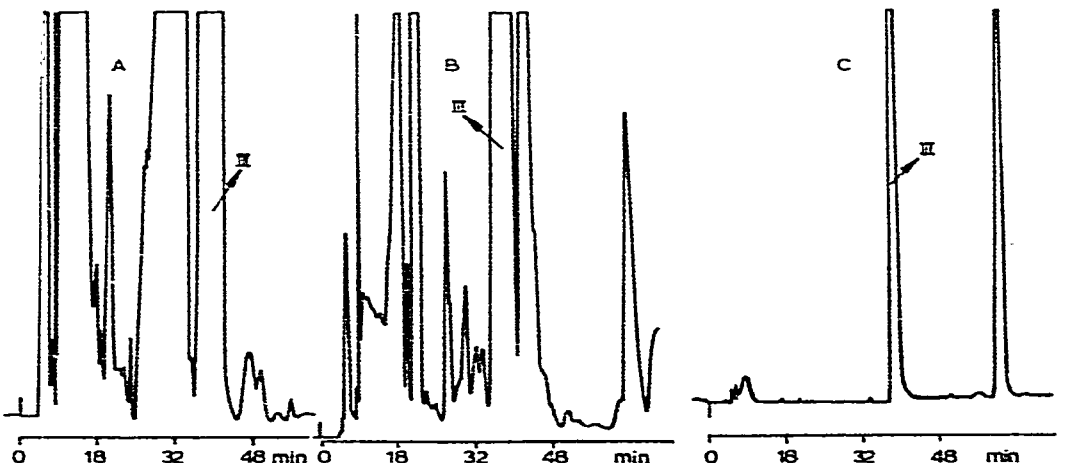

Fig. 5. Semi-preparative HPLC chromatograms of 24-h rat urine extracts after administration of 500 mg/kg of Sunset Yellow FCF (A), Orange GGN (B) and ANSA derivatized with acetic anhydride (C).

Fig. 6. UV spectrum of the common rat urine metabolite identified as N-acetyl-ANSA.

Chromatographic procedure

Without a counter ion, the retention of the standard aromatic sulphonic acids was inadequate for acceptable resolution. Addition of TPA^+ as the counter ion to the solvent system markedly increased the retention and resolution of these compounds. Small changes in pH produced marked changes in their retention times. A decrease in the pH resulted in a marked reduction of the retention time and resolution, and an increase in the pH resulted in the opposite effect. A pH of 3.50 was optimal for resolution and retention and was used in all analyses. The concentration of the TPA^+ ion was also varied at constant pH. A reduced concentration caused a small reduction in the retention times and an increased concentration produced an increased retention, although this effect was not as marked as that of the pH[12–14]. A TPA^+ counter-ion concentration of 0.005 *M* was determined to be optimal.

The chromatographic conditions were optimized with respect to the resolution and selectivity between endogenous peaks of urine blanks and the metabolites in

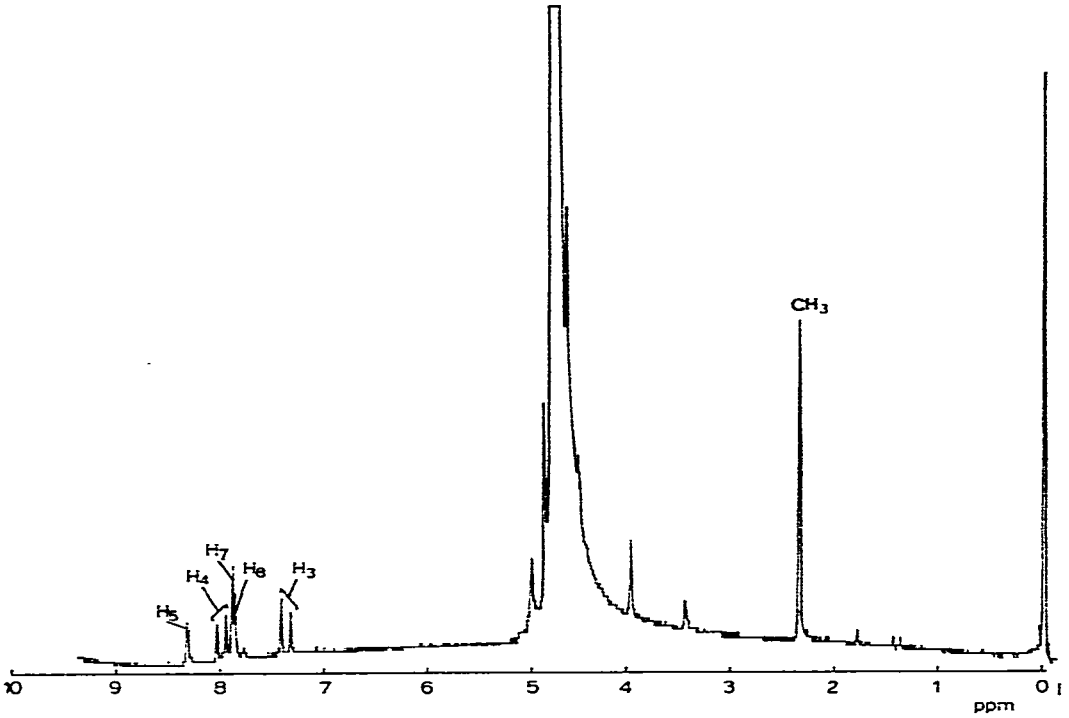

Fig. 7. PMR spectrum of the common rat urine metabolite N-acetyl-ANSA isolated after administration as well of Sunset Yellow FCF, Orange GGN and ANSA [*cf.* Fig. 1 (IV)].

urine extracts of rats that had received Orange GGN and Sunset Yellow FCF. A mixture of standard sulphanilic acid, metanilic acid and their N-acetyl derivatives was also injected under identical chromatographic conditions.

The choice of 240 and 260 nm as detection wavelengths was to check if the individual peak-height ratios of the sulphonic acid standards and the corresponding metabolites found in the urine extracts were constant (Fig. 4). At 260 nm endogenous urine peaks interfered weakly on the chromatogram, the common metabolite, however, being less sensitively detected than at 240 nm. When the dose of the administered dye was enhanced, higher peaks corresponding to the metabolites in the urine extracts were recovered. Urine extracts of rats receiving the same amounts of dyes showed metabolite peaks with nearly the same intensities.

In urine samples collected after 72 h from rats that had received the highest doses (500 mg/kg), only poor traces of the metabolites could be obtained.

PMR spectroscopic examination

Apart from the signal of H_2O, situated at 4.76 ppm, a peak at 2.35 ppm and a series of peaks in the aromatic zone are observed. Protons H_3 and H_4 (Fig. 1, IV) have chemical shifts of 7.35 (doublet) and 7.97 ppm (doublet), respectively. The coupling constant $J_{3,4}$ is 9 Hz. Proton H_5 at 8.3 ppm is weakly coupled with protons H_7 and H_8 ($J_{5,7} = 1.5$ Hz and $J_{5,8} \approx 0.8$ Hz). Protons H_7 and H_8 have chemical shifts of 7.83 and 7.85 ppm ($J_{7,8} = 1$ Hz), respectively. Integration of the spectrum

indicates that the peak at 2.35 ppm corresponds to a CH_3 group and its position suggests that a monoacetyl derivative of ANSA is involed. Indeed, the PMR spectrum of ANSA standard, dissolved in ^2H_2O and stabilized by addition of ascorbic acid (vitamin C), is similar to that of the isolated common metabolite, apart from the absence of the CH_3 peak at 2.35 ppm and a weak shift of proton H_8 to lower frequency. This indicates that the metabolite is acetylated at the NH_2 function and not at the OH function. Acetylation at the OH function should produce an important shift of protons H_3 and H_4.

After acetylation of the ANSA standard, followed by semi-preparative HPLC isolation and cation-exchange purification, the compound with the same HPLC retention time and the same UV spectrum as the common metabolite also has an identical PMR spectrum.

CONCLUSION

The combined system of ion-pair extraction and ion-pair reversed-phase HPLC allows the identification of sulphanilic acid and methanilic acid and their N-acetyl derivatives as metabolites in the urine of rats after administration of the dyes Sunset Yellow FCF and Orange GGN. This technique also allows the detection in rat urine of a common Orange GGN, Sunset Yellow FCF and ANSA metabolite, identified as N-acetyl-ANSA by a combination of PMR spectroscopy and semi-preparative HPLC isolation and purification of rat urine extracts and acetylated ANSA standard.

ACKNOWLEDGEMENTS

We thank Prof. Dr. A. Lafontaine and Ir. J. Gosselé for their interest in this work.

REFERENCES

1 R. Walker, *Food Cosmet. Toxicol.*, 8 (1970) 659.
2 H. G. Bray, R. C. Clowes and W. V. Thorpe, *Biochem. J.*, 49 (1951) 1XV.
3 J. W. Daniel, *Toxicol. Appl. Pharmacol.*, 4 (1962) 572.
4 R. Jones, A. J. Ryan and S. E. Wright, *Food Cosmet. Toxicol.*, 2 (1964) 447.
5 R. R. Scheline and B. Longberg, *Acta Pharmacol. Toxicol.*, 23 (1965) 1.
6 E. S. Stevenson, K. Dobriner and C. P. Rhoads, *Cancer Res.*, 2 (1942) 160.
7 J. C. Larsen and F. Tarding, *Arch. Toxicol., Suppl.*, 1 (1978) 251.
8 J. C. Larsen and F. Tarding, *Acta Pharmacol. Toxicol.*, 39 (1976) 525.
9 G. Schill, *Separation Methods for Drugs and Related Organic Compounds*, Apothekarsocietetan, Stockholm, 1978.
10 C. Van Peteghem and J. Bijl, *J. Chromatogr.*, 210 (1981) 113.
11 K. T. Muir, J. H. G. Jonkman, D.-S. Tang, M. Kunitani and S. Riegelman, *J. Chromatogr.*, 221 (1980) 85.
12 J. H. Knox and G. R. Laird, *J. Chromatogr.*, 122 (1976) 17.
13 R. Gloor and E. L. Johnson, *J. Chromatogr. Sci.*, 15 (1977) 413.
14 H. U. Ehmcke, H. Kelker, K. H. Kōnig and H. Ullner, *Z. Anal. Chem.*, 294 (1979) 251.