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Gas chromatography of urinary anthranilic acid

It has been reported that an increase in the amount of urinary anthranilic acid, excreted as one of intermediary metabolites of tryptophan, is associated with some diseases[1,2], *e.g.* congenital hypoplastic anemia. Recently, analysis of tryptophan metabolites by gas chromatography (GC) was reported by NOGUCHI *et al.*[3]; the anthranilic acid was converted to its methyl trifluoroacetyl amino derivative, with diazomethane and trifluoroacetic anhydride, and separated successfully by GC. However, there is no report dealing with the quantitative determination of anthranilic acid in urine. In the present paper, a procedure has been developed for the determination of urinary anthranilic acid for clinical and diagnostic purposes.

Experimental

Apparatus and conditions. A Shimadzu Model GC-4APF gas chromatograph equipped with a hydrogen flame ionisation detector (HFID) and a Shimadzu Model GC-4APE gas chromatograph equipped with an electron capture detector (ECD) were used. The 1.5 m long × 3 mm I.D. stainless-steel columns were packed with 5 % GE-XF1105 on Gas-Chrom Z (80–100 mesh) and 30% OV-17, respectively. The column oven temperature was 120°, and the detector and injection port temperatures were 160° and 150°, respectively. The gas flow rates of nitrogen, hydrogen and air were 30, 65, and 1000 ml/min, respectively. The sample size was 1.0 μl and the chart speed was 0.5 cm/min.

Standard procedure. 1 ml of conc. HCl and 1 ml of 0.1 *M* $NaNO_2$ were added to 10 ml of sample solution containing more than 20 μg of anthranilic acid, and the mixture was allowed to stand for 20 min in an ice-bath. After this period of time, 1.1 ml of 0.1 *M* sulphamic acid were added and the reaction mixture was heated on a bath at 80° under reflux for 35 min to hydrolyse the diazonium salt and to produce salicylic acid. After cooling in an ice-bath, the contents were quantitatively transferred to a 30-ml centrifuge tube and 5 g of sodium chloride were added. The salicylic acid produced was then extracted for 1 min with two 5-ml portions of chloroform, followed by centrifuging at 2000 r.p.m. for 3 min. After centrifuging, the chloroform layers were sucked up by a syringe equipped with a long needle and a 0.5-ml aliquot of a chloroform solution containing a known amount of an internal standard (α-phenoxypropionic acid or α,α'-dichloro-*p*-xylene) was added to the combined chloroform extracts. Then, a few drops of dimethoxypropane (DMP) were added to dehydrate the mixture, which was shaken vigorously. The solution mixture was transferred to a 5-ml pear-shaped flask, evaporated to dryness at room temperature at a reduced pressure and the residue was dissolved in about 1 ml of freshly prepared ethereal diazomethane and allowed to stand at room temperature for 10 min; a small volume of chloroform was then added, the solvent was removed under reduced pressure until the volume was about 0.1–0.2 ml and 1.0 μl of the solution was injected onto the gas chromatograph. At the same time, aliquots of a standard chloroform solution containing known amounts of salicylic acid and an internal standard in various ratios were evaporated to dryness and the contents were treated as described above, preparatory to GC analysis.

Results and discussion

The optimum reaction conditions for the standard procedure as described under *Experimental* were established from preliminary experiments. It was found desirable to keep the acid concentration of the reaction medium above 0.4 *N* for the diazotisation step with nitrous acid. Below 0.4 *N*, unreacted anthranilic acid remained, which caused considerable variability in analytical results. Appreciable amounts of sulphamic acid were added to destroy the excess of nitrous acid, which interferes seriously with the quantitative conversion of the diazonium salt to salicylic acid. The effects of temperature and time on hydrolysis of the diazonium salt were examined under standard conditions, and the formation of salicylic acid was observed to be quantitative and constant at 80° for a hydrolysis of 30–40 min. Longer heating or a higher temperature lead to a decrease in the formation of salicylic acid. When the temperature was below 60°, the hydrolysis proceeded slowly and it was time-consuming to reach quantitative hydrolysis. The salicylic acid produced was readily extracted with chloroform in an acid medium. Loss of salicylic acid and α-phenoxypropionic acid during the evaporation to dryness at room temperature at reduced pressure was

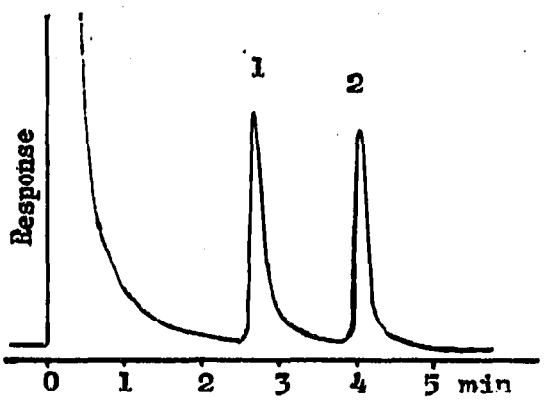

Fig. 1. Separation of (1) methyl salicylate (derived from anthranilic acid) and (2) methyl α-phenoxypropionate (internal standard). Column: 1.5 m × 3 mm I.D. stainless-steel packed with 5% GE-XF1105 on Gas-Chrom Z (80–100 mesh); temperature, 110°. Detection with a hydrogen flame ionisation detector.

TABLE I

RECOVERY OF ANTHRANILIC ACID

	Taken (μg)	*Found* (%)
1	100	97.8
2	100	95.2
3	100	98.5
4	100	95.7
av.		96.8
5	20	98.4
6	20	95.6
7	20	99.0
8	20	95.4
av.		97.1

negligible, but their methyl esters are volatile and therefore special attention was paid to concentration of the solutions containing them. DMP was found to be an excellent and elegant reagent for dehydration of the organic solvent; it reacted readily with water to produce methanol and acetone at room temperature and did not create any interference. A gas chromatogram obtained from 10 ml of a 0.1 *M* HCl solution containing 20 μg of anthranilic acid is illustrated in Fig. 1 and calibration curves were linear and passed through the origin in the range of 10 to 30 μg of anthranilic acid.

The recovery over the entire procedure was computed to be 96.8 $\pm$ 1.6 % (S.D.) and 97.1 $\pm$ 1.9 % (S.D.) for 100 μg and 20 μg of anthralinilic acid, respectively, as shown in Table I.

Urinary anthranilic acid was assayed by this technique from normal human urine (Fig. 2A). Peak X with a retention time of 2.0 min agreed with that of methyl salicylate and was completely separated from the coexistent urinary components using a 3 % OV-17 column. A 5 % GE-XF1105 column did not show a satisfactory separation of methyl salicylate from other urinary substances. It is of interest whether peak X is due to the presence of anthranilic acid in urine and investigations are currently being performed in our laboratory. When authentic anthranilic acid was added to parallel urine samples and carried through the procedure, the height of peak X (namely, peak MS in Fig. 2B) was found to increase in direct proportion to the quanti-

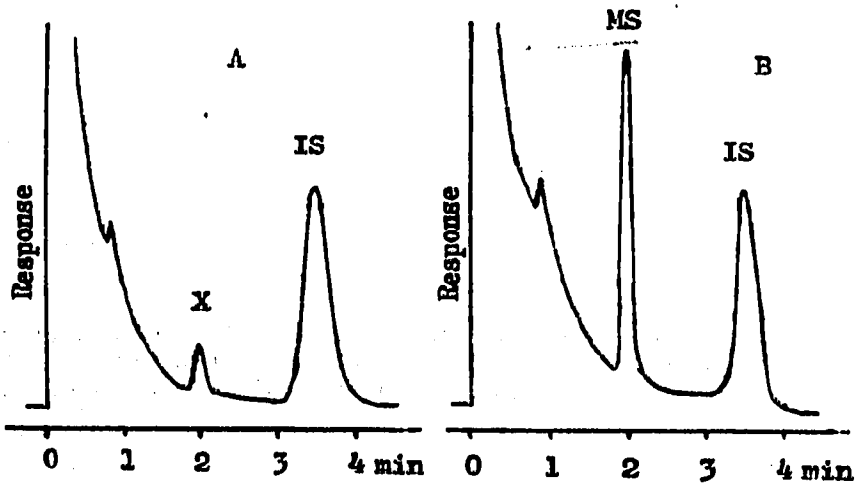

Fig. 2. Chromatogram of a sample from 10 ml of normal urine (A) compared to a parallel sample (B) to which 100 μg of anthranilic acid was added. Column: 1.5 m × 3 mm I.D. stainless-steel packed with 3% OV-17; temperature, 120°. Detection with a hydrogen flame ionisation detector. MS = Methyl salicylate; IS (internal standard) = methyl α-phenoxypropionate.

TABLE II

RECOVERY OF ADDED ANTHRANILIC ACID FROM IDENTICAL URINE SAMPLES

	Added (μg)	*Found* (%)
1	100	89.1
2	100	86.9
3	100	90.8
av.		89.1

ty of added anthranilic acid. Fig. 2B shows a chromatogram of a urine sample containing 100 μg of additional anthranilic acid and the reproducibility determined by carrying out three identical analyses is shown in Table II.

Trifluoroacetylation of methyl salicylate led to a more sensitive derivative and a satisfactory response was obtained from 0.1 ng of this using an electron capture detector. The derivative was readily prepared by treating methyl salicylate with trifluoroacetic anhydride in the presence of a small amount of pyridine at room temperature. α,α'-Dichloro-*p*-xylene was used as an internal standard and a GC separation was achieved successfully using a glass column, as shown in Fig. 3.

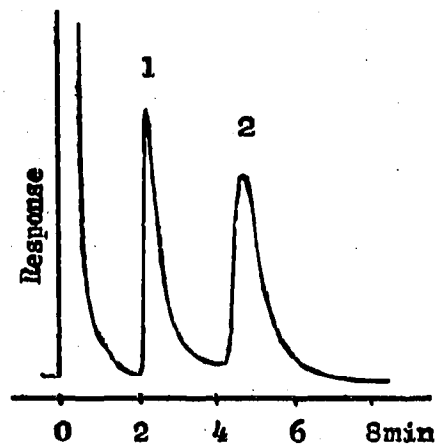

Fig. 3. Separation of (1) trifluoroacetylsalicylic acid methyl ester and (2) α, α'-dichloro-*p*-xylene (internal standard). Column: 1.5 m × 3 mm I.D. glass packed with 3% OV-17; temperature, 120°. Detection with an electron capture detector.

3-Hydroxyanthranilic acid seems to form the corresponding *o*-diazo-oxide[4] on reaction with nitrous acid and the resulting yellow product was not extractable with chloroform. The procedure is somewhat cumbersome. As can been seen from the recovery studies, however, it is reproducible and specific for the determination of anthranilic acid under the given operating conditions.

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