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Note

Determination of thiazinamium sulphoxide in urine by means of Amberlite XAD-2 column chromatography and thin-layer densitometry

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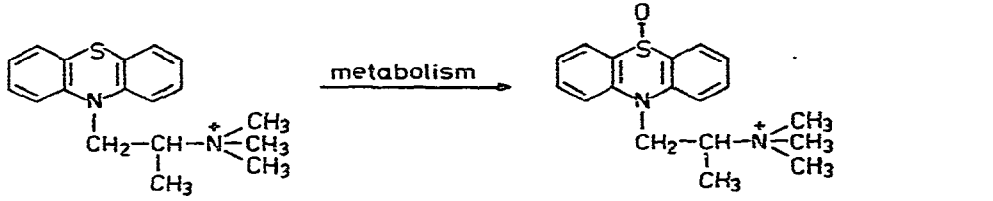
Hydrophilic organic compounds are difficult to isolate from body fluids, particularly components that are ionized over a wide pH range, such as quaternary ammonium compounds, amino acids and amphoteric sulphonic acids. In many instances, ion-pair extraction can be used[1–5], provided that the molecule has a sufficiently lipophilic moiety. However, if the latter moiety becomes more polar, *e.g.*, owing to metabolic changes, ion-pair liquid–liquid batch extraction may no longer be adequate. However column chromatography using Amberlite XAD-2 seems to be a possibility for isolation of such compounds.

We report here the successful application of Amberlite XAD-2 for the isolation from urine of the quaternary metabolite thiazinamium sulphoxide, the main metabolite of the anticholinergic drug thiazinamium methylsulphate (Multergan®). After the isolation two-dimensional thin-layer chromatography has been used for separation of the parent drug (I) and the metabolite (II) (see Fig. 1). The spots are visualized by immersion in an oxidative mixture and quantitation is done by measuring transmission, using an *in situ* method.



THIAZINAMIUM CATION — THIAZINAMIUM SULPHOXIDE CATION

I — II

Fig. 1. Formation of thiazinamium sulphoxide cation by metabolic oxidation.

MATERIALS AND METHODS

Apparatus

All densitometric measurements were performed on a Vitatron TLD 100 flying-

spot densitometer, using a filter of 525 ± 2 nm, and connected with a Vitatron UR 402 combined recorder–integrator.

The thin-layer plates were Fertigplatten Kieselgel 60 (Merck) (silica thin layers, 0.25 mm on 200 × 200 mm glass plates, without fluorescence indicator).

Glass columns of length 15 cm and I.D. 8 mm, provided with a stopcock, were used.

Chemicals

Amberlite XAD-2, particle size 300–1000 μm (Serva, Heidelberg, G.F.R.), was purified by extraction with methanol in a Soxhlet apparatus for 3 h and stored in a refrigerator (4°) under a mixture of methanol–water (30:70)[6].

Methanol, ammonium acetate, 25% ammonia solution, iron(III) chloride, acetone, absolute ethanol and 96% sulphuric acid were of reagent grade (Pro Analysi, Merck, Darmstadt, G.F.R.) and were used without purification.

Solutions of thiazinamium sulphoxide iodide (Specia, Paris, France) containing 0.5, 1.0 or 2.0 μg per 20 μl were used as reference solutions.

Column chromatographic procedure

The glass column was provided with a layer of glass-wool and filled with Amberlite XAD-2 suspension to a height of 6 cm, and was washed with distilled water until 30 ml has passed through the column. Then 1.0 ml of urine (or sufficient to give a concentration of thiazinamium sulphoxide of 0.5–2.0 μg in 20 μl of the final methanol solution) was transferred dropwise from a separation funnel on to the column (10 drops per minute). The separation funnel was washed with 10 ml of distilled water, which was allowed to pass through the column. Finally, the column was washed with *ca.* 50 ml distilled water (20 drops per minute). The column was then allowed to dry and remaining traces of water were removed by a stream of nitrogen. All aqueous eluates were discarded. The stopcock was closed and methanol transferred into the column to a height of 2 cm above the resin. The adsorbent was stirred vigorously in the methanol by means of a metal rod in order to remove air bubbles and was allowed to settle for 10 min. Methanol was added and elution was carried out (1 ml/min) until 15 ml of methanol eluate had been collected in a graduated conical glass tube. The methanol was evaporated in a water-bath at 70° under a gentle stream of nitrogen and the dry residue was dissolved in methanol (200 μl).

Thin-layer chromatographic procedure

A 20-μl volume of the methanol solution was applied as a spot to a thin-layer plate (in the lower left-hand corner, 2 cm from each side) and the plate was developed in methanol to a height of 15 cm in an unsaturated chamber. All developments and drying procedures (15 min) were carried out at 20° in the dark. After development, the plate was dried and the reference spots (0.5, 1.0 and 2.0 μg of thiazinamium sulphoxide iodide in 20 μl methanol) were applied to it above the position where the sample was spotted. The plate was turned through 90° and developed in a mixture of ammonium acetate (8 g), water (42 ml), methanol (200 ml) and ammonia solution (6 ml) in an unsaturated chamber; the pH of this mixture was 9.0. The plate was dried and immersed for 30 sec in an oxidative mixture of water (150 ml), sulphuric acid

(150 ml), iron(III) chloride (4 g), acetone (800 ml) and absolute ethanol (800 ml); this mixture is stable for 1 week. After immersion, the back of the plate was rinsed; then the plate was dried and heated in an oven at 140° for 4 min in order to develop the colours of the spots. After cooling, the transmission of the pink spots was measured immediately on the densitometer at 525 nm.

RESULTS AND DISCUSSION

The thiazinamium cation (I) can easily be isolated from body fluids by ion-pair liquid–liquid batch extraction[7], but attempts to isolate its only human metabolite, the sulphoxide, from urine in this way were unsuccessful because of the increased polarity of the lipophilic moiety (phenothiazine group). However, thiazinamium and its sulphoxide were found to be adsorbed very well from urine by Amberlite XAD-2, a synthetic macroreticular copolymer of styrene and divinylbenzene.

This resin has already been used successfully in the isolation of several drugs and drug metabolites[8–12] but not for quaternary ammonium compounds. After the adsorption process, subsequent elution with methanol was almost complete and, as a result, Amberlite XAD-2 column chromatography gave excellent recoveries. Other column materials were tested, including aluminium oxide (acidic, neutral and alkaline), silica gel, cellulose and charcoal, but their adsorption and elution characteristics were unacceptable (low recoveries). When using the Amberlite XAD-2 procedure, I and II can be transferred quantitatively from urine into an organic solvent (methanol), which allowed a 75-fold concentration step. This extract still contained too many interfering substances to allow direct UV quantitation. Gas chromatography could be performed with the parent drug, but failed with the sulphoxide because of its low volatility and on-column adsorption. Thin-layer chromatography with a mixture of ammonium acetate, methanol, water and ammonia (modified according to Korczak-Fabierkiewicz *et al.*[13]) gave an adequate separation of I and II, but was hampered by high values for the urine blanks. However, if the plate was first developed in methanol, followed by development with ammonium acetate–methanol–water–ammonia in a two-dimensional way, the interfering urine substances moved with the methanol and no longer interfered in the second development. I and II remained at the starting point with methanol, but in the second development I had an R_F value of 0.54 and II an R_F value of 0.36. After the development with methanol, three quantitative reference solutions of thiazinamium sulphoxide iodide were spotted on the plate so as to permit the construction of a calibration graph and to correct for variability in the plates.

For quantitation, we preferred to measure the transmission of the coloured spots because of the high sensitivity (reflection measurements were also possible, but were found to be much less sensitive because a major proportion of the substance accumulates at the surface of the glass plate rather than at the surface of the silica layer).

For quantitative *in situ* measurements, a smooth application of the reagent is required, which can be achieved better by immersion than by spraying. We decided to modify the reagent described by Chan and Gershon[14] by decreasing the water content so as to avoid abrasion of the silica from the glass plate. After immersion, heating at 140° gave pink-coloured spots with an absorption maximum at 525 nm. The sulphoxide spots were measured with the Vitatron TLD 100 flying spot den-

sitometer[15–18]. The amount in the sample can be calculated from the calibration graph, constructed from measurements with the reference solutions on each plate. The parent drug thiazinamium cation can also be determined in this way, but for routine analysis we preferred the quicker gas chromatographic method[7].

Selectivity, sensitivity, recovery and reproducibility

When normal human urine was examined, no blank values were observed, and so far no other drugs have been found to interfere in the procedure. The method can be used for urine samples up to 25 ml; the actual volume necessary to give an adequate supply to the column depends on the concentration of the drug in urine after therapeutic application. The detection limit of thiazinamium sulphoxide (calculated as iodide) on the plate is about 100 ng, which results in a detection limit for the whole procedure of 40 ng/ml in urine. Accurate quantitation can be achieved at concentrations of 250 ng/ml and above. The recovery at a therapeutic concentration of 10 μg/ml in urine was $90.6 \pm 6.5\%$ ($n = 7$).

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