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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF ^{14}C-LABELLED TOLOXATONE AND ITS METABOLITES

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SUMMARY

A method for the analytical and micropreparative separation of toloxatone and its urinary metabolites in man is described. Toloxatone was given as an aqueous solution and was labelled with ^{14}C. Following solvent extraction of urine, before and after enzymatic hydrolysis, one-step thin-layer chromatography on silica gel in combination with reversed-phase high-performance liquid chromatography, gave a good micropreparative separation for mass spectrometric analysis. After lyophilization of the high-performance liquid chromatographic fractions, the purity of the metabolites was checked by thin-layer chromatography. Acetic acid was chosen to regulate the pH of the mobile phase (acetonitrile—water) because it can be easily removed by lyophilization when a preparative separation is desired. The retention times as a function of the pH have been evaluated. Formic acid is also proposed for the optimization of the high-performance liquid chromatographic analysis. The quantitative analysis of ^{14}C-labelled toloxatone and its metabolites was carried out, after solvent extraction of 2 ml of urine, using the same high-performance liquid chromatographic method with off-line and flow-through radioactivity detection.

INTRODUCTION

Toloxatone, 5-(hydroxymethyl)-3-(3-methylphenyl)-2-oxazolidinone (Fig. 1) is a reversible inhibitor of monoamine oxidase A [1, 2] which possesses an antidepressant activity [3—5]. Previous studies in man [6] have shown that the drug is extensively metabolized, the majority of the dose being excreted in the urine. Two metabolites have been isolated from urine: 5-(hydroxymethyl)-3-(3-carboxyphenyl)-2-oxazolidinone (metabolite 1) and 5-(hydroxymethyl)-3-(4-hydroxy-3-methylphenyl)-2-oxazolidinone (metabolite 2). Toloxatone and metabolite 2 were also excreted in urine as conjugates with glucuronic acid. The structure of these metabolites was confirmed by comparison with the

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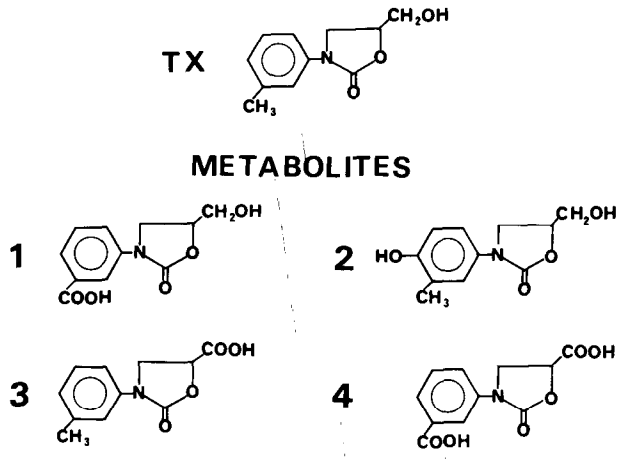

Fig. 1. Chemical structures of toloxatone (TX) and its metabolites.

synthetic compounds. Two other metabolites were isolated by thin-layer chromatography (TLC) but remained unidentified. Further studies showed that, even with successive TLC analyses, the purity of the two unknown metabolites was not suitable for identification by mass spectrometry (MS). Therefore, combined TLC and high-performance liquid chromatography (HPLC) were attempted for the qualitative and quantitative analysis of the metabolites.

MATERIALS AND METHODS

Chemicals and reagents

Toloxatone labelled with ^{14}C in the carbonyl group of the oxazolidinone ring was synthesized by the chemical and radioisotope division of ICN. The radiochemical purity was > 99% after TLC analysis with solvent system 1 (see Table I) and with the system benzene—methanol—acetone (90:10:5, v/v/v). The specific activity was 0.344 μCi/mg.

HPLC-grade water for preparative HPLC analysis was used (Fisons Scientific Apparatus, Loughborough, U.K.). Pro analysi acetic acid, formic acid, orthophosphoric acid (E. Merck, Darmstadt, F.R.G.), acetonitrile HPLC S grade (Rathburn Chemicals, Walkerburn, U.K.), ethyl acetate "RPE-ACS" grade and chloroform RPE (Carlo Erba, Milan, Italy) were used.

Enzymatic hydrolysis was carried out with β-glucuronidase—arylsulphatase (H_1, Sigma, St. Louis, MO, U.S.A.) (2 mg/ml of urine).

HPLC analysis

HPLC was performed with a Micromeritics system (752 gradient programmer, 750 pump, 786 variable-wavelength detector), a WISP 710B automatic injector (Waters Assoc., Milford, MA, U.S.A.), coupled to a Hewlett-Packard 3390A integrator and a Perkin-Elmer 56 recorder. A 25 × 0.46 cm CP-TM-Microspher ODS 3-μm column (Chrompack, Middelburg, Netherlands) was used at a flow-rate of 1 ml/min. The ultraviolet (UV) detector was set at 240 nm. A guard column of 7.5 × 0.21 cm (Chrompack packed guard column, reversed-phase type B) was mounted directly on the head of the analytical column.

UV detection was combined with off-line radioactivity measurements (0.5—1 ml fractions) by liquid scintillation counting (Intertechnique SL 3000, Roche Bioelectronique, Velizy, France) with 10 ml of Unisolve (Koch-Light Labs., Colnbrook, U.K.).

Radioactivity was also determined with a flow-through radioactivity detector (Flo-One HS, Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.) coupled to the UV detector of the HPLC system. Lumapack (Kontron Analytique, Trappes, France) was used as scintillation fluid at a flow-rate of 3 ml/min.

Sample preparation for isolation of metabolites

Toloxatone and its metabolites were isolated from urine (0—12 h fraction) after oral administration of an aqueous solution of [^{14}C]toloxatone (200 mg, specific activity 0.344 μCi/mg) to six healthy adult volunteers. Urine (200 ml) was extracted at pH 1 (1 *M* hydrochloric acid) with ethyl acetate (3 × 500 ml) before and after enzymatic hydrolysis (37°C, pH 5.5, 16 h). The solvent was evaporated at 30°C under reduced pressure (Rotavapor, Büchi, Flawil, Switzerland). The dry residue was dissolved in methanol and applied on silica gel F_{254} plates 20 cm × 20 cm (0.25 mm, Merck). Toloxatone, 150 μgequiv./cm, was spotted in a band of 18 cm using a Linomat III (Camag, Müttenz, Switzerland) and the plates were developed with solvent system 1 (Table I).

TABLE I

SOLVENT SYSTEMS FOR THE TLC SEPARATION

1 Chloroform—methanol—water	65:35:5
2 Toluene—methanol—acetone	50:50:10
3 Toluene—ethanol	50:50
4 Toluene—methanol—acetone	80:20:5
5 Chloroform—acetone	50:50

After detection of the radioactive bands on NS-2T film (Kodak, Rochester, NY, U.S.A.), the silica gel corresponding to the bands was scraped off the plates and toloxatone and its metabolites were recovered from the silica gel with methanol (3 × 5 ml). The fractions isolated by TLC (system 1) were further purified by HPLC; methanol was evaporated at 30°C under reduced pressure, the dry residue was dissolved in water (≈5 μgequiv./μl) and 250 μgequiv. of toloxatone were injected onto the HPLC column. The HPLC fractions containing the radioactivity were collected and the mobile phase was frozen and freeze-dried. The dry residue was dissolved in acetonitrile to a concentration of 1 μgequiv./μl toloxatone for identification by MS. Before MS analysis, the purity of each fraction was verified by TLC analysis (single spot with the solvent systems given in Table I).

Sample preparation for quantitative analysis of labelled drug and metabolites

Analysis of unconjugated compounds. A 2-ml volume of urine (0—12 h fraction) was dispensed into a conical tapered glass tube and the pH was adjusted to 1 with 1 *M* hydrochloric acid. The sample was extracted successively with 5 ml of chloroform and with 3 × 5 ml of ethyl acetate on a mechanical

shaker (15 min). After centrifugation (10 min at 1000 *g*), the organic phase was transferred into a separate tube and evaporated to dryness in a water bath at 40°C under a stream of nitrogen. The two dry residues (chloroform and ethyl acetate) were dissolved in 1 ml of water and 50—100 μl were injected into the HPLC system.

Analysis of conjugated compounds. The same procedure was followed after enzymatic hydrolysis (37°C, pH 5.5, 16 h) to determine the metabolites conjugated with glucuronic acid.

RESULTS AND DISCUSSION

Optimization of the HPLC analysis for isolation of metabolites

It has been shown that reversed-phase HPLC with gradient elution is a rapid and powerful method for the separation of urinary acids [7]. Off-line identification by MS with direct inlet methods is possible with a few micrograms of the compound, but of high purity. For this type of preparative separation, analytical columns can be used with a high efficiency and capacity [8]. However, only volatile salts and acids can be used for the separation, to assure the recovery of the compound from the HPLC fraction [9]. Acetic acid has been used to adjust the pH of the mobile phase in the reversed-phase mode for the preparative separation of polar metabolites [10]. The limitations of its use, however, are worth noting.

The effect of acetic acid on the isocratic mobile phase (water—acetonitrile,

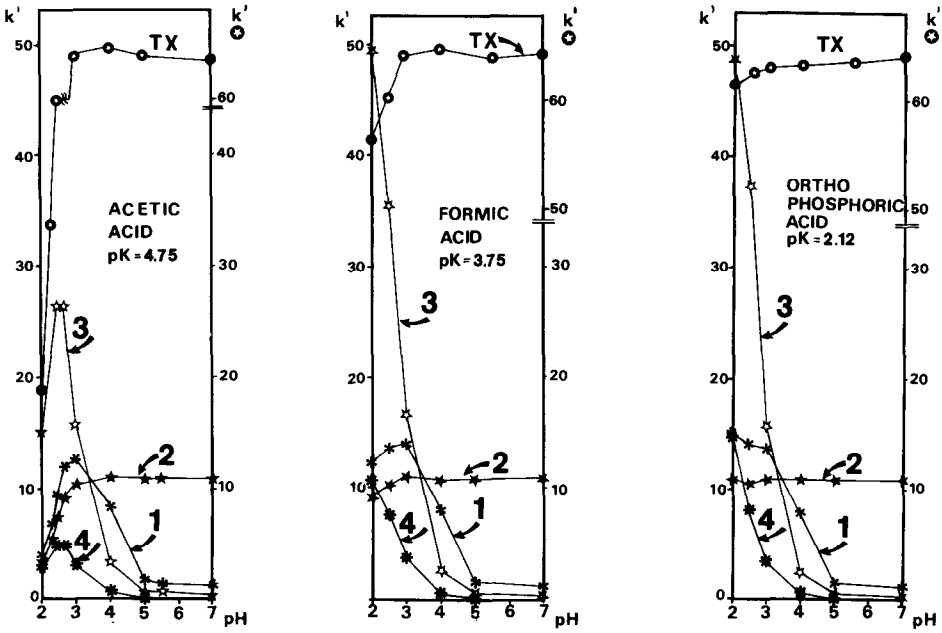

Fig. 2. Effect of pH on the separation (k') of toloxatone (TX) and its metabolites 1, 2, 3 and 4. HPLC analysis was carried out in isocratic conditions (10% acetonitrile); the pH of the water (90%) was adjusted with acetic, formic and orthophosphoric acid. The k' values of toloxatone are given on the right-hand ordinate. $k' = (t_R - t_0)/t_0$.

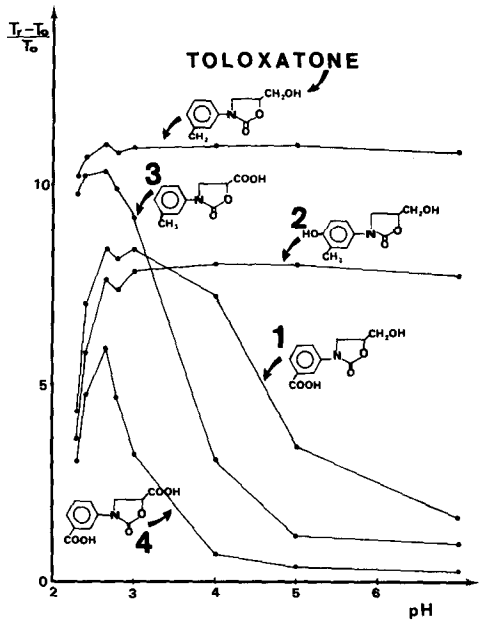

Fig. 3. Effect of pH on the separation of toloxatone and its metabolites 1, 2, 3 and 4. HPLC analysis was carried out with a concave gradient of acetonitrile—water (10:90, v/v, to 40:60, v/v) over 10 min. The pH of the water was adjusted with acetic acid.

90:10, v/v), was investigated. The capacity factor [$k' = (t_R - t_0)/t_0$] of toloxatone and the four metabolites isolated, are shown (Fig. 2) in the pH range 2—7. As expected, no major changes were observed for toloxatone and metabolite 2 between pH 3 and 7, whereas a consistent reduction of k' values was observed for the carboxylic acid derivatives (metabolites 1, 3 and 4). For the acid metabolites, the sigmoid curve predicted by Horváth et al. [11] was not found, since k' values decreased below pH 3. Furthermore, this effect was more pronounced with a lower proportion of acetonitrile in the mobile phase (Figs. 2 and 3).

Since acetic acid is moderately strong (pK 4.75), low pH values (< 3) are reached only with relatively high concentrations of the acid [12]. At this pH most of the acetic acid is in the unionized form, which increases the elution of the compounds analysed and results in a reduction of k' values. This hypothesis was confirmed by the analysis of toloxatone and the four metabolites with formic acid (pK 3.75) and with orthophosphoric acid (pK_1 2.12) (see Fig. 2).

Formic acid, which is also volatile, can be used over a larger pH range than acetic acid, whereas orthophosphoric acid is not recommended because of difficulty in its removal from the sample.

In the present study, optimization of the HPLC analysis was obtained with a concave gradient (No. 9 on the 752 gradient programmer; see Fig. 4) of water (pH 2.7) and acetonitrile (10 to 40% acetonitrile in 10 min). The pH 2.7 (≈1%, v/v, acetic acid in water) corresponded to a good separation of the five compounds (Fig. 3).

Urinary excretion of toloxatone and metabolites

Between 86% and 95% of the dose was excreted by the subjects in the 0—12 h urine fraction after administration of toloxatone. The micropreparative separation of toloxatone and its metabolites was carried out on this fraction. The combination of silica gel TLC and reversed-phase HPLC allowed a good separation of toloxatone and its metabolites whose purity was confirmed by TLC (solvent systems showed in Table I) either with autoradiography or UV detection at 254 nm.

Before enzymatic hydrolysis, 60—65% of the radioactivity administered was extracted with chloroform and ethyl acetate and four major compounds were isolated after TLC (solvent system 1): metabolites 1 (R_F 0.35), 2 (R_F 0.52), 3 (R_F 0.22) and 4 (R_F 0.09).

After hydrolysis with β-glucuronidase—arylsulphatase a fifth band corresponding to toloxatone (R_F 0.62) was isolated after TLC; in this case, the radioactivity extracted from urine corresponded to 75—80% of the dose. After enzymatic hydrolysis, the radioactivity extracted with chloroform and ethyl acetate did not account for the total (86—95%). The difference could be attributed to incomplete enzymatic hydrolysis or to a metabolite which is not conjugated with glucuronic and/or sulphuric acid [6].

Fig. 4 shows typical HPLC chromatograms of samples containing approx. 200 μg of toloxatone equivalents of metabolites 1 and 3. MS analysis of the HPLC radioactive fractions by direct probe insertion into the mass spectrometer, in the electron-impact or chemical-ionization (ammonia) mode, or by gas chromatography—mass spectrometry (GC—MS) after derivatization with trimethylanilinium hydroxide (methylation) or N,O-bis(trimethylsilyl)tri-

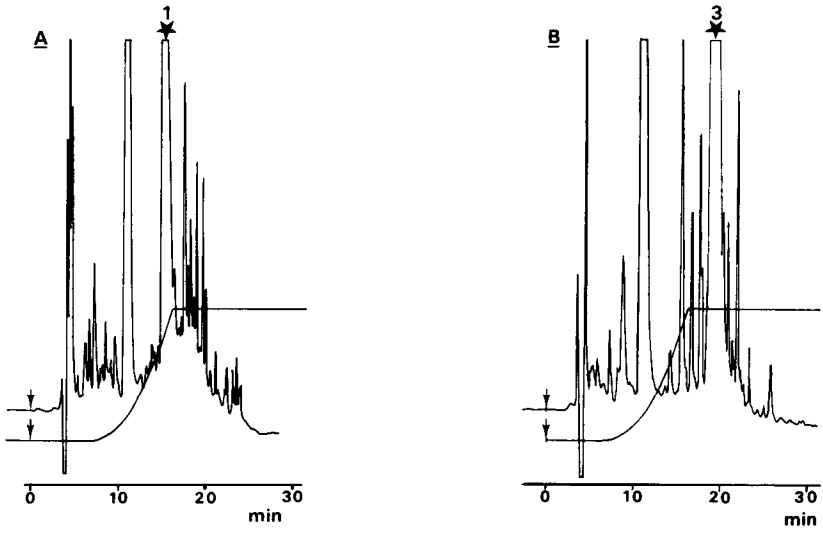

Fig. 4. HPLC chromatograms obtained after TLC separation of urine extracts with ethyl acetate. Urine was hydrolysed with β-glucuronidase—arylsulphatase. The fractions analysed contained ≃ 200 μg of toloxatone equivalent of metabolite 1 (A) and ≃ 200 μg of toloxatone equivalent of metabolite 3 (B). The concave gradient (10 min) was started at the time of injection. The isocratic run (6 min) corresponds to the volume between the gradient programmer and the column at 1 ml/min. Radioactivity peaks in the UV signal are starred (★).

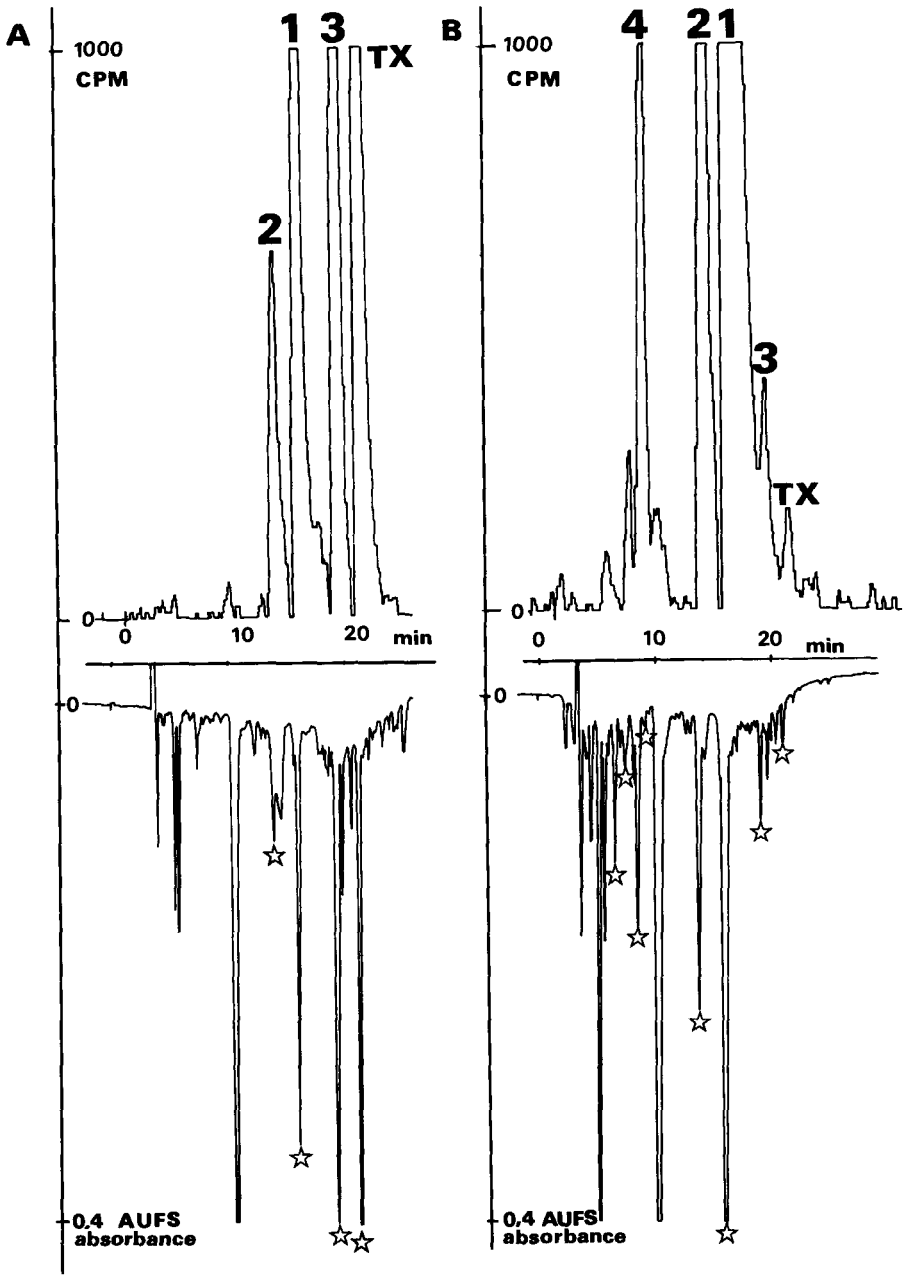

Fig. 5. HPLC chromatograms of urine after hydrolysis with β-glucuronidase—arylsulphatase, extracted with chloroform(A) and with ethyl acetate (B). The peaks of radioactivity, relative to toloxatone (TX) and its metabolites 1, 2, 3 and 4, determined in the UV signal are starred (☆).

fluoroacetamide (silylation), allowed the identification of the metabolites shown in Fig. 1.

The structures were also confirmed by high-resolution MS analysis. MS analysis of the carboxylic acid metabolites 1, 3 and 4 was not possible by

direct introduction into the ion source when HPLC separation of the metabolites was carried out with distilled water. The presence of unidentified impurities prevented the desorption of these compounds, whereas, after methylation or silylation, the same samples were easily analysed by GC—MS. This problem was avoided using commercial HPLC-grade water.

Quantitative analysis of ^{14}C-labelled toloxatone and its metabolites

Optimization of the HPLC analysis allowed the quantitation of toloxatone and its metabolites after the extraction of 2-ml urine samples; 50—100 μl of the aqueous solution of the urine extracts (see sample preparation) were injected into the HPLC system. Both the chloroform and the ethyl acetate extracts were chromatographed and the radioactivity was determined by liquid scintillation counting in each HPLC fraction (0.5—1 ml). Toloxatone and metabolite 3 were extracted principally with chloroform, whereas metabolites 1, 2 and 4 were well extracted with ethyl acetate (Fig. 5).

After the HPLC analysis of each 0—12 h urine fraction the recovery of the total radioactivity injected on the HPLC column was > 98%. The quantitative analysis was obtained without internal standard. The radioactivity counted for toloxatone and its metabolites, after HPLC analysis of the chloroform and the ethyl acetate extracts, was converted to percentage of the dose excreted for each subject; the results are shown in Fig. 6.

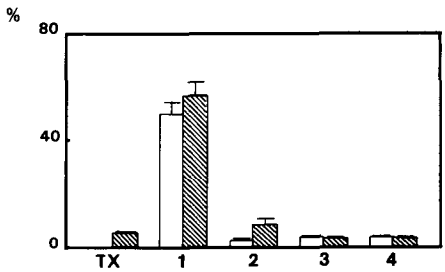

Fig. 6. Urinary excretion profile of ^{14}C-labelled toloxatone (TX) and its metabolites 1, 2, 3 and 4 in 0—12 h post dose urine. Mean ± S.D. values in six subjects, before (open bars) and after (hatched bars) hydrolysis with β-glucuronidase—arylsulphatase, are shown.

Toloxatone and metabolite 2 were excreted in urine mostly as conjugates, whereas metabolites 1, 3 and 4 were principally excreted in the free form. The determination of the radioactivity was repeated with a flow-through radioactivity detector (Flo-One HS). The values obtained with both techniques were similar but direct quantitation with the flow-through detector resulted in a considerable reduction of the overall time of analysis. In addition, a smaller volume of scintillation fluid was used.

In conclusion, this work describes a simple and efficient technique for the isolation of urinary metabolites of ^{14}C-labelled toloxatone in man. The combination of one-step silica gel TLC with reversed-phase HPLC purification is proposed as a general approach for the isolation of polar metabolites in urine, particularly for carboxylic acid derivatives.

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