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High-performance liquid chromatographic analysis of hippuric acid in human blood plasma

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ABSTRACT

A method has been developed for the isocratic high-performance liquid chromatographic analysis of hippuric acid in human blood plasma. After the addition of an internal standard (3-methoxysalicylic acid), plasma samples (1 ml) were made alkaline and extracted stepwise with methylene chloride and ethyl acetate. The detection limit was 50 pmol of hippuric acid per ml of plasma. The concentrations of hippuric acid in plasma from house painters (n = 8), with long-term exposure to solvent vapours from alkyd paints, were in the range 1–21 nmol/mol (median 11 nmol/ml). These values were statistically significantly higher than those for controls (n = 9) 2–8 nmol/ml (median 3 nmol/ml)

INTRODUCTION

Occupational long-term exposure to low levels of organic solvents results in several deleterious effects on the organism [1,2]. Some of the more important effects of solvent exposure are functional changes in the central nervous system, embryotoxicity and carcinogenicity [3–5]. Workers with long-term low-level exposure to solvent mixtures such as white spirit (17–22% aromatics) have shown disturbancies in the function of the central nervous system and in blood cell count [6]. In organic solvent mixtures, such as Solvesso 100 (95–98% aromatics), some components are known to exert toxic effects on the blood-forming organs in the human body [1,7].

In connection with our studies on the impact of solvents on human blood platelets we have used viable platelets as models for serotonergic neurons. In those studies we found that the platelet function, estimated as the uptake of serotonin in platelets (V_{max}), was altered in platelets from workers with long-term

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exposure to organic solvents, at levels below the Swedish exposure limits for the specific components included [8–10].

Because of these results and the fact that serotonin uptake can be influenced by inhibitors of the platelet membrane function [11], it became of interest to evaluate whether or not acidic metabolites form aromatic organic solvents may affect the cell membrane function of the platelet.

Various methods for the analysis of organic acids in blood have been published [12–19]. For biological monitoring of exposure to aromatic solvents (ethylbenzene, styrene, toluene, xylene), several methods have been developed for the determination of the corresponding urinary metabolites (mandelic and phenylglyoxylic acids, both from the two first solvents, and hippuric and methylhippuric acids, respectively) [18–21]. To our knowledge there is no published method for isocratic high-performance liquid chromatographic (HPLC) analysis of these metabolites in blood plasma in a low concentration range (50–500 pmol/ml of plasma).

The aim of this investigation was to develop an isocratic HPLC method for the analysis of aromatic acids (preferably hippuric acid) in blood plasma in concentrations below 500 pmol/ml. Painters are occupationally exposed to organic solvents (toluene) in the paints. Since toluene is metabolized in the body to benzoic acid, which in turn biotransforms into hippuric acid, analysis of hippuric acid was preferred. Such a method would make it possible to study the impact of acid metabolites from aromatic organic solvents on the function of human platelets. Furthermore, it would be possible to study the relation between the uptake of aromatic organic solvents and the amount of their acid metabolites in the blood, *e.g.* during occupational exposure.

EXPERIMENTAL

Reagents and chemicals

Ethyl acetate was HPLC grade (Rathburn, Walkerburn, U.K.). Methanol, methylene chloride and tetrahydrofuran, as well as hippuric, mandelic and phenylglyoxylic acids, disodium tetraborate (borax), hydrochloric acid (32%) and phosphoric acid (85%) were of p.a. quality (Merck, Darmstadt, F.R.G.). 3-Methoxysalicylic acid was p.a. quality (Aldrich Chemie, Steinheim, F.R.G.). RBS-25 detergent in cleaning solution for glassware was from Fluka (Buchs, Switzerland). Glass-redistilled water was used.

Blood samples

Eight randomly chosen house painters (men; age range 18–51 years) participated in the study. Their long-term occupational exposure consisted of low levels of organic solvent vapours from alkyd paints. They had been employed as painters for two to ten years. The painters' daily exposures to aromatic solvents were measured by diffusion sampling and gas chromatographic analysis. The total

exposures were calculated to be comparable with 1–35 ppm toluene [22]. The exposure to toluene was 0.2–16 ppm. Nine subjects (seven men and two women; age range 22–48 years) were chosen at random as a control group. They were taken from an investigation on the hematological parameters of healthy people who were not occupationally exposed to solvents. All subjects gave informed consent to participate in the study.

Blood (5 ml) was sampled from a cubital vein with a stainless-steel cannula (Wassermann needle; 1.4 mm I.D.) and collected in glass test-tubes containing 0.048 ml of 0.34 *M* tripotassium ethylenediaminetetraacetic acid (K_3EDTA; Vacutainer No. 606452). After centrifugation for 15 min at 200 × *g*, the plasma supernatant was removed with a pipette (polypropylene tip) and immediately stored at −70°C. The analysis were performed within a month after blood sampling.

Extraction of metabolites from blood plasma

All glass test-tubes used were cleaned before use by boiling for 1 h in a detergent solution (RBS-25; 2%, v/v), and rinsed with glass-redistilled water. The extraction procedure is outlined in Fig. 1. The frozen plasma samples (1 ml) were allowed to thaw at room temperature prior to extraction. A 100-μl volume of internal standard (3-methoxysalicylic acid, 11.8 nmol) and 3 ml of saturated borax solution were added, and the mixture was extracted with 4 ml of methylene chloride for 1 min on a vortex mixer.

During the extraction, an emulsion was formed between the phases. The methylene chloride phase, including the emulsion, was re-extracted with borax solution (2 ml). The aqueous phase from this separation was collected. After centrifugation at 1500 *g*, the aqueous phase from the first extraction step was re-extracted with 4 ml of methylene chloride, which was then used to extract the collected aqueous phase form the emulsion separation. Between each extraction step the samples were centrifuged at 1500 *g*.

The combined aqueous phases (5 ml) from the first separation, and from the emulsion separation, were acidified to pH 1 with hydrochloric acid (2.0 *M*) and extracted three times with 2-ml portions of ethyl acetate. The combined ethyl acetate phases were evaporated to dryness under a nitrogen stream at 35°C. The residue was dissolved in 500 μl of methanol and again evaporated to dryness. This residue was dissolved in 200 μl of the mobile phase and centrifuged for 15 min at 2700 *g*. A 20-μl aliquot of the supernatant was analysed by HPLC.

Chromatography

A Waters liquid chromatographic system was used (Model 510 pump, Model 990 diode array detector; Millipore-Waters Chromatography Division, Milford, MA, U.S.A.). The UV absorbance was recorded at 205 nm. The column was a Hibar LiChroCART 125-4 RP-18 Supersphere (125 mm × 4 mm I.D.; average particle size 4 μm; Merck). The mobile phase was methanol–water–

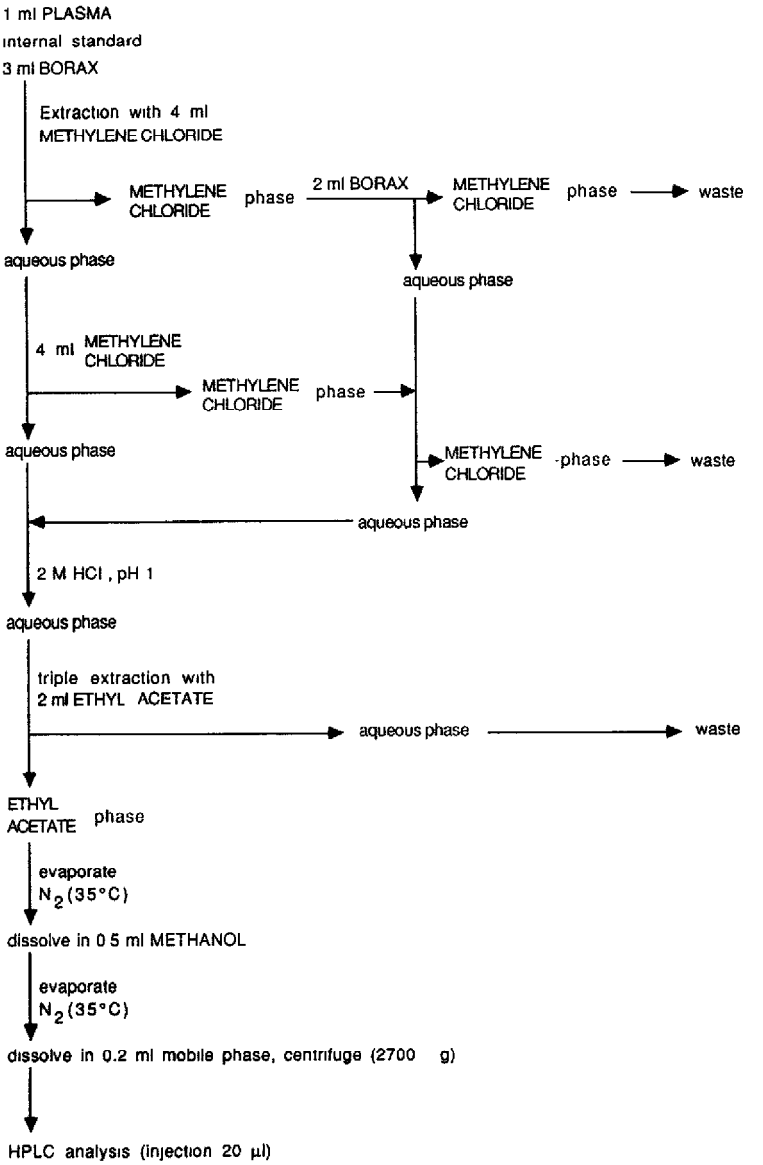

Fig. 1. Extraction procedure for hippuric, mandelic, 3-methoxysalicylic and phenylglyoxylic acids in blood plasma.

tetrahydrofuran–phosphoric acid (concentrated) (350:635:10:5, v/v). The flow-rate was 1.0 ml/min. A Rheodyne injector with a loop volume of 20 µl was used. The column was reconditioned overnight by recycling 1 l of methanol–water (35:65, v/v) through the column at 0.5 ml/min.

RESULTS AND DISCUSSION

Fig. 2 shows a separation of hippuric, mandelic, 3-methoxysalicylic and phenylglyoxylic acids. Owing to the low concentration of metabolites normally present in plasma, it was necessary to set the detector wavelength at 205 nm, close to the solvent cut-off limit. The standard curves were linear in the range tested (50–500 nmol). The detection limits were 50 pmol/ml plasma at a signal-to-noise ratio of 2 for hippuric and 3-methoxysalicylic acids. The recoveries of these acids in the extraction procedure were 69% (S.D. 5%) and 70% (S.D. 4%), respectively. The precision of the analysis was checked by injecting 10 nmol of hippuric acid ten times, which gave a within-day variation of ± 0.43%.

A randomly chosen chromatogram, obtained from the HPLC analysis of plasma from a control subject, is shown in Fig. 3. This plasma blank showed several peaks early in the chromatogram. The first two peaks were impurities in the extraction solvent. Peaks appearing later were of low intensity and none of them interfered with the acids analysed. The most important peak in the chromatogram corresponded to hippuric acid (2 nmol/ml of plasma), which is also an

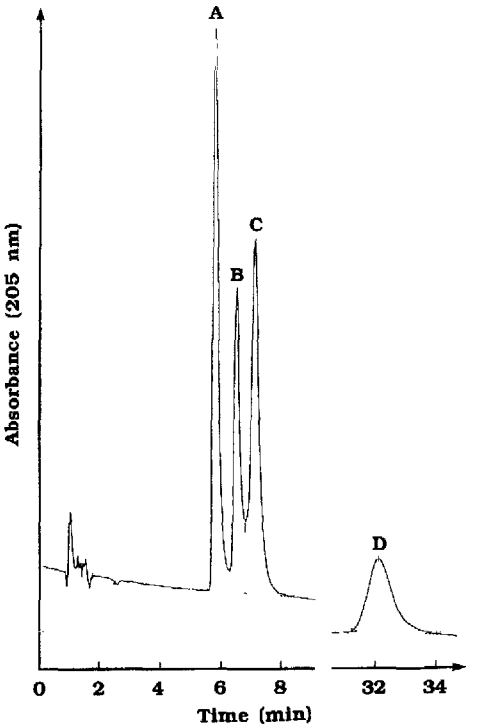

Fig. 2. HPLC separation of three aromatic acids dissolved in the mobile phase Peaks A = mandelic acid (1 51 nmol), B = hippuric acid (0 250 nmol), C = phenylglyoxylic acid (1.20 nmol); D = 3-methoxysalicylic acid (0.295 nmol) Ordinate, 0 066 a.u

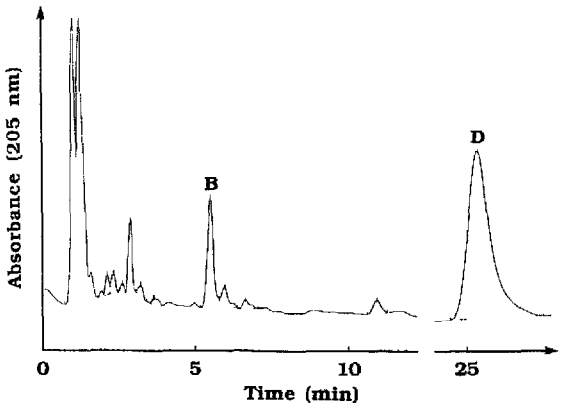

Fig 3 Chromatogram of an extracted plasma sample (1.0 ml) obtained from a non-exposed control subject. Peaks B = hippuric acid (2 nmol/ml of plasma); D = internal standard Ordinate, 0.052 a.u

endogenous metabolite. Co-chromatography with reference substances was used to identify hippuric, mandelic, 3-methoxysalicylic and phenylglyoxylic acids. Overlapping of peaks in the chromatogram was checked by using the diode array detector.

Table I presents the results from the analyses of extracts of plasma samples from house painters and the control group. The amount of hippuric acid in

TABLE I

HIPPURIC ACID CONCENTRATION IN PLASMA FROM HOUSE PAINTERS AND CONTROLS

Painters			Controls		
No.,	Age (years)	Hippuric acid[a] (nmol/ml)	No	Age (years)	Hippuric acid (nmol/ml)
E1	51	21 (p)	C1	22	3
E2	45	16 (p)	C2	45	3
E3	31	10 (m, p)	C3	49	8
E4	42	19 (m)	C4	34	3
E5	37	6 0 (m)	C5	43	6
E6	44	13 (p)	C6	32	6
E7	43	1.3 (m, p)	C7	35	2
E8	18	4 8 (p)	C8	32	3
			C9	29	4
Median		11.5			3
Range		1.3–21			2–8

Mann–Whitney *U*-test (hippuric acid): 1-tailed, $U = 14$, $0\,01 < p < 0\,02$

[a] p = phenylglyoxylic acid detected; m = mandelic acid detected.

plasma (11.5 nmol/ml) was greater than the normally occurring levels of hippuric acid (3 nmol/ml) from non-exposed subjects. The hippuric acid concentration in plasma from the painters was significantly higher ($p < 0.02$).

No measurable concentration of mandelic acid or phenylglyoxylic acid was detected in plasma from the controls. Plasma samples from six of the eight painters (E1–E3 and E6–E8) contained traces of phenylglyoxylic acid. In plasma from four painters (E3–E5 and E7) traces of mandelic acid were detected No significant linear correlation was detected between the hippuric acid concentration in plasma and the total exposure ($r = -0.504$) or the toluene exposure ($r = -0.571$). This may be due to rapid excretion of the metabolites or to the varying working tasks of the painters.

A randomly chosen chromatogram, obtained from the HPLC analysis of plasma from a painter, is shown in Fig. 4. In this chromatogram peaks corresponding to mandelic, hippuric and phenylglyoxylic acids can be seen. Several other peaks, which are not found in the plasma extracts from non-exposed subjects (Fig. 3), occur in the chromatograms from the painters (Fig. 4). These peaks were not identified.

Several methods of extraction of solvent metabolites from blood plasma have been published [12–19,23,24]. Some of these were tested using blood samples containing added solvent metabolites in the concentration range below 500 pmol/ml of plasma. However, emulsions were formed and phase separation could not be achieved.

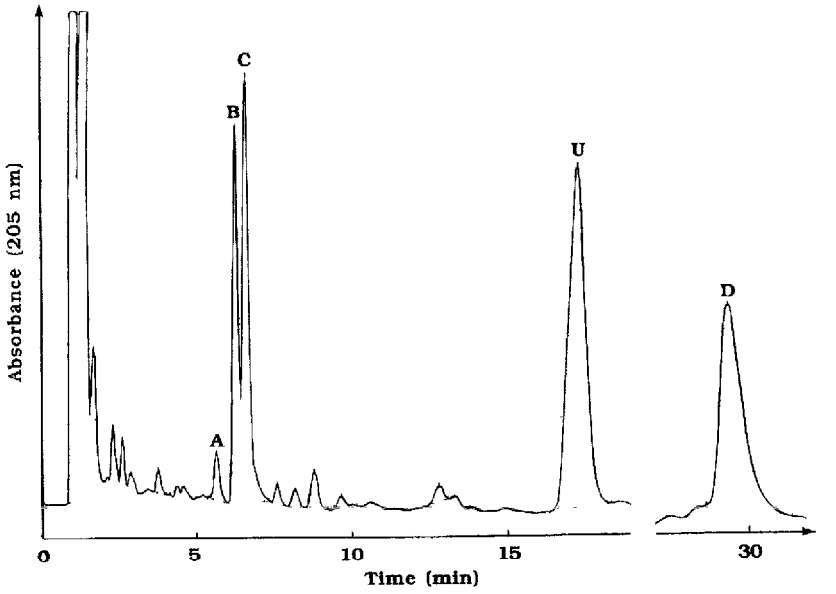

Fig 4 Chromatogram of an extracted plasma sample (1 0 ml) obtained from a house painter Peaks A = mandelic acid, B = hippuric acid (10 nmol/ml of plasma), C = phenylglyoxylic acid, U = unknown, D = internal standard. Ordinate, 0 045 a.u.

A problem that frequently arises when analysing low-molecular-mass compounds in biological samples is the interference from proteins in extraction procedures. The sensitivity of available methods for the analysis of small amounts of urinary metabolites was not sufficient for analysing these substances in blood or plasma. Other methods for the analysis of acids in plasma, involving acid precipitation of proteins, do not enable the detection of acids in the low concentration range.

It was found that some commercially available solvents used in the extraction procedure contained impurities that interfered with the analysis of the aromatic acids in the picomole range. The solvents selected and eventually used throughout this study were found to be acceptable, and were used without further purification.

Blood plasma taken from unexposed control subjects was analysed. In these samples the concentrations of mandelic and phenylglyoxylic acids were below the detection limit. The plasma samples were spiked with hippuric, mandelic or phenylglyoxylic acid. When the plasma sample was first washed with methylene chloride, an emulsion was formed in the organic phase (Fig. 1). This emulsion remained during subsequent extraction. However, with the described extraction method, the formation of emulsions did not affect the recovery of the acid metabolites. Thus this extraction method gives better results than other previously published methods for the analysis of hippuric acid in plasma using acid precipitation of proteins.

CONCLUSIONS

The described analysis can be used for the estimation of hippuric acid concentrations down to 50 pmol/ml of plasma. An improved resolution of the early eluting components in the chromatogram may be achieved by gradient elution. However, we have found that isocratic analysis is sufficient for determination of hippuric acid in blood plasma. Other metabolites structurally related to hippuric acid might be analysed by this procedure in order to estimate the dose of inhaled aromatic solvents in humans. The impact of aromatic solvent metabolites on the function of human platelets might therefore be explored.

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