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High-performance liquid chromatographic determination of phenol, 4-nitrophenol, β-naphthol and a number of their glucuronide and sulphate conjugates in organ perfusate

Melanie J. Thompson, Linsay N. Ballinger, Sheree E. Cross, Michael S. Roberts*

Department of Medicine, University of Queensland, Princess Alexandra Hospital, Brisbane 4102, Qld., Australia

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Abstract

This paper describes a simple and more sensitive reversed-phase HPLC method for the quantification of phenol, 4-nitrophenol and β-naphthol and some of their glucuronide and sulphate conjugates in aqueous solution and liver perfusate buffer. Methanol–water mobile phases with ion-pairing agents for each phenolic group are detailed. The assay showed good recovery, accuracy and precision and is suitable for the quantification of these phenolic compounds in liver perfusion experiments.

Keywords: Phenol; 4-Nitrophenol; β-Naphthol

1. Introduction

Many phenolic compounds (steroids, drugs, drug metabolites) are detoxified by hepatocytes by the formation of sulphate or glucuronide conjugates. In order to investigate the metabolism and disposition of a series of these compounds in the liver, a rapid, sensitive and precise assay is required to quantitate the phenolic compounds and their metabolites in the perfusate resulting from isolated perfused rat liver (IPRL) preparations. The compounds of interest were phenol (Phe), 4-nitrophenol (PNP) and β-naphthol (β-N) (Fig. 1), which were chosen to examine the effect of lipophilicity on the outflow profiles of the parent phenolic compound and the extent of metabolism to their glucuronide and sulphate conjugates by the liver. Commercial standards of both sulphate and glucuronide metabolites were not available for all three compounds; however, assays were developed to quantify: (i) Phe and its β-D-glucuronide conjugate (PG); (ii) PNP along with 4-nitrophenylsulphate (PNPS) and 4-nitrophenyl-β-D-glucuronide (PNPG); and (iii) β-N and β-naphthyl sulphate (β-NS). Conformation of the presence of phenyl sulphate and β-naphthol glucuronide was conducted for organ perfusions using specific enzymatic procedures, sulphatase and glucuronidase to convert sulphate and glucuronide to their respective parent phenols [1]. In the present study, we were particularly interested in the effect of lipophilicity on the transit time and disposition of phenolic structures and their glucuronide and sulphate conjugates in the liver. HPLC methods have been published for the analysis of phenols [2,3], nitrophenols [3,4], α-naph-

*Corresponding author.

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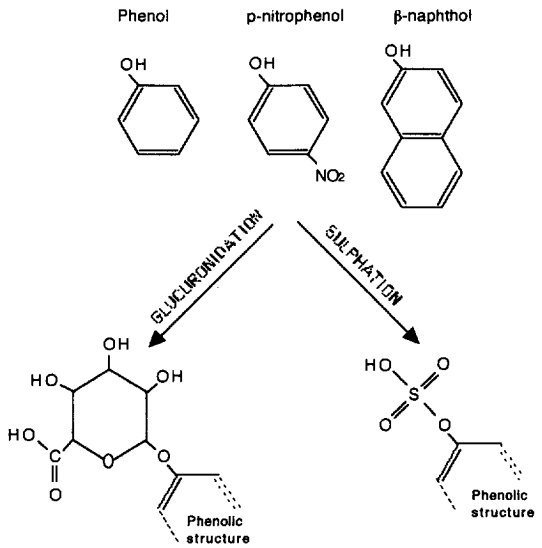

Fig. 1. Molecular structures and metabolic schemes of phenol, 4-nitrophenol and β-naphthol.

thol and its sulphate [5], 3-nitrophenol [6] and some chlorophenols [7]. A recent publication by Ghabrial et al. [8] describes an ion-pair method for the quantitation of PNP; however, the method lacked sensitivity and was not validated for other phenolic compounds.

2. Experimental

2.1. Chemicals

Phenol (Phe), phenyl-β-D-glucuronide (PG), 4-nitrophenol (PNP), 4-nitrophenyl-β-glucuronide (PNPG), 4-nitrophenyl sulphate (PNPS), β-naphthol (β-N), β-naphthyl sulphate (β-NS), *p*-cresol, albumin (bovine-fraction V; BSA) and triethylamine (TEA) were all obtained from Sigma/Aldrich (Sydney, Australia). Methanol (HPLC-grade), orthophosphoric acid and glacial acetic acid were obtained from BDH chemicals (BDH, Melbourne, Australia).

2.2. HPLC conditions and instrumentation

The HPLC system (Millipore Waters, Brisbane, Australia) consisted of a dual system (Model 480 LC), with data collected and integrated using Maxima 820 software on a Powermate 1 (NEC) personal computer. A Brownlee stainless-steel column, 300 mm×4.6 mm I.D., packed with C_{18} silica Spherisorb 5 μm was employed for all separations (Alltech, Baulkham Hills, Australia). All components of the system were coupled via a system interface module (SIM). The sample volume was 20 μl, the flow-rate was 1 ml min^{-1} and detection was effected at 254 nm.

2.3. Analytical conditions employed for phenol and phenylglucuronide

The mobile phase used consisted of methanol–water (40:60, v/v), 0.1% orthophosphoric acid at a pH of 2.7 (±0.02). The retention times of phenol, phenyl-β-D-glucuronide and *p*-cresol were 4.4, 6.5 and 10.3 min, respectively (Fig. 2A).

2.4. Analytical conditions employed for p-nitrophenol and conjugates

The mobile phase used consisted of methanol–water (30:70, v/v), 1% TEA (triethylamine). The pH of the aqueous portion (water and TEA) was adjusted with glacial acetic acid to approximately 5.3 before the addition of methanol. The final pH (with methanol added) was adjusted with glacial acetic acid to 5.6 (±0.02). The retention times of PNPG, PNPS, PNP and *p*-cresol were 6.4, 12.5, 22.4 and 26.8 min respectively (Fig. 2B).

2.5. Analytical conditions employed for β-naphthol and β-naphthol sulphate

The mobile phase used consisted of methanol–water (50:50, v/v) with 1% TEA. The pH of the water with TEA added was adjusted with glacial acetic acid to approximately 5.3 before the addition of methanol. The final pH was adjusted with glacial acetic acid to 5.6 (±0.02). The retention times of β-NS, *p*-cresol and β-N were 11.3, 12.5 and 27.6 min, respectively (Fig. 2C).

2.6. Sample preparation

The following protein precipitation method was employed for sample preparation before analysis. A

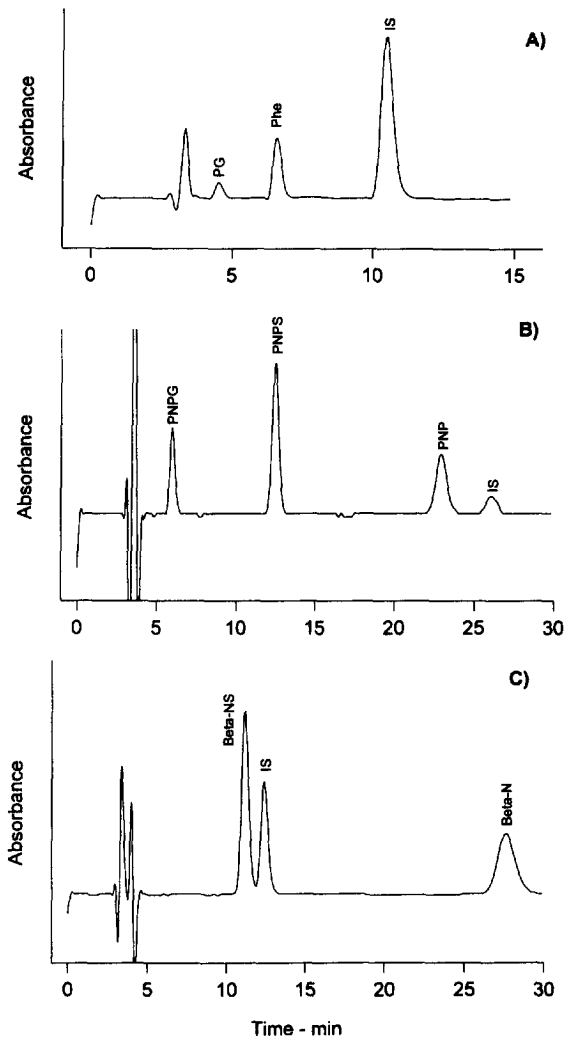

Fig. 2. Typical chromatograms of analytes extracted from liver perfusate. (A) Phenol and PG with *p*-cresol as I.S. (B) PNP, PNPG and PNPS with *p*-cresol as I.S. (C) β-N and β-NS with *p*-cresol as I.S. Chromatographic conditions as in text, peaks as labelled.

100-μl aliquot of each sample of liver perfusate (Kreb's Henseleit buffer containing 1% w/v BSA) was taken and to it 200 μl methanol containing *p*-cresol (internal standard, 5 μg ml^{-1}) was added. The sample was vortex-mixed for 20 s and then centrifuged at 10 000 *g* for 10 min to sediment the precipitated protein. An amount of 100 μl of supernatant was then transferred to a microvial and 20 μl injected onto the HPLC system.

2.7. Preparation of standard curves

Standard solutions were prepared by the addition of known concentrations of each solute, to drug-free perfusate. Protein from these samples was then precipitated and supernatant analysed as described above. Standard curves of peak-height ratio (PHR) (analyte/internal standard) against concentration were plotted.

2.8. Precision of method

Ten replicate injections were made of the standard solutions at 1 μg ml^{-1}, 10 μg ml^{-1} and 50 μg ml^{-1}. The coefficient of variation (C.V.) was calculated to assess intra-assay precision. The same refrigerated stock solution was used to prepare the standard curve for five consecutive days and the C.V. calculated to assess inter-day assay variation.

2.9. Recovery

Solutions of the same concentration as those prepared for the standard curve were made in Kreb's Henseleit buffer without BSA. These were extracted and analysed in the same manner. Recovery of each solute was calculated as:

$$\%\text{Recovery} = \left(\frac{\text{PHR in perfusate sample}}{\text{PHR in aqueous sample}}\right) \cdot 100$$

Each extraction was performed in triplicate on solutions at three different concentrations and was repeated on four consecutive days. The C.V. was then calculated to assess the reproducibility of the extraction procedure.

2.10. Analysis of perfusate samples from the isolated perfused rat liver preparation

The livers of female, Sprague–Dawley rats (190–250 g) were perfused by cannulation of the portal vein (inflow) and the hepatic vein (outflow) in a similar manner to the method of Gores et al. [9]. The livers (8.43±0.3 g) were perfused at 30 ml min^{-1} in

Table 1
Validation range for linear response for each solute

Compound	Validation range (μg ml^{-1})	Equation of line (slope and intercept±S.D.; n=3)
Phe	0.5–100	y=0.027 (±0.0012)x−0.773 (±0.0183) r^2=0.999
PG	0.5–50	y=0.088 (±0.0132)x−0.220 (±0.132) r^2=0.999
PNPG	1–100	y=0.014 (±0.0203)x+0.074 (±0.002) r^2=0.996
PNPS	1–100	y=0.028 (±0.0134)x−0.145 (±0.028) r^2=0.999
PNP	1–100	y=0.020 (±0.0063)x−0.1134 (±0.098) r^2=0.999
β-N	1–100	y=0.009 (±0.0055)x−0.00005 (±0.00004) r^2=0.999
β-NS	1–100	y=0.0141 (±0.0032)x−0.0084 (±0.0042) r^2=0.999

Lowest concentration is the quantitation limit; x=slope of the line; y=concentration of solute.

a single-pass system with carbogenated Krebs Henseleit buffer (pH 7.4) containing 1% (w/v) BSA and 37 μg ml^{-1} PNP. Each liver was perfused for 20 min with the perfusate containing PNP and for 20 min with PNP-free perfusate. Samples of the outflowing perfusate were collected every minute for the duration of the perfusion. The fractions were then analysed for PNP, PNPS and PNPG as described above.

3. Results and discussion

A simple mobile phase was employed for the assay of phenol and its phenyl glucuronide conjugate as the solutes were eluted with short retention times and good peak shape. However, the sulphate metabolites of PNP and β-N, though showing stable retention times gave highly tailed peaks due to their ionic nature. This was counter-acted by the use of 1% TEA in the mobile phase to act as an ion-pairing agent. The glucuronide conjugates studied are known to be extremely stable [10] and not affected by the assay conditions employed. By adjusting the organic phase content, the mobile phase could be adapted for a series of phenolic compounds with different polarities. A recent paper describes an assay for PNP and its metabolites [8]; however, the assay developed within our laboratory showed greater sensitivity (detection limit 0.5–1 μg ml^{-1}, compared to approx. 300 μg ml^{-1} [8]) and employed a simple mobile phase.

Table 2
Intra- and inter-day variation of peaks for each solute at high and low concentrations

Solute	Concentration (μg ml^{-1})	Mean intra-day C.V. (n=10) (%)	Inter-day C.V. (n=5) (%)
Phe	1	5.7	6.6
	100	1.6	2.3
PG	1	4.5	5.8
	30	1.4	3.1
PNG	1	7.9	8.8
	50	3.3	4.6
PNS	1	6.9	8.4
	50	3.0	4.2
PNP	1	6.2	8.2
	50	2.7	3.1
β-N	1	5.3	6.1
	100	1.3	2.4
β-NS	1	3.2	5.1
	100	1.1	2.7

Table 3
Percentage recoveries for each solute at high and low concentrations

Solute	Concentration (μg ml^{-1})	Mean recovery (%)	Intra-day C.V. (%)
Phe	1	78.9	2.3
	30	95.1	1.15
PG	1	91.2	2.4
	30	91.5	1.0
PNG	1	97.4	2.2
	50	100.5	2.1
PNS	1	108.2	2.4
	50	98.9	2.3
PNP	1	99.1	1.9
	50	101.5	1.8
β-N	1	101.3	2.4
	100	102.4	2.8
β-NS	1	96.4	2.7
	100	95.2	2.6

A linear response was observed for the calibration of all compounds between the concentration limits (Table 1). The calibration ranges were chosen based on the expected solute concentrations in the perfusate. Any samples with concentrations exceeding the range were diluted appropriately.

Inter-assay variation was shown to be less than 3% and inter-day precision was shown to be less than 9% in all cases. Values calculated for C.V. at high and low concentrations are shown below in Table 2. Excellent values for recovery were observed for all solutes from the perfusate showing that the extraction method could be used reliably on a daily basis (Table 3). The sensitivity of the assays were good and easily allowed quantification of the solutes in the perfusate.

Fig. 3 shows typical results from the IPRL for perfusate containing PNP. The concentration of substrate (PNP) and sulphate and glucuronide metabolites could be measured accurately in the perfusate samples.

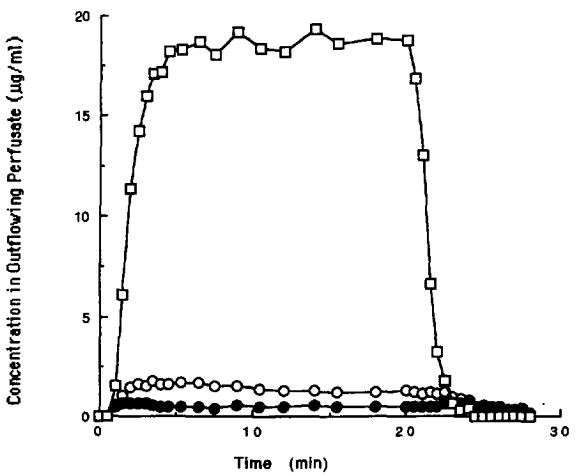

Fig. 3. Levels of PNP (□), PNPG (○) and PNPS (●) in outflow perfusate during isolated rat liver perfusion with Krebs Henseleit buffer (pH 7.4) containing 1% (w/v) BSA and PNP (37 μg ml^{-1}).

4. Conclusion

The assay described here shows good values for recovery, accuracy and precision and has been found suitable for the quantitation of Phe and PG; PNP, PNPG and PNPS; β-N and β-NS in IPRL experiments.

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