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High-performance liquid chromatographic analysis of *p*-nitrophenol and its conjugates in biological samples

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ABSTRACT

p-Nitrophenol (pNP) and its conjugated metabolites, generated in a perfused rat liver preparation, are readily separated and quantitated in serum perfusate and bile samples using a reversed-phase high-performance liquid chromatographic method Serum perfusate samples can be analyzed following protein precipitation with acetonitrile; following protein precipitation with 1 5 *M* perchloric acid (1 part to 2 parts serum) there was degradation of pNP sulfate to pNP when samples were stored at room temperature pNP can also be analyzed in blood perfusate samples following extraction with a number of organic solvents including ethyl acetate or isobutanol–methylene chloride (4 1, v/v) Rat liver perfusions at a constant input concentration of 40 μM demonstrated a high hepatic extraction ratio of pNP (mean of 0.90) due to the formation of the sulfate and glucuronide conjugates, no pNP glucoside was detected in perfusate or bile samples.

INTRODUCTION

p-Nitrophenol (pNP) is widely used as a model substrate to evaluate the influence of drug therapy, disease, nutrient deficiencies and other physiologically altered conditions on conjugative drug metabolism in animal studies both *in vivo* and *in vitro* [1–7]. The analysis of the loss of pNP provides a convenient method for the *in vitro* assay of phenol sulfotransferase and UDP-glucuronyltransferase activities [1–4,8]. As well, the analysis of the formation of pNP from its conjugated metabolites is used to measure β-glucuronidase and sulfatase activities [3,8,9]. pNP is metabolized predominantly in rats to one sulfate (pNPS) and one glucuronide (pNPG) conjugate; pNP glucoside is a minor metabolite (generally $<2\%$ of dose) which has been identified in rat liver perfusions [5,10,11]. This simple and well characterized metabolic profile makes pNP a desirable model substrate in drug conjugation studies.

A number of assay procedures have been used for the analysis of pNP [5,12–19]. pNP has been most commonly analyzed by spectrophotometric methods [1–5,10]; in some cases the metabolites have been quantitated following enzymatic hydrolysis to pNP [5,6,12] There are only a few published high-performance liquid chromatographic (HPLC) methods that have been used to analyze both of

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the conjugated metabolites in addition to pNP [14,15,17,19]. Two of these assays have not been used to analyze biological samples [14,17]; another assay used a reversed-phase system to analyze pNP, pNPS and pNPG in rat plasma and urine samples but did not include an internal standard in the assay [15] while the last system incorporated an ion-pair reagent in the mobile phase and was used for plasma, urine and bile samples [19]. In none of these published assays was the assay reproducibility or detection limits for the compounds specified, nor were the HPLC assays used in the analysis of blood samples.

We have developed a reversed-phase HPLC assay to quantitate pNP, pNPG and pNPS in blood, perfusate serum and bile samples generated in a perfused rat liver preparation. Studies performed using the assay have established the sensitivity and reproducibility of the assay as well as the conditions for the extraction of pNP and pNPS and the stability of pNP and its conjugates in biological fluids.

EXPERIMENTAL

Chemicals

pNP and potassium phosphate monobasic were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A), pNPS and pNPG from Sigma (St. Louis, MO, U.S.A), phenol from Allied Chemical Co. (Morristown, NJ, U.S.A) and salicylamide from Eastman (Rochester, NY, U.S.A.). pNP glucoside was a gift from Dr W. D. Conway, Department of Pharmaceutics, State University of New York at Buffalo. All solvents were HPLC grade.

Assay procedure

The assay was developed using a Model 710B WISP autoinjector, a Model 6000A pump, a Model 440 ultraviolet absorbance detector (all from Waters Assoc., Milford, MA, U.S.A) and a Hewlett-Packard Model 3392A integrator (Avondale, PA, U.S.A.). The mobile phase consisted of 6% methanol and 1% acetic acid in 0.05 *M* monobasic potassium phosphate solution The pH was adjusted to 4.16 with potassium hydroxide Samples were injected onto a Whatman guard column and Partisil-5-ODS 3 (5 μm particle size) analytical column (Whatman, Clifton, NJ, U.S.A), and pNP and its conjugates were detected at a wavelength of 280 nm. The flow-rate of the mobile phase was 1 ml/min and the inlet pressure of the system was approximately 80 bar. Additionally, we have used a μBondapak C_{18} column (Waters Assoc.) and a gradient system composed of 0.05 *M* KH_2PO_4 and 1% acetic acid with 16–25% of methanol to separate pNP and its conjugates.

Sample preparation

Perfusate serum samples were analyzed following protein precipitation using acetonitrile. A 50-μl aliquot of serum, 50 μl of distilled, deionized water (DDW) and 200 μl of acetonitrile (containing 500 μM phenol as the internal standard)

were vortex-mixed and centrifuged (15 000 *g* for 4 min). The supernatant (25 μl) was used as the injection sample. Standards were prepared from blank perfusate serum (50 μl), known amounts of pNP, pNPS and pNPG in DDW (50 μl) and acetonitrile (200 μl).

Bile samples were prepared for injection by combining 10 μl of bile and 120 μl of a 20% solution of methanol in water that contained phenol as the internal standard. Calibration curves were prepared by substituting standards in DDW for the bile. The injection volume was 10 μl.

Blood perfusate samples, which consisted of 20% washed bovine red blood cells, 300 mg% glucose and 1% bovine serum albumin in Krebs Henseleit bicarbonate buffer, were analyzed for pNP after extraction with ethyl acetate or other organic solvent systems A 1-ml volume of 0.1 *M* phosphate buffer (pH 4.2) was added to 2 ml of blood perfusate. This was followed by the addition of 0.1 ml of salicylamide (3.34 m*M*), the internal standard, and 0.1 ml of DDW or standard solution and 5 ml of organic solvent. The samples were vortex-mixed for 1 min and centrifuged at 160 *g* for 10 min. A 4-ml aliquot of organic layer was removed and evaporated to dryness under nitrogen gas at room temperature. The samples were reconstituted with 0.2 ml of a solution containing 40% methanol in water and analyzed by HPLC.

Stability testing

Perfusate serum samples were refrigerated, frozen or repeatedly frozen and thawed (up to four times) and then assayed to determine the stability of pNP and its conjugates under each of these conditions. To determine the intra-day variability, a 100-μl aliquot of perfusate serum, 100 μl of DDW and 400 μl of acetonitrile containing 500 μ*M* phenol was prepared and injected four times. To determine the inter-day variability, new mobile phase and injection samples were prepared on three separate days. In each case, the peak-area ratios of all compounds were examined at low (20 μ*M*) and high (200 μ*M*) concentrations.

The stability of the prepared injection samples was examined at room temperature in the autoinjector sample tray over a time period up to 12 h, as well as after refrigeration for up to 18 h. Injection samples were prepared by combining 100 μl of blank perfusate serum, 100 μl of DDW containing known concentrations of all three standards and 400 μl of acetonitrile containing 500 μ*M* phenol. The supernatant obtained after centrifugation was then divided into six separate injection samples and injections were made over a 12-h period starting immediately after centrifugation. Other samples were refrigerated at time zero and analyzed about 18 h later. Additional studies examined the use of 1.5 *M* perchloric acid instead of acetonitrile for protein precipitation. In these studies, samples were prepared using 300 μl of blank perfusate serum, 120 μl of known concentrations of standards in DDW and 150 μl of 1.5 *M* perchloric acid containing phenol as the internal standard. The stability of pNP and its conjugates after storage at room or refrigerated temperatures was examined with these samples in a similar fashion as that of the acetonitrile-containing solutions.

Perfused liver studies

The HPLC assay was used to analyze bile and perfusate samples obtained using a single-pass *in situ* perfused rat liver preparation, as described by Pang [20]. The liver donors consisted of three adult Sprague–Dawley male rats (Harlan Sprague–Dawley, Indianapolis, IN, U.S.A) The perfusion medium consisted of 20% (v/v) bovine red blood cells, 1% bovine albumin and 300 mg% glucose in Krebs-Henseleit bicarbonate buffer, which was infused into the portal vein at a constant rate of 10 ml/min. pNP was infused at input concentrations (C_{in}) of 40 μM and bile and perfusate samples were obtained under steady state conditions Serum perfusate and not blood perfusate was analyzed in these experiments since preliminary studies demonstrated negligible uptake into red blood cells in our perfusion medium (hematocrit = 0.085).

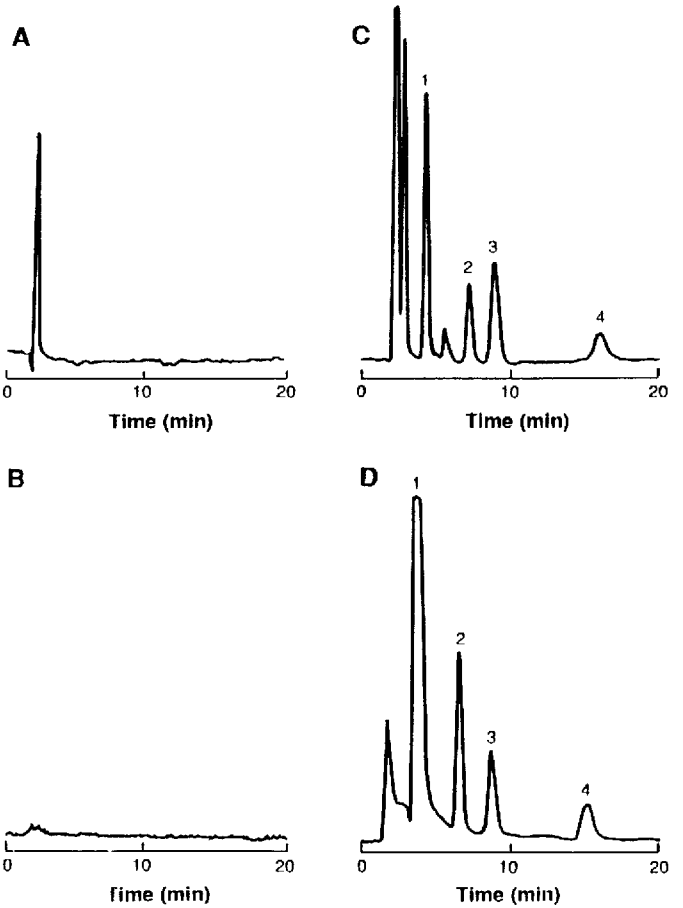

Fig 1 HPLC of (A) blank perfusate serum, (B) blank bile, (C) perfusate serum and (D) bile obtained under steady state conditions following the administration of *p*-nitrophenol. 1000 and 400 nmol/min, respectively, to a perfused rat liver preparation. Peaks 1 = pNPG; 2 = phenol, the internal standard, 3 = pNPS, 4 = pNP. The full scale signal voltage for the *y*-axis is 16 mV

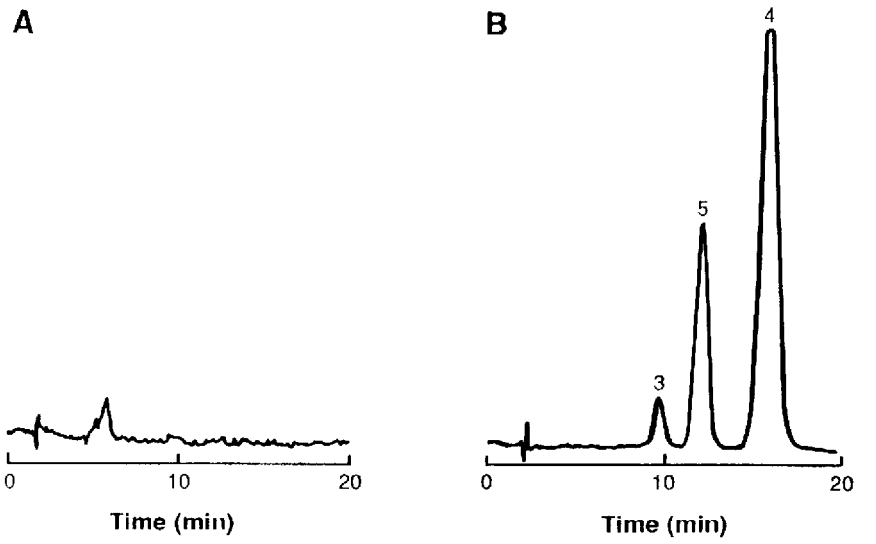

Fig 2 HPLC of (A) blank blood perfusate and (B) blood perfusate with added *p*-nitrophenol, *p*-nitrophenol sulfate and *p*-nitrophenol glucuronide (200 μM concentrations of each) following extraction with ethyl acetate. Peaks as described for Fig. 1 with peak 5 representing salicylamide, the internal standard used for the blood perfusate extractions The full scale signal voltage for the *y*-axis is 16 mV.

RESULTS AND DISCUSSION

The HPLC assay provided baseline separation of pNP and its conjugates in perfusate serum, bile and blood samples (Figs. 1 and 2). Chromatograms of blank serum, bile and blood samples demonstrated the absence of any interfering peaks. pNP glucoside eluted between the glucuronide and sulfate conjugates with the two mobile phases that we used and therefore would not interfere with the quantitation of the other conjugates. Linearity was achieved in all calibration curves in bile and perfusate serum in the concentration range of pNP, pNPG and pNPS of 5–250 μM (r = 0.999 for all linear regression calculations). Since pNPG was eliminated in high concentrations in the bile, standard curves for pNPG in bile were extended up to concentrations of 25 mM; these calibration curves also exhibited linearity.

The detection limits for pNP, pNPG and pNPS in terms of both the amount injected and the corresponding perfusate serum concentration for a 25-μl injection volume are given in Table I. The detection limit was taken as the injection amount that produced a signal-to-noise ratio of 3. The reproducibility of the assay is given in Table II. At a 20 μM concentration of pNP and its conjugates, the intra-day variability was $<6.0\%$ while the inter-day variability was $\leq 8.2\%$; the variability was lower at higher concentrations of pNP, pNPS or pNPG.

No changes in the concentrations of pNP or its conjugates in perfusate serum samples were seen after freezing or following repeated freezing and thawing (up

TABLE I

ASSAY SENSITIVITY FOR *p*-NITROPHENOL AND ITS GLUCURONIDE AND SULFATE CONJUGATES

Compound	Amount detected (nmol)	Sample concentration of perfusate serum (μM)
pNP	0 02	4.8
pNPS	0 033	8.0
pNPG	0.017	4 0

to four times) over a six-month period (data not shown). Following protein precipitation with 1.5 *M* perchloric acid, there was loss of pNPS over time when samples were left at room temperature; this was accompanied by an increase in pNP concentrations over time (Fig. 3). No alterations in the peak-area ratios of pNP or its conjugates to the internal standard, phenol, were seen over the 12-h period at room temperature when acetonitrile was used for protein precipitation. In both investigations, the peak area of phenol was constant when analyzed over the 12-h time period or after refrigeration. As well, the chromatograms of the samples refrigerated for 18 h produced similar peak-area ratios of pNP and its conjugates as was seen with the zero time samples. All compounds present in bile samples were stable at room temperature over the 12-h study period.

Numerous organic solvent systems were investigated for their ability to extract pNP from blood perfusate. pNP was well extracted (>90%) in most solvent systems including that of ethyl acetate and of isobutanol–methylene chloride (4:1, v/v) (Table III). The extraction efficiency of pNP was unchanged in the presence

TABLE II

INTRA-DAY AND INTER-DAY VARIABILITY FOR THE DETERMINATION OF *p*-NITROPHENOL AND ITS GLUCURONIDE AND SULFATE CONJUGATES IN PERFUSATE SERUM

The internal standard area did not vary by more than ±2.17%

Compound	Concentration (μM)	Variability (%)	
		Intra-day (n = 4)	Inter-day (n = 4)
pNP	20	5.85	8 19
	200	1 51	2 19
pNPS	20	4.20	6.65
	200	0.84	2 57
pNPG	20	2.78	3 59
	200	0.66	1 14

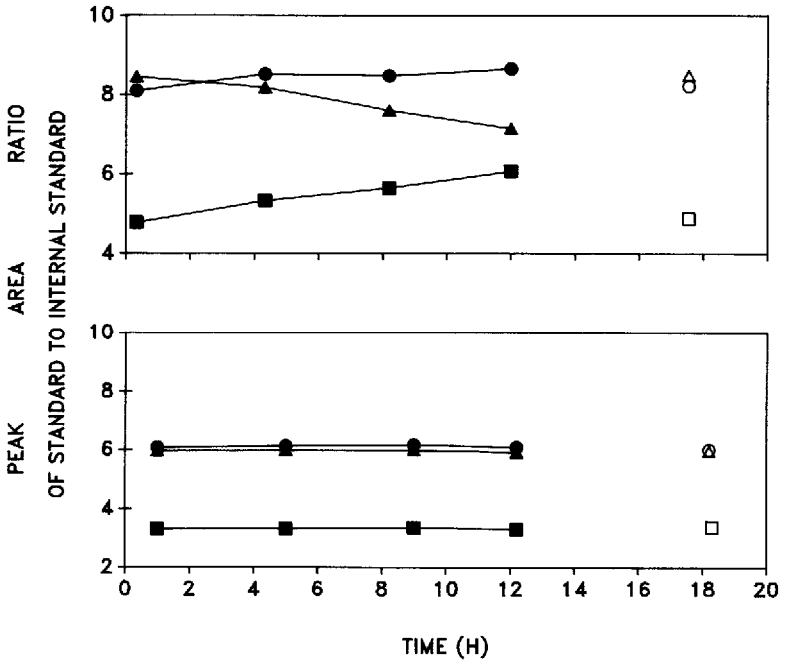

Fig. 3 HPLC peak-area ratio of compound to internal standard for *p*-nitrophenol (■), *p*-nitrophenol sulfate (▲) and *p*-nitrophenol glucuronide (●) present in perfusate serum samples which were stored at room temperature following protein precipitation using 1.5 *M* perchloric acid (upper panel) or acetonitrile (lower panel) The open symbols represent samples stored in the refrigerator for 18 h and then analyzed by HPLC The standard deviation bars for all data points are contained within the symbol

of its conjugated metabolites The best solvent system for the extraction of pNPS was that of isobutanol–methylene chloride (4:1, v/v) in which the efficiency of extraction was approximately 50% (Table III). The percentage of pNPS extracted into ethyl acetate was low and appeared to be concentration-dependent. pNPG was poorly extracted in all solvent systems examined; its efficiency of extraction in the isobutanol–methylene chloride solvent system was $<2\%$.

The perfused rat liver studies demonstrated that pNP was highly metabolized by the liver at an input concentration (C_{in}) of 40 μM (Fig. 4). The formation of the glucuronide and sulfate conjugates accounted for the total metabolism of pNP; at a C_{in} of 40 or 200 μM, no peak with the same retention time as the glucoside conjugate was detected in perfusate or bile samples. At a C_{in} of 40 μM, 7.2% of the dose was eliminated by biliary excretion. pNP, pNPS and pNPG were all present in bile samples but the glucuronide conjugate comprised 85% of the total drug and metabolite content of bile samples.

To summarize, a reversed-phase HPLC assay for the measurement of pNP and its conjugates has been developed to quantitate these compounds in samples of perfusate blood, serum and bile generated in a perfused rat liver system. The

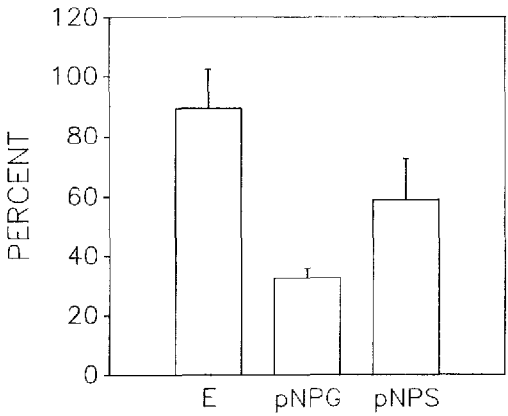

Fig 4 Percentage extraction of pNP and percentage of the input concentration of pNP metabolized to pNPS and pNPG under steady state conditions in perfused rat liver preparations The C_{in} of pNP was 40 μM The results are expressed as mean ± S.D for three rat liver preparations

assay demonstrates good sensitivity and reproducibility for the analysis of pNP and its conjugated metabolites.

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TABLE III

MEAN EXTRACTION EFFICIENCIES OF *p*-NITROPHENOL AND ITS CONJUGATES FROM BLOOD PERFUSATE

n = 2 or 3 N D = not detected The extraction efficiency of the internal standard, salicylamide, was 90 0 ± 4 2% (mean ± S D)

Solvent system	Concentration (μM)	Extraction efficiency (%)		
		pNP	pNPS	pNPG
Isobutanol–methylene chloride (4 1, v/v)	20	97.9	45 0	N D.
	100	91.5	51 5	2.0
	250	95 8	47.8	1.0
Ethyl acetate	20	89 1	11.4	N D
	50	96.1	6.7	N D.
	100	96 0	5.3	N D
	200	99 3	4.4	N D
	300	97 0	3 9	N.D

REFERENCES

1 H K Watanabe and M. Matsui, *J Pharm Dyn.*, 7 (1984) 641
2 P Vega, C. Gaule, E. Sanchez and E Del Villar, *Gen. Pharmacol*, 17 (1986) 641
3 L K Griffeth, G. M Rosen and E J. Rauckman, *Drug Metab Dispos*, 13 (1985) 391
4 M. H Morrison, H E Barber, P G Foschi and G. M Hawksworth, *J Pharm Pharmacol.*, 38 (1986) 188
5 L A Reinke, S A Belinsky, R K Evans, F C Kauffman and R. G Thurman, *J Pharmacol. Exp. Ther*, 1981 (217) 863
6 L A Reinke, F. C Kauffman, R K Evans, S A Belinsky and R G. Thurman, *Res Commun Chem. Pathol Pharmacol*, 23 (1979) 185
7 J H N. Meerman, C. Nijland and G J. Mulder, *Biochem Pharmacol.*, 36 (1987) 2605.
8 G. W Lucier, B R Sonawane and O. S. McDaniel, *Drug Metab Dispos*, 5 (1977) 279
9 M -P Kung, S. W Spaulding and J. A. Roth, *Endocrinology*, 122 (1988) 1195
10 K. Minck, R R. Schupp, H P A Illing, G F Kahl and K. J Netter, *Arch Pharmacol*, 279 (1973) 347
11 N Hamada and T Gessner, *Drug Metab Dispos*, 3 (1975) 407
12 P Moldeus, H Vadi and M Berggren, *Acta Pharmacol Toxicol*, 39 (1976) 17.
13 J D Gregory and F Lipmann, *J Biol Chem*, 229 (1957) 1081
14 M W. Anders and J P Latorre, *J Chromatogr*, 55 (1971) 409
15 G Diamond and A J Quebbemann, *J. Chromatogr*, 177 (1979) 368.
16 B Schultz, *J Chromatogr.*, 269 (1983) 208
17 A. Karakaya and D E Carter, *J. Chromatogr*, 195 (1980) 431
18 R. T. Coutts, E. E Hargesheimer and F M Pasutto, *J Chromatogr*, 195 (1980) 105
19 M Machida, Y Morita, M Hayashi and S Awazu, *Biochem. Pharmacol*, 31 (1982) 787
20 K S. Pang, in J. R. Mitchell and M G Horning (Editors), *Drug Metabolism and Toxicity*, Raven Press, New York, 1984, p 331