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High-performance liquid chromatographic assay for acetaminophen glucuronide in human liver microsomes

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Abstract

A rapid and specific high-performance liquid chromatographic assay was developed for the determination of acetaminophen glucuronide formed by human liver microsomes. In addition, incubation conditions were systematically evaluated. Conditions that yielded the optimal rate of acetaminophen glucuronide formation over various concentrations of acetaminophen (0.15–30 m*M*) consisted of the following: 0.1 *M* potassium phosphate buffer, 1 m*M* magnesium chloride, 30 μg/mg alamethicin, 4 m*M* uridine 5′-diphosphoglucuronic acid at a pH of 7.1. Alamethicin produced higher and more consistent APAPG formation rates compared to Brij-58. Adding saccharolactone to the incubation medium reduced the velocity of the reaction. Acetaminophen glucuronide, acetaminophen, and the internal standard (paraxanthine), were analyzed on a C_{18} column with UV detection at 250 nm. The mean correlation coefficient (r^2) of the standard curves for acetaminophen glucuronide was >0.99 over the range of 0.1–25 nmol. The intra- and inter-day coefficients of variation were <4%. This method is suitable for in vitro studies using acetaminophen glucuronide formation as an index reaction for UGT activity.

Keywords: Acetaminophen glucuronide

1. Introduction

Glucuronidation is a major drug biotransformation reaction in the body. This reaction catalyzes the transfer of glucuronic acid from uridine 5′-diphosphoglucuronic acid (UDPGA) to a multitude of endogenous and exogenous lipophilic aglycon substrates through the activity of UDP-glucuronosyltransferases (UGTs). UGTs are glycoproteins that are mainly localized within the endoplasmic reticulum (ER) [1]. Acetaminophen (*N*-acetyl-*P*-aminophenol, 4-acetamidophenol, APAP) is a widely used analgesic drug that undergoes significant glucuronidation (Fig. 1). UGT1A6 is the major UGT isoenzyme catalyzing APAP glucuronide (APAPG) formation in vivo; UGT1A9 also glucuronidates APAP but with much lower affinity [2,3]. Few assay reports have been published to quantify APAPG formation in liver microsomes [4,5]. Pacifici et al. utilized tritium-labeled acetaminophen and determined APAPG formation as radioactivity remaining in the aqueous portion after an ethyl acetate-extraction [4]. Miners

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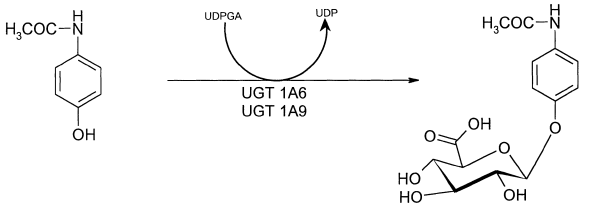

Fig. 1. Structures of acetaminophen and acetaminophen glucuronide.

et al. isolated APAPG using solid-phase extraction with subsequent evaporation and reconstitution in mobile phase; analysis was by HPLC with UV detection [5]. Although these techniques were suitable for APAPG determination, a non-radioactive assay that does not require sample extraction was desired.

Interest in phase II metabolic pathways, notably glucuronidation, has recently increased due to their role in drug disposition and disease pathogenesis [6–8]. Indeed, APAP glucuronidation in human liver microsomes shows marked variability between individuals [4,5]. Thus, a thorough evaluation of methods that are commonly utilized to measure glucuronidation activity in vitro is needed. In this report, we describe a simple, sensitive, and specific HPLC assay for the determination of APAPG using human liver microsomes. We also evaluated the effect of several incubation conditions on APAP glucuronidation in an attempt to optimize the rate of glucuronide formation under physiologic conditions.

2. Experimental

2.1. Reagent and chemicals

APAP, APAPG, alamethicin, Brij-58 (polyoxyethylene [20]-cetyl ether), UDPGA, magnesium chloride, Trizma hydrochloride (Tris [hydroxymethyl] aminomethane hydrochloride), saccharolactone (D-saccharic acid 1,4-lactone), paraxanthine (1,7-dimethylxanthine) were purchased from Sigma (St. Louis, MO, USA). Potassium phosphate, ethanol, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Acetic acid was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). All utilized chemicals were of the highest purity available for analytical research.

Human liver samples were obtained from patients undergoing partial hepatectomy for hepatic metastatic cancer. All patients granted written informed consent and the procedure was approved by the University of Pittsburgh Institutional Review Board. Liver samples were carefully removed from the unaffected area adjacent to the resected tumor and were normal by both macroscopic and microscopic examinations. The samples were frozen immediately in liquid nitrogen and stored at −80°C until used for preparation of microsomes. Human liver microsomes were prepared by differential centrifugation and protein concentration was quantified using Lowry's method [9].

2.2. Instrumentation

The HPLC system consisted of a Waters model 717 autosampler, model 501 HPLC pump, and model 486 ultraviolet detector set at 250 nm (Waters Corp., Milford, MA, USA). The mobile phase consisted of 10% methanol and 1% acetic acid and was delivered at a flow-rate of 1 ml/min through a Waters Symmetry® C_{18}, 5 μ, 3.9×150 mm column. Signal output was captured using Millennium^{32} software, version 3.05 (Waters Corp., Milford, MA, USA).

2.3. 2.3.Stock solutions

A 0.1 *M* potassium phosphate or Tris buffer (pH 7.4 and 7.1) was prepared in deionized water. UDPGA, Brij-58, magnesium chloride, and saccharolactone stocks were prepared in either phosphate or Tris buffer solution. Alamethicin was prepared in phosphate or Tris buffer containing 10% ethanol. For calibration standards, a 10 nmol/μl stock of APAPG was prepared in methanol and further diluted with methanol to a final concentration of 1 and 0.1 nmol/μl.

2.4. Incubations and HPLC assay

Human liver microsomes (0.5 mg/ml final protein concentration) and alamethicin (0–300 μg/mg protein) or Brij-58 (0–0.3 mg/mg protein) were preincubated on ice for 5 min. The incubation mixture

consisted of magnesium chloride (1 or 10 m*M*), and APAP (over a 0.15 to 30 m*M* range of concentrations), in the presence and absence of 5 m*M* saccharolactone. Final incubation volume was 250 μl. The reaction was started by adding 4 m*M* of UDPGA and placing tubes in a 37°C water bath (preliminary experiments indicated that UDPGA 4 m*M* was a saturating concentration with a K_m of 0.7 m*M*). After 1 h of incubation, the reaction was terminated by adding 25 μl of 6% perchloric acid containing 25 μg paraxanthine (internal standard), followed by vortex-mixing and cooling on ice. The tubes were then centrifuged at 2000 *g* for 5 min, and the supernatant was transferred into autosampler vials. A 75 μl aliquot of the supernatant was injected into the HPLC column.

2.5. Calibration and linearity

Calibration curves were obtained daily for 3 days using seven different amounts of APAPG (0.1, 0.5, 1.25, 2.5, 5, 12.5 and 25 nmol) prepared in incubation buffer containing all incubation constituents except microsomes; curves were constructed by calculating the peak-height ratios of APAPG to that of paraxanthine. Calibration curve data points were fit using linear regression analysis and a $1/y^2$ weighting scheme.

2.6. Precision and accuracy

The precision and accuracy of the assay was determined using quality control (QC) samples of known APAPG amounts (i.e., 1.25 and 12.5 nmol) prepared in incubation buffer containing all incubation constituents except microsomes. Six replicates of each QC were analyzed on 3 days, after which the intra- and inter-day means, standard deviations and coefficients of variation (C.V.) were calculated.

2.7. Optimization of incubation conditions

Initial experiments were conducted to evaluate the effect of buffer selection (Tris vs. Phosphate) and magnesium chloride concentration (1 vs. 10 m*M*) on APAP glucuronidation. The rate of APAP glucuronide conjugation was also studied at two different pHs, 7.4 and 7.1. Saccharolactone (a β-glucuronidase inhibitor) is sometimes used in the incubation medium, presumably to reduce the rate of enzymatic hydrolysis of the conjugate. We evaluated the effect of adding saccharolactone (5 m*M*) on APAPG formation.

The active site of the UGTs resides within the lumenal side of the ER. Thus, specific microsomal treatment is often needed to remove enzyme latency and to obtain maximum activity for in vitro experiments. Traditionally, various types of detergents (e.g., Brij-58, Triton×100, and CHAPS) have been used for this purpose, but they may yield variable activity. More recently, alamethicin (an antibiotic fungal peptide) has been shown to demonstrate better efficiency, but the optimal concentration has not been determined [10,11]. Therefore, we characterized the profile of APAPG formation under various concentrations of Brij-58 and alamethicin. Variability (inter-day C.V.) in the incubation procedure was assessed by measuring APAPG formation in three different microsomal sources over 3 days.

3. Results

3.1. Chromatographic separation

Representative chromatograms of microsomal incubations are shown in Fig. 2. APAP, APAPG, and the internal standard (paraxanthine) were separated within 10 min of the chromatographic run. The retention times for APAPG, APAP and paraxanthine were approximately 3.1, 4.5 and 9.3 min, respectively. APAPG was stable in processed microsomal incubates for at least 24 h at room temperature.

3.2. Calibration, precision and accuracy

Standard curves for APAPG were linear over the range of 0.1–25 nmol. The mean correlation coefficient (r^2) for the standard curves was >0.99. Intra- and inter-day C.V. for APAPG QC samples were less than 4% (Table 1). Calibration curve and QC samples were prepared in buffer containing all incubation constituents except microsomes, since preliminary experiments showed that APAPG recovery was not affected by the presence of inactivated microsomes.

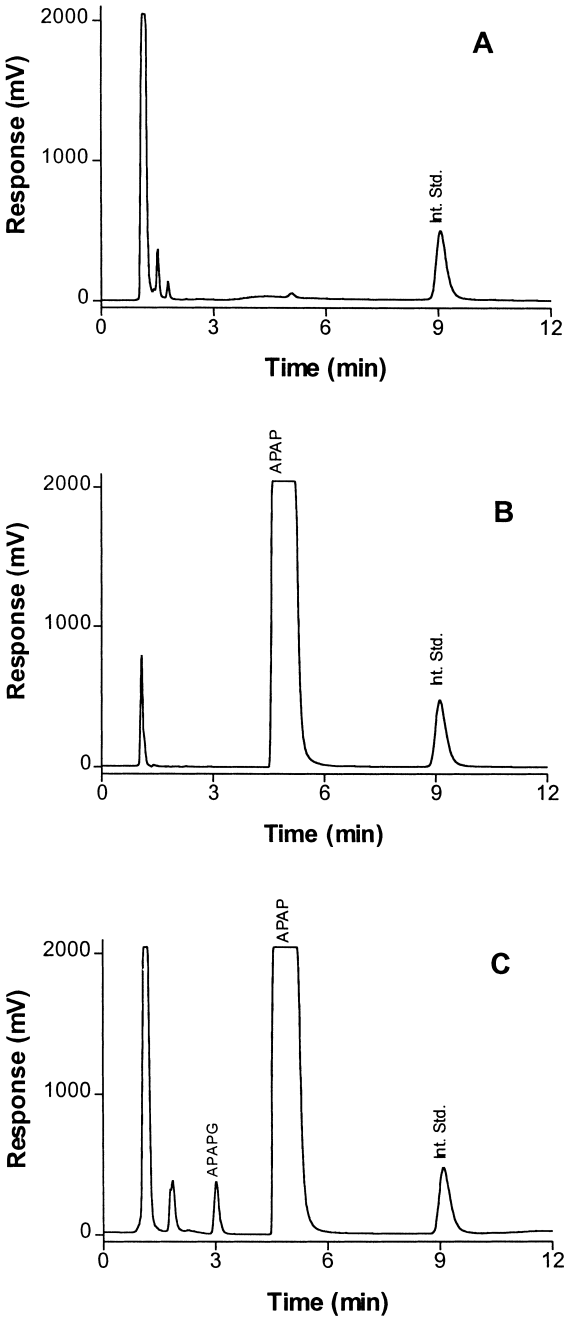

Fig. 2. Representative chromatograms of acetaminophen and acetaminophen glucuronide in human hepatic microsomes. Panel A — microsomal incubation in the absence of acetaminophen. Panel B — microsomal incubation in the absence of UDPGA. Panel C — microsomal incubation containing 30 m*M* acetaminophen and 4 m*M* UDPGA in the presence of 30 μg/mg alamethicin.

Table 1
Intra- and inter-day precision and accuracy for acetaminophen glucuronide in incubation buffer

APAPG	Amount added (nmol)	Amount found (Mean±SD, nmol)	C.V. (%)
Intra-day	1.25	1.29±0.03	3.5
(n=6)	12.50	12.75±0.12	0.9
Inter-day	1.25	1.29±0.02	1.7
(n=18)	12.50	12.71±0.16	1.3

3.3. Incubation conditions

Similar rates of APAPG formation at a given concentration were produced when either phosphate or Tris buffer was used (data not shown). Likewise, rates of APAPG formation were similar using 1 or 10 m*M* magnesium chloride. Since the concentration of magnesium chloride within the endoplasmic reticulum (ER) has been reported to be approximately 1 m*M* [12,13], this concentration was used in our experimental conditions.

The rate of APAPG formation was approximately 15% lower at pH 7.1 as compared to pH 7.4. However, we adjusted our experimental conditions to pH 7.1 to approximate the reported pH of the ER [14]. A significant reduction (45% at pH 7.1) in the APAPG formation velocity was found when the most commonly used concentration of saccharolactone (5 m*M*) was added to the medium. Therefore, saccharolactone was not included in the final incubation conditions.

Alamethicin treatment was associated with a 2-fold increase in APAP glucuronidation compared to Brij-58 under the same conditions (Fig. 3). A concentration as low as 15 μg/mg microsomal protein was found to be sufficient to produce an increase in UGT activity relative to controls. Brij-58 treatment was associated with a bell-shaped curve of activity; maximum activity was obtained at 0.1 mg/mg protein (Fig. 3).

3.4. Incubation of APAP with human liver microsomes

APAPG formation was linear up to 90 min and up to 1.5 mg/ml microsomal protein under the conditions tested. APAPG formation was assessed in three different microsomal sources over 3 days. The

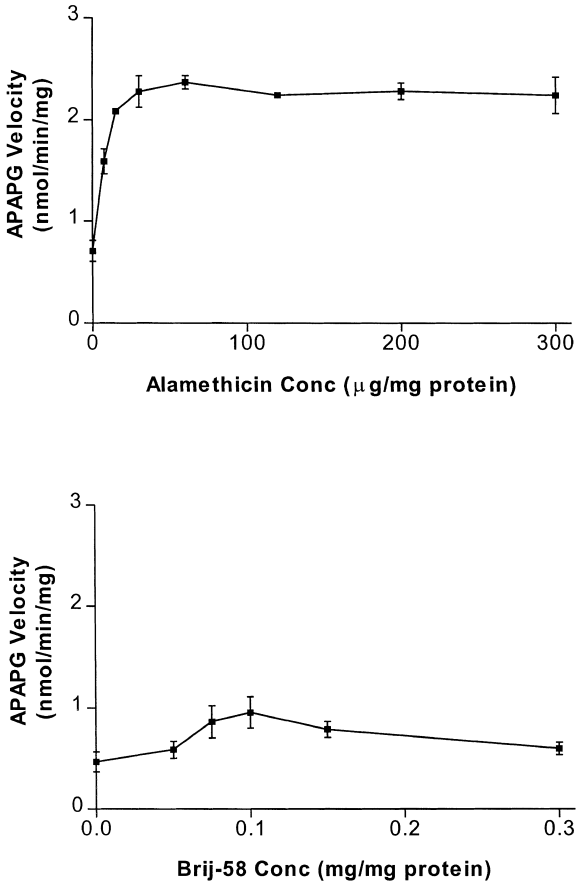

Fig. 3. The effect of microsomal treatment with different concentrations of alamethicin (upper panel) or Brij-58 (lower panel) on acetaminophen glucuronide formation.

inter-day variability (C.V.) of APAPG formation rates was less than 13% over the APAP concentration range of 0.15 to 30 m*M*. Eadie–Hofstee plots exhibited biphasic kinetics (Fig. 4) with apparent K_m values of 0.97±0.30 and 10.78±1.61 m*M* and V_{max} values of 0.28±0.07 and 2.28±0.30 nmol/min/mg.

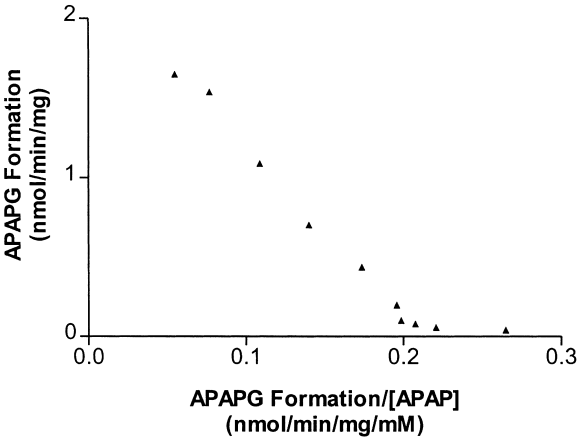

Fig. 4. Eadie–Hofstee plot of acetaminophen glucuronide formation velocity following incubation of acetaminophen with human liver microsomes (n=3).

4. Discussion

APAP glucuronidation represents about 55% of the drug's primary biotransformation in the body. APAP has been proposed as an in vivo probe of UGT activity in part due to its safety at therapeutic doses [15]. This paper describes an HPLC assay for measuring APAP glucuronidation in human liver microsomes. The method described provides rapid, sensitive and specific detection of APAPG with a total run time of 10 min.

An additional objective was to evaluate different incubation conditions on the rate of APAPG formation. UGT activity was affected by the pH of the incubation medium. It has been reported previously that UGT activity increases in a graded fashion when microsomes are pretreated briefly at alkaline pH [16]. This may result from conformational changes of enzyme subunits and better substrate interaction within the plane of the membrane. Pacifici et al. showed that APAPG formation is maximal at pH 7.5. We found that the rate of formation of APAP conjugate is slightly higher at pH 7.4 compared to pH 7.1. However, since Kim et al. have reported recently that the pH of the ER is approximately 7.1 [14], we adjusted our incubation conditions to be consistent with physiological pH.

β-glucuronidase is widely distributed in mammalian tissues, particularly in the liver. It functions to deconjugate the glucuronide molecule from a multitude of endo- and xenobiotics. Several investigators have added saccharolactone to the incubation medium to inhibit any spontaneous enzymatic hydrolysis of the conjugate during in vitro experiments. However, the importance of using β-glucuronidase inhibitors appears to be substrate and/or microsomal source dependent. Benet et al. have suggested that

acyl glucuronides are more susceptible to effects of β-glucuronidase than phenolic glucuronides [17,18], although this has been questioned [19]. More recent work by Brunelle et al. have suggested that the precaution of inhibiting glucuronide enzymatic hydrolysis may not be necessary when investigating in vitro UGT reactions using human liver microsomes due to their much lower β-glucuronidase activity [20]. Furthermore, Meunier and Verbeeck studied APAPG stability in rat hepatic microsomal incubations and found that there is negligible hydrolysis of APAPG under the conditions of in vitro glucuronidation experiments [21]. In our study, we found that the addition of saccharolactone reduced the velocity of the reaction by about 45% at pH 7.1. This effect was still evident when a stronger buffer (i.e., 0.3 *M*) was used, but to a lesser extent. Our data indicate that saccharolactone should not be included when evaluating APAP glucuronidation in vitro as it interferes with UGT activity. The mechanism by which saccharolactone affects APAPG formation is likely due to a shift in the pH of the incubation medium.

Microsomal treatment with various detergents is commonly used to remove UGT latency for in vitro experiments. This step significantly improves the rate of conjugate formation of various UGT substrates. We evaluated APAPG formation in the presence and absence of alamethicin or Brij-58. Consistent with previous reports, Brij-58 produced a nearly 2-fold increase in APAPG velocity as compared to untreated microsomes. Recently, alamethicin (a 20 amino acid peptide antibiotic produced by the fungus *Trichoderma viride*) has been used to activate glucuronidation in microsomes. The mechanism by which alamethicin works is believed to be by inserting peptide molecules through the membrane and forming well defined pores that allow the free movement of substrates and cofactors without affecting enzyme intrinsic activity [22]. Alamethicin treatment was associated with a 3-fold increase in APAPG formation over non-treated microsomes. Furthermore, Brij-58 was associated with a bell-shaped curve of activity, while alamethicin produced a more consistent effect. The variable activity of Brij-58 at higher concentrations may be due to its inhibitory effect on UGT even as it permeabilizes the membrane [11]. Therefore, alamethicin has better and more consistent effect on maximizing UGT velocity at potentially lower concentration (i.e., 15–30 μg/mg protein) than has been reported.

In conclusion, optimal incubation conditions for APAP glucuronidation in human hepatic microsomes are presented. A simple and sensitive HPLC method was developed and validated for direct analysis of APAPG in human hepatic microsomes.

References

[1] A. Radominska-Pandya, P.J. Czernik, J.M. Little, E. Battaglia, P.I. Mackenzie, Drug Metab. Rev. 31 (1999) 817.
[2] K.W. Bock, A. Forster, H. Gschaidmeier, M. Bruck, P. Munzel, W. Schareck, S. Fournel-Gigleux, B. Burchell, Biochem. Pharmacol. 45 (1993) 1809.
[3] M.H. Court, D.J. Greenblatt, Biochem. Pharmacol. 53 (1997) 1041.
[4] G.M. Pacifici, D.J. Back, M.L. Orme, Biochem. Pharmacol. 37 (1988) 4405.
[5] J.O. Miners, K.J. Lillywhite, K. Yoovathaworn, M. Pongmarutai, D.J. Birkett, Biochem. Pharmacol. 40 (1990) 595.
[6] S.N. de Wildt, G.L. Kearns, J.S. Leeder, J.N. van den Anker, Clin. Pharmacokinet. 36 (1999) 439.
[7] A.M. Batt, J. Magdalou, M. Vincent-Viry, M. Ouzzine, S. Fournel-Gigleux, M.M. Galteau, G. Siest, Clin. Chim. Acta 226 (1994) 171.
[8] C.P. Strassburg, M.P. Manns, R.H. Tukey, Cancer Res. 57 (1997) 2979.
[9] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[10] M.B. Fisher, K. Campanale, B.L. Ackermann, M. van den Branden, S.A. Wrighton, Drug Metab. Dispos. 28 (2000) 560.
[11] J.M. Little, P.A. Lehman, S. Nowell, V. Samokyszyn, A. Radominska, Drug Metab. Dispos. 25 (1997) 5.
[12] T. Sugiyama, W.F. Goldman, Am. J. Physiol. 269 (1995) C698.
[13] C.L. Berg, A. Radominska, R. Lester, J.L. Gollan, Gastroenterology 108 (1995) 183.
[14] J.H. Kim, L. Johannes, B. Goud, C. Antony, C.A. Lingwood, R. Daneman, S. Grinstein, Proc. Natl. Acad. Sci. USA 95 (1998) 2997.
[15] K.W. Bock, J. Wiltfang, R. Blume, D. Ullrich, J. Bircher, Eur. J. Clin. Pharmacol. 31 (1987) 677.
[16] A. Dannenberg, T. Wong, D. Zakim, Arch. Biochem. Biophys. 277 (1990) 312.
[17] H. Spahn, S. Iwakawa, E.T. Lin, L.Z. Benet, Pharm. Res. 6 (1989) 125.
[18] C. Volland, L.Z. Benet, Pharmacology 43 (1991) 53.
[19] J. Tomasic, D. Keglevic, Biochem. J. 133 (1973) 789.
[20] F.M. Brunelle, R.K. Verbeeck, Xenobiotica 26 (1996) 123.
[21] C.J. Meunier, R.K. Verbeeck, Drug Metab. Dispos. 27 (1999) 26.
[22] K. He, S.J. Ludtke, W.T. Heller, H.W. Huang, Biophys. J. 71 (1996) 2669.