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Note

High-performance liquid chromatographic analysis of harmol and its conjugated metabolites after enzyme hydrolysis in biological fluids

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Harmol is a phenolic substrate which is conjugated in the liver with glucuronic acid and sulphate, and is not subjected to Phase I metabolism [1]. It has therefore been used widely as a model compound in the investigation of conjugation processes in vivo in rats [1—3], as well as in vitro in rat liver enzyme homogenates [4], isolated rat hepatocytes [5] and isolated perfused rat liver [2, 6]. In these studies, harmol has been measured in biological fluids by liquid scintillation spectrometry of radiolabelled drug or by fluorimetry after thin-layer chromatography. These methods are cumbersome and lack selectivity, or rely on the use of potentially hazardous radioactive materials. This report describes a rapid, sensitive and selective high-performance liquid chromatographic (HPLC) analysis of harmol in biological samples, which is also suitable for the measurement of its conjugated metabolites. The method has been applied to the measurement of harmol in perfusate and bile samples derived from a single bolus dose study in the isolated perfused rat liver.

EXPERIMENTAL

Reagents

Harmol hydrochloride, D-saccharic-1,4-lactone and the internal standard, warfarin, were obtained from Sigma (St. Louis, MO, U.S.A.). Analytical-grade triethylamine (Ajax, Sydney, Australia), orthophosphoric acid (85%, v/v) (Mallinckrodt, St. Louis, MO, U.S.A.), HPLC-grade acetonitrile (Waters Assoc., Milford, MA, U.S.A.), ascorbic acid (BDH, Poole, U.K.),

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β-glucuronidase—arylsulphatase enzyme (partially purified powder from *Helix pomatia*, Type H-1, Sigma) and singly glass-distilled water were also used.

Instrumentation

The method was developed on a constant-flow high-performance liquid chromatograph (Waters Assoc.) which consisted of a solvent delivery system (Model 6000A), an injector (Model U6K) and a fixed-wavelength UV absorbance detector operating at 254 nm (Model 441). The reversed-phase plastic column was obtained prepacked (Rad-Pak A, C_{18}, 10-μm particles, 100 mm × 8 mm I.D., Waters Assoc.) and was housed in a radial compression module (Model RCM-100). An in-line precolumn (packed with μBondapak C_{18}, 10-μm particles, Waters Assoc.) was used to protect the main analytical column.

Chromatography

The mobile phase consisted of water—acetonitrile (72:28, v/v) containing triethylamine (1%) adjusted to pH 7.0 with orthophosphoric acid. Chromatography was carried out at a flow-rate of 3 ml/min, which gave a back-pressure of 9.6 MPa.

Calibration standards and sample treatment

To produce a standard curve, drug-free perfusate (225 μl) without red blood cells (vide infra) was pipetted into 1.5-ml microcentrifuge tubes (Treff, Degersheim, Switzerland) and spiked with 25 μl of concentrated aqueous harmol solution (which also contained 20 m*M* ascorbic acid to retard harmol degradation during storage) to yield final drug concentrations corresponding to 12.5, 25.0, 50.0, 100.0 and 200.0 μ*M* in electrolyte perfusate. Internal standard (warfarin, 1 mg/ml, 50 μl) and acetonitrile (250 μl) were added, and the mixture was then vortexed (10 s) and centrifuged (13 000 *g*) for 1 min. A 20-μl sample of the supernatant was injected into the liquid chromatograph. A calibration curve was prepared by plotting the relationship between the peak-height ratio of harmol to warfarin and the harmol concentration in each standard. To determine the harmol concentration in isolated perfused rat liver perfusate samples (vide infra), 250-μl aliquots of each sample were processed along with the spiked perfusate standards as described above.

Conjugated metabolite determination

Conjugated metabolites of harmol were quantified by standard β-glucuronidase and arylsulphatase enzyme hydrolysis methods [7]. A 300-μl aliquot of perfusate sample was added to 700 μl of 0.1 *M* acetate buffer (pH 5.0) which contained 1250 U of β-glucuronidase activity and between 50 and 140 U of arylsulphatase activity per 700 μl. Another 300-μl aliquot of perfusate sample was added to 700 μl of the enzyme—buffer mixture that also contained saccharic-1,4-lactone (at a final concentration of 40 m*M*), which totally inhibits β-glucuronidase activity but does not affect arylsulphatase activity [1, 7]. These mixtures were incubated in an oscillating water bath at 37°C for 90 min. A 250-μl aliquot of each enzyme-hydrolysed mixture was assayed for harmol by HPLC as outlined above. Perfusate concentrations of total conjugated metabolites were calculated as the difference in harmol concentrations between

non-hydrolysed samples and samples enzyme-hydrolysed without saccharic-1,4-lactone present, after appropriate corrections for the dilutions involved in the hydrolysis procedure. Perfusate concentrations of harmol sulphate were determined as the difference in harmol concentrations between non-hydrolysed samples and samples enzyme-hydrolysed with saccharic-1,4-lactone. Concentrations of harmol glucuronide were calculated as the difference between total conjugated metabolite level and harmol sulphate level in each sample.

Enzyme hydrolysis for up to 6 h of a perfusate sample containing harmol-conjugated metabolites showed that liberation of free harmol reached a maximum level by 60—90 min. Hydrolysis was therefore complete by 90 min. Furthermore, incubation of a stock solution of harmol in acetate buffer for 120 min at 37°C, with and without the presence of enzyme, showed no degradation of harmol over this time period.

Analytical recovery and assay precision

The analytical recoveries of harmol and warfarin from perfusate were estimated by comparing the peak height of an acetonitrile-precipitated perfusate sample containing a known amount of the substance with the peak height of an aqueous acetonitrile (50:50) solution containing the same amount of each compound. The within-day assay precision was determined by replicate assays of a pool of spiked perfusate.

Isolated perfused rat liver study

An isolated perfused rat liver was established using standard techniques described previously [8]. In brief, the recycling 100-ml circuit (which perfused the liver at a constant flow-rate of 16 ml/min), perfusate reservoir, peristaltic pump and oxygenator were housed in a cabinet at 37°C. The perfusate consisted of Krebs—Henseleit electrolyte solution (pH 7.4) with 0.1% (w/v) glucose, 1% (w/v) bovine serum albumin and 10% washed human red blood cells added. A 20-μmol bolus dose of harmol was added to the reservoir and the perfusate (1.5 ml) was sampled predose, then at 5, 10, 15, 20, 25, 30, 40, 50 and 60 min for determination of harmol and metabolite concentrations. An equal volume of perfusate was added to the reservoir to replace that removed by sampling. Bile was collected in 30-min aliquots. After separation of red blood cells, the perfusate samples, together with the bile samples, were stored at −20°C until assayed for harmol and its conjugates.

Calculations

Coefficients of variation were calculated from the ratio of the standard deviation to the mean. Pharmacokinetic parameters were calculated by standard model-independent pharmacokinetic formulae [9].

RESULTS AND DISCUSSION

Chromatograms of blank perfusate and perfusate containing harmol (100 μM) are shown in Fig. 1. Both harmol (retention time, t_R = 2.7 min) and warfarin (t_R = 4.5 min) peaks were well resolved to baseline. An endogenous component in perfusate eluted with a retention time of 5.5 min, but it was

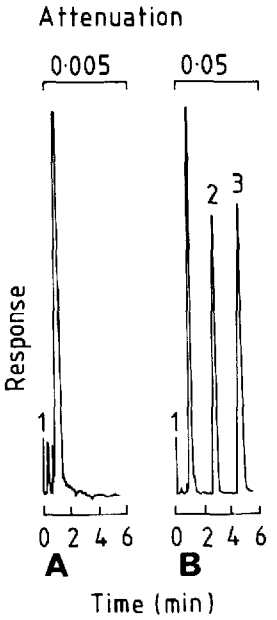

Fig. 1. Chromatograms of a blank perfusate extract (A) and an extract of perfusate containing harmol (concentration = 100 μM) (B). The peaks are the injection event (1), harmol (2) and the internal standard, warfarin (3).

baseline-separated from the internal standard, warfarin, and samples could be injected at 6-min intervals. Calibration curves were linear ($r > 0.99$) in the range of 0—200 μM and the analytical recovery from perfusate was 106 ± 9% ($n = 5$) for harmol and 103 ± 3% ($n = 5$) for warfarin. The minimum detectable level (defined as a peak four times that of baseline noise) of harmol was 0.5 μM on the highest detector sensitivity used (0.005 a.u.f.s.).

The within-day coefficient of variation was 0.2% ($n = 6$) at 12.5 μM and 1.4% ($n = 6$) at 200 μM. Harmol in aqueous solution stored at −20°C was only stable for about seven days. Aqueous concentrated stock solutions of harmol (used to produce standard curves) therefore contained 20 mM of the antioxidant, ascorbic acid, to minimize degradation of these solutions. Fresh concentrated stock solutions were made every three weeks, during which time no appreciable degradation of drug was noted. As perfusate samples were not stabilized with ascorbic acid, they were assayed within two days of performing an experiment.

Large amounts of the conjugated metabolites of harmol are excreted into the bile [2, 3, 7] and this assay can also be used for the analysis of rat liver bile samples. In the analysis of perfusate samples, column effluent was monitored at 254 nm, which closely corresponds to the λ_{max} of harmol (≈ 248 nm). At 254 nm, however, there were interfering peaks in the chromatogram of blank bile which precluded the analysis of bile at this wavelength. Therefore, an alternative detection wavelength at 313 nm, which closely corresponds to another λ_{max} of harmol (≈ 330 nm), was preferred in the analysis of bile. At 313 nm, there were no interfering peaks at the retention times of harmol nor

warfarin in the chromatogram of blank bile. Although assay sensitivity was reduced at the higher wavelength (lowest detectable limit in bile was 1.0 μM), this was of little consequence owing to the large amounts of harmol in enzyme-hydrolysed bile samples.

The assay was applied to the analysis of samples obtained from an isolated perfused rat liver preparation that was dosed with 20 μmol of harmol. The perfusate levels declined monoexponentially (Fig. 2) with a rapid elimination half-life of 2.9 min. Harmol glucuronide and harmol sulphate concentrations reached peak values at approximately 20—25 min and declined with pseudo half-lives of 12.3 and 43.6 min, respectively. At the conclusion of the experiment (t = 60 min), there were no detectable levels of harmol in perfusate, though 0.5% of the dose was present as harmol glucuronide and 3.6% as harmol sulphate in perfusate plasma. At this time, 0.6% of the administered dose had been excreted into bile as unchanged harmol, 46.0% as harmol glucuronide and 14.4% as harmol sulphate. In total, 65.1% of the dose had been accounted for harmol and its metabolites in perfusate and in bile, the remainder was presumably localized in liver tissue. These data are in broad agreement with previous findings of the disposition of harmol in isolated perfused rat liver [6].

In summary, this method overcomes the limitations imposed by previous assay procedures, and is both sufficiently sensitive and selective for the quantitation of harmol and its conjugated metabolites in biological fluids such as isolated rat liver perfusate and bile.

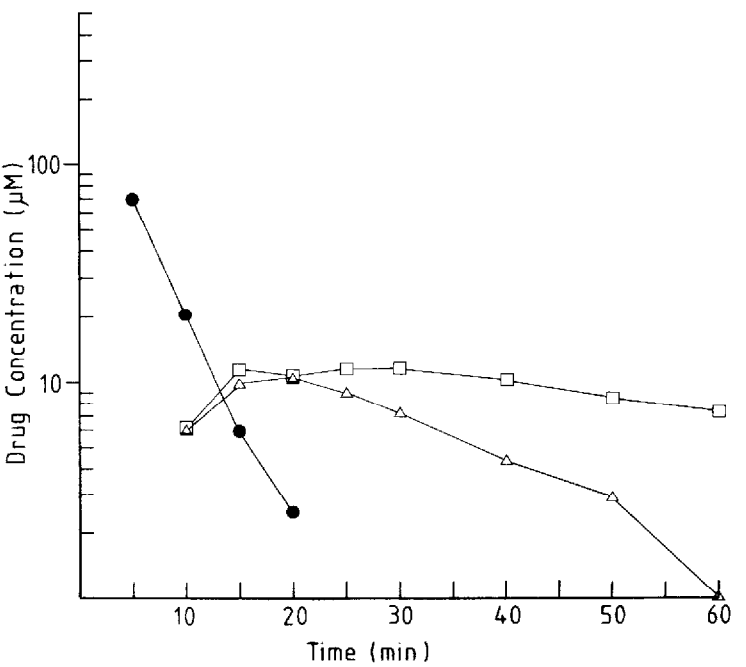

Fig. 2. Semilogarithmic plot of perfusate harmol concentrations (•) and glucuronide (△) and sulphate (□) metabolite perfusate concentrations versus time in a single isolated perfused rat liver preparation dosed with 20 μmol of harmol.

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