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Journal of Chromatography B, 707 (1998) 347–350

JOURNAL OF CHROMATOGRAPHY B

Short communication

Assessment of microsomal tolbutamide hydroxylation by a simple thin-layer chromatography radioactivity assay

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Received 15 September 1997; received in revised form 21 November 1997; accepted 21 November 1997

Abstract

A radio thin-layer chromatographic method is described for in vitro measurement of tolbutamide methylhydroxylation as an alternative to the commonly used HPLC assay. After the incubation experiments of [^{14}C]tolbutamide with human liver microsomes, the supernatants were directly spotted onto standard silica gel TLC plates and developed in a horizontal chamber using a solvent system consisting of toluene–acetone–formic acid (60:39:1, v/v). Dried TLC plates were exposed to a phosphor imager plate and quantificated by use of a phosphor imager. Reaction rates were calculated from the ratio of labelled metabolite to the total radioactivity. The correlation coefficient between HPLC and the TLC method was 0.978 (n=14). The described method provides a valuable tool for the determination of tolbutamide hydroxylation activity in human liver microsomes. © 1998 Elsevier Science B.V.

Keywords: Tolbutamide

1. Introduction

In vitro investigations for the identification of cytochrome P450 enzymes that are involved in the metabolism of drug candidates are an important issue for rational drug development. Therefore, robust routine methods are needed to measure the in vitro metabolism of standard test drugs that are regarded to be specific for a single cytochrome P450 enzyme. Cytochrome P450 2C9 (CYP 2C9) is involved in the metabolism of many xenobiotics [1–4]. Methylhydroxylation of the hypoglycaemic agent tolbutamide (Fig. 1) has been proposed as a specific probe for CYP 2C9 [5]. In the present study we have developed a thin-layer chromatographic method for in vitro measurement of CYP 2C9 activity as an alternative to the commonly used high-performance liquid chromatography (HPLC) assay. This thin-layer chromatography (TLC) assay employs [^{14}C]tolbutamide, a commercially available radiolabelled compound, is easy to establish, and allows a rapid and sensitive measurement of hydroxytolbutamide formation.

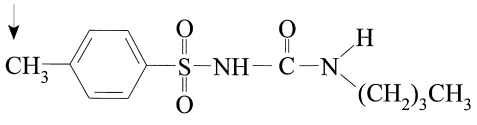

Fig. 1. Chemical structure of tolbutamide. The arrow indicates the site of hydroxylation by CYP 2C9.

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0378-4347/98/$19.00
PII S0378-4347(97)00603-8

2. Experimental

2.1. Reagents and chemicals

Tolbutamide was purchased from Sigma (Deisenhofen, Germany) and hydroxytolbutamide from Ultrafine Chemicals (Manchester, UK). [Ring-U-^{14}C]tolbutamide (60 mCi/mmol) was obtained from Amersham (Buckinghamshire, UK), and NADPH from Boehringer Mannheim (Mannheim, Germany). Solvents of analytical grade (Merck, Darmstadt, Germany) were used for HPLC and development of TLC plates (silica gel 60 F-254, 10×10 cm, Merck). All other reagents were of the highest quality available.

Human liver microsomes (Reaction Phenotyping Kit) were obtained from XenoTech LLC (Cambridge, KS, USA), and stored at −80°C until use.

2.2. Incubation conditions

Incubations were performed as duplicates in 1.5-ml microcentrifuge tubes (Eppendorf, Germany) for 45 min at 37°C and contained 0.1 mg microsomal protein, 100 m*M* Tris buffer (pH 7.4 at 37°C), 5 m*M* $MgCl_2$, 1 m*M* NADPH and 20 to 1200 μ*M* tolbutamide in a total volume of 0.5 ml. Radioactive samples contained 0.67 μCi of [ring-U-^{14}C]tolbutamide. Labelled tolbutamide, delivered as a solution in ethanol, was evaporated to dryness under a gentle stream of nitrogen before adding a solution of unlabelled tolbutamide in acetonitrile–water (25:75, v/v). The final acetonitrile concentration in the incubations did not exceed 0.5% (v/v). The reaction was terminated by addition of 0.25 ml 10% (w/v) trichloroacetic acid, vortex mixing, and cooling of the incubation tubes on ice. After centrifugation (3 min at 10 000 *g*) aliquots of the supernatants were directly injected into the HPLC system or spotted onto TLC plates.

2.3. Chromatographic separation

2.3.1. Thin-layer chromatography

Two μl of the supernatant were spotted onto TLC plates and run in a horizontal TLC chamber (Camag, Berlin, Germany) (vertical development in standard glass TLC tanks is also feasible) that was saturated with solvent vapour. The mobile phase consisted of toluene–acetone–formic acid (60:39:1, v/v/v). The TLC plates were dried (10 min, 120°C) and then exposed for up to four days to a phosphor imager plate and quantified by use of a phosphor imager (Fuji BAS 2000). The reaction rates were calculated from the ratio of labelled metabolite to the total radioactivity. The radioactive metabolite was positively identified as hydroxytolbutamide by comparison of the R_F value with authentic non-labelled standard.

2.3.2. High-performance liquid chromatography

HPLC analysis was performed using fully automated sample extraction by the column switching technique [6]. The HPLC system consisted of an ISS 200 advanced LC sample processor (Perkin-Elmer, Überlingen, Germany), an isocratic pump (Gynkotek, Germering, Germany), an HP 1090 pump with ternary gradient system, diode array detection system, column switching equipment, and a HP Vectra 486/66XM with HPLC 3D chemstation (Hewlett-Packard, Waldbronn, Germany). For automated sample extraction, a μBondapak C_{18} (37–55 μm) column (17×4.6 mm I.D.) with a mobile phase of 0.25% (w/v) aqueous ammonium acetate was used and for separation a Hypersil ODS (5 μm) column (12.5×4.6 mm I.D.) with a linear gradient rising from water–methanol (70:30, v/v) (sample transfer onto analytical column, time point 0 min) to water–methanol (5:95, v/v) (time point 10 min). Water as well as methanol was supplemented with 0.5% (w/v) ammonium acetate. The flow-rate was 1 ml/min. Under these conditions hydroxytolbutamide and tolbutamide had retention times of 6.9 and 9.6 min, respectively. Formation of hydroxytolbutamide was monitored by UV absorbance at 230 nm. Hydroxytolbutamide was quantified by comparison of peak areas with authentic standards, measured over a concentration of 0.05 to 40 nmol/injected sample (equivalent to 0.25 nmol/ml) hydroxytolbutamide. Linear calibration curves were obtained with an $r^2 >$ 0.998 by triplicate measurement of five concentrations. Radioactivity was measured off-line with a Topcount microplate scintillation counter after collecting the eluate in 24-well microplates for liquid scintillation counting (Canberra Packard, Meriden, CT, USA).

2.4. Method validation

For comparison of the HPLC and the TLC assay, samples were run as duplicates in HPLC and TLC ($n=14$). The results of both assays were compared using linear regression analysis. The intra-day and inter-day precision of the TLC assay was determined by analyzing two samples, five times on the same day and daily for three days, respectively. The limit of detection was estimated by dilution of a representative incubation experiment.

Table 1
Intra-day variability of the TLC assay

Day	Donor	Mean rate of hydroxytolbutamide formation (nmol/min/mg protein)	S.D.	C.V. (%)
1	2	350.63	7.63	2.18
1	16	396.75	2.13	0.54
2	2	375.00	7.94	2.12
2	16	408.00	8.56	2.10
3	2	372.75	4.28	1.15
3	16	404.63	15.2	3.76

Two different samples (each $n=5$) were analyzed by TLC daily for three days.

3. Results and discussion

Fully automated solid-phase sample extraction showed a mean recovery of hydroxytolbutamide from incubation mixtures performed with heat inactivated microsomes (30 min, 80°C) of 101±2.45% ($n=4$) over the concentration range of 1 to 12 nmol. Total recovery of ^{14}C radioactivity in incubation mixtures (100 μ*M* tolbutamide, 1 mg/ml microsomal protein) was 98.9% ($n=2$). The rate of formation of hydroxytolbutamide was linear with incubation time up to 2 h and with microsomal protein concentration from 0.1 to 1 mg protein/ml (the apparent K_m of hydroxytolbutamide formation was about 160 μ*M*).

The results of this HPLC assay were subsequently used to develop an uncomplicated TLC based assay. Using standard silica gel TLC plates, a good separation of tolbutamide and hydroxytolbutamide was achieved using a solvent system consisting of toluene–acetone–formic acid (60:39:1, v/v/v). Since supernatants of incubation experiments were directly spotted onto TLC plates, no additional sample extraction step was necessary. Because [^{14}C]hydroxytolbutamide was directly quantified using the specific radioactivity of the substrate, there was no need for calibration samples, either. Quantification was done using a phosphor imaging system that requires sometimes several days of exposure time. However, quantification by other more rapid TLC radioactivity scanning systems is also feasible.

Thus, a rapid assay principle was available for the assessment of tolbutamide hydroxylation activity in microsome preparations. The assay conditions were subsequently optimized and compared to HPLC results. No additional radioactive metabolites were detected either with HPLC or TLC. There was no detectable metabolite formation in the absence of NADPH. An amount of 0.67 μCi [ring-U-^{14}C]tolbutamide was chosen for the total incubation mixture in order to obtain sufficient amounts of radioactivity in incubations, when only 1% of the substrate was metabolized. Intra- and inter-day variability was between 0.5 and 3.8% (Tables 1 and 2). The limit of detection was 150 pmol (equivalent to 30 pmol/min/mg protein) hydroxytolbutamide.

A direct comparison of TLC and HPLC results of tolbutamide hydroxylation activities of 14 individual human liver microsome samples revealed nearly identical enzyme activities for both assays. The correlation coefficient between both methods was found to be 0.978 (Fig. 2). It was concluded therefore, that the TLC assay yielded nearly identical results as conventional HPLC analysis and was regarded to provide valid results of tolbutamide hydroxylation activities.

The advantage of TLC method is the very small sample volume (2 μl). In contrast, HPLC analysis requires sample volumes of 200 to 500 μl. There-

Table 2
Inter-day variability of the TLC assay

Donor	Mean rate of hydroxytolbutamide formation (nmol/min/mg protein)	S.D.	C.V. (%)
2	365.92	13.83	3.78
16	403.13	5.77	1.43

Two different samples were analyzed by TLC five times on the same day.

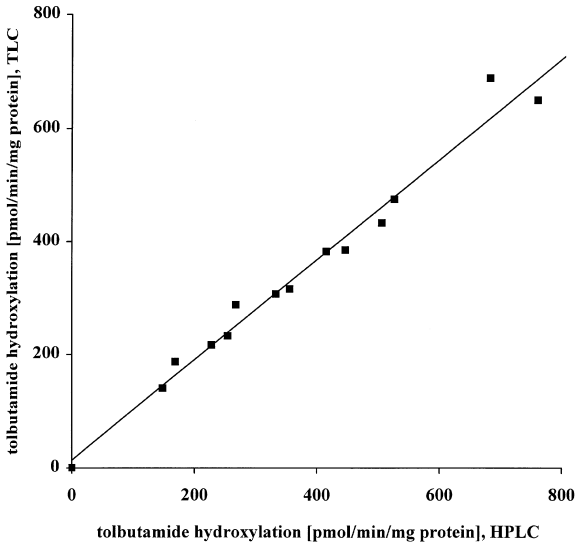

Fig. 2. Correlation between TLC and HPLC determinations of tolbutamide hydroxylation by 14 individual human liver microsomal samples (r^2=0.978). The data points represent the average of duplicate determinations.

fore, a reduction of the total incubation volume to 200 μl containing 40 μg microsomal protein is possible. This results in a significant reduction in the consumption of sometimes sparse microsomal material. In addition, the TLC method proved to be robust and eliminated the need for time consuming quantification by using calibration curves and equilibration of the analytical system, which is necessary if HPLC is used together with UV detection. Using horizontal TLC development, up to 14 samples were run on a single 10×10 cm TLC plate.

Taken together, the TLC method described herein together with labelled tolbutamide provides a valuable tool for the determination of tolbutamide hydroxylation activity in human liver microsomes. Because of its flexibility, this TLC approach is probably also applicable for other cytochrome P450 test reactions.

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