Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 814 (2005) 259–262
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of Rhodamine 123 in cell lysate by HPLC with visible wavelength detection

Tahira Iqbal, Minori Kinjo, Thomas C. Dowling*

Renal Clinical Pharmacology Laboratory, Department of Pharmacy Practice and Science, University of Maryland, School of Pharmacy, 100 N. Penn St., AHB Rm. 540-D, Baltimore, MD 21201, USA

Received 13 August 2004; accepted 14 October 2004
Available online 10 November 2004

Abstract

Rhodamine 123 (R123) is widely used to quantify P-glycoprotein (P-GP) functional efflux activity in vitro. We developed a rapid and specific high-performance liquid chromatography (HPLC) method to quantify Rhodamine 123 for use in experimental cell culture studies. The R123 standards (2.5–250 ng/mL) and quality controls (QCs) (5, 75, 200 ng/mL) were prepared in cell lysis buffer consisting of 0.75% Triton 100X and 0.2% sodium chloride. The mobile phase consisted of acetonitrile, 1.5 mM tetrabutyl ammonium bromide in 20 mM sodium acetate buffer (pH 4.0) (50:20:30) delivered at a rate of 1.0 mL/min. Samples (50 μL) were injected onto a C_{18} reversed-phase HPLC column with detection at 500 nm. Analyte retention times were 1.4 and 4.3 min for R123 and internal standard (R6G), respectively. Intra- and inter-day coefficients of variation were ≤4.2%. Samples were stable for at least three freeze-thaw cycles at room temperature for 24 and 48 h. This method was used to evaluate the functional activity of P-glycoprotein in renal tubule cell models including human kidney (HK-2), Madin–Darby canine kidney (MDCK) and multi-drug resistance gene-transfected MDCK cells (MDR1-MDCK).

Keywords: P-glycoprotein; Rhodamine 123; High-performance liquid chromatography (HPLC)

1. Introduction

Rhodamine 123 (2-(6-amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester, $C_{21}H_{16}N_2O_3$·HCl, F.W. 380.82, Fig. 1) is membrane-permeable fluorescent dye that is widely used to evaluate the functional transport activity of P-glycoprotein (P-GP) [1–5]. P-glycoprotein is an MDR-encoded membrane transporter that is physiologically expressed in normal tissues associated with excretory functions, including kidney proximal tubules. P-GP appears to play a critical role in multi-drug resistant cancer cells and P-GP modifying agents are being evaluated for use in combination with anti-cancer agents [6–8]. The development of P-GP modifying drugs raises important therapeutic and toxicological issues, as such agents may alter the clearance and distribution of other drugs.

Previously reported methods for the determination of R123 utilized either fluorometric analysis using luminescence spectrometer [9], fluroscene spectrophotometry after extraction [1,3,4], a Cytoflour fluorescence multiwell plate reader [10], flow cytometery [11] or high-performance liquid chromatography (HPLC) with fluorometric detection [2]. Pavek et al. [5] reported an HPLC method for R123 using diamond fuchsin as internal standard in perfusion media. This method required solid phase extraction prior to injection to column, with detection at 500 nm for R123 and 550 nm for diamond fuchsin. Here, we describe a rapid and sensitive HPLC method for determining R123 concentrations in cell lysis buffer obtained from cell culture experiments conducted in human kidney (HK-2), Madin–Darby canine kidney (MDCK) and multi-drug resistance gene transfected MDCK cells (MDR1-MDCK).

* Corresponding author. Tel.: +1 410 706 6590; fax: +1 410 706 6580.
E-mail address: tdowling@rx.umaryland.edu (T.C. Dowling).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.10.037

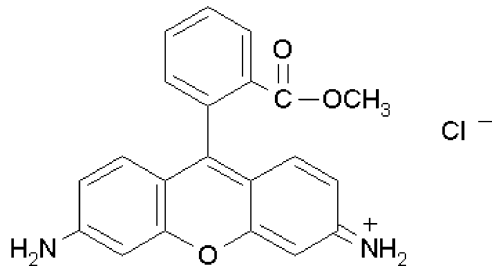

Fig. 1. Structure of Rhodamine 123.

2. Experimental methods

2.1. Regents and chemicals

Rhodamine 123, Rhodamine 6G (R6G, internal standard), sodium acetate, sodium chloride, tetrabutylammonium bromide (TBA) and Triton 100X were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA), and deionized distilled water was obtained from a Picopure system (Hydro-Picosystem Plus, Rockville, MD).

2.2. Equipment/instrumentation

The HPLC system consisted of a Waters 2690 separation module (Waters Corp., Milford, MA) and a Model 2487 dual wavelength absorbance detector set at 500 nm. The chromatographic data was collected and analyzed using Millenium Chromatography Manager (Version 3.2, Waters Corp.). Separation was achieved at ambient temperature with a Waters Nova-Pak C18 (3.9 mm × 150 mm), reversed phase HPLC column preceded by an Alltech guard column with C18 Bondapak/Corasil (37–50 μm) packing. The mobile phase consisted of acetonitrile, 20 mM sodium acetate buffer pH 4.0, and water containing 1.5 mM TBA (50:20:30). The mobile phase was filtered through a 0.45 μm filter and was delivered at an isocratic rate of 1.0 mL/min with a pump pressure of approximately 1450 psi.

2.3. Preparation of stock solutions and standards

Stock solutions of 1.0 and 0.1 mg/mL for R123 and 1 mg/mL and 50 μg/mL for internal standard were made in methanol, stored at −4 °C and were used to spike determined quantity in cell lysis buffer for the preparation of standards and quality controls (QCs).

2.4. Preparation of samples

MDCK and MDR1-MDCK cells were grown in standard culture media (DMEM) and HK-2 cells were grown in phosphate-enhanced media (RPMI) supplemented with penicillin (100 U/mL), streptomycin (100 ug/mL), 5% fetal bovine serum, epidermal growth factor (2 ng/mL) and bovine pituitary extract (0.01 mg/mL). All cells were grown to confluence in T-75 flasks at 37 °C with 5% CO_2 and subcultured by trypsinization. Confluent cells were exposed to R123 (5.0 μM) for 2 h, alone and in the presence of tacrolimus (0.1 μM) and sirolimus (0.1 μM). Cells were then washed three times with ice-cold buffer containing 20 μM verapamil. The cell layers were then solubilized and removed from tissue plates by adding 2 mL of a solution of 0.2 NaCl and 0.5% Triton X-100. The lysate aliquots (100 μL) of standards, QCs and, experimental samples were then prepared by adding 20 μL of internal standard, vortex-mixed for 10 s, and 50 μL was injected onto the column.

Table 1
Intra- and inter-day precision and accuracy for Rhodamine 123

Reproducibility	Concentration (ng/mL)		CV (%)	Difference (found vs. added)
	Added	Found (mean ± S.D.)		
Intra-assay reproducibility[a]				
Quality controls	5	5.06 ± 0.037	0.74	1.1
	75	76.3 ± 1.7	2.3	1.7
	200	202 ± 3.8	1.9	0.79
Inter-assay reproducibility[b]				
Quality controls	5	5.09 ± 0.096	1.9	1.8
	75	78.0 ± 0.30	0.38	4.0
	200	201 ± 2.0	1.0	0.34
Standards	2.5	2.49 ± 0.033	1.3	−0.26
	10	10.2 ± 0.20	2.0	2.0
	50	50.8 ± 1.7	3.4	1.6
	100	96.8 ± 4.1	4.2	−3.2
	150	154 ± 6.1	3.9	2.4
	250	247.6 ± 7.55	3.1	−0.95

[a] Seven quality control sample per concentration per day for four days.
[b] Twenty-eight quality control samples or two standards per day per concentration for four days.

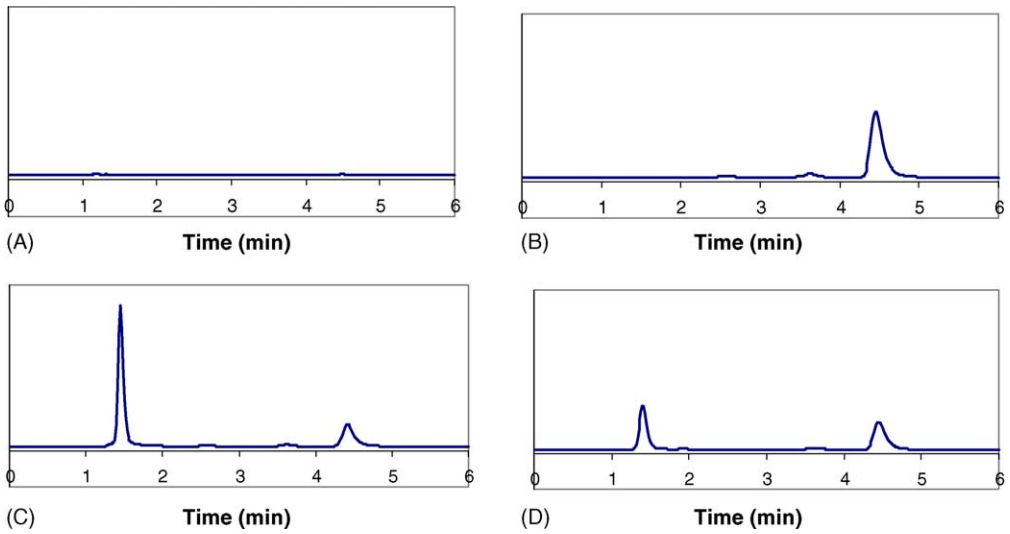

Fig. 2. Representative chromatograms of: (A) blank cell lysis buffer; (B) cell lysis buffer with R6G; (C) standard containing R123 (250 ng/mL); (C) sample from positive control (DEX) in HK-2 cells.

2.5. Calibration and linearity

Calibration curves were constructed using six standard concentrations in cell lysis buffer that were run in duplicate. Curves were obtained daily for four days by plotting the peak–height ratios of R123 to the internal standard (R6G) against the corresponding concentration of R123. Calibration curves were generated using linear regression analysis and obtained over the respective standard concentration range. The standard concentrations ranged from 2.5 to 250 ng/mL and are shown in Table 1. All standards and QC samples were stored at $-20\,^{\circ}C$ until analysis.

2.6. Precision and accuracy

The precision and accuracy of the assay was ascertained based on analysis of QC samples. QC sample concentrations for R123 were 5, 75 and 200 ng/mL. Seven replicate QC samples at each concentration were analyzed on four consecutive days, after which inter- and intra-day means, standard deviations, coefficients of variation (CV%) and average percent difference were calculated by standard methods (FDA Guidance for Industry, Bioanalytical method validation, 2001).

2.7. Stability assay

Low, medium and high QC in triplicate were tested for freeze-thaw effects (three cycles) and stability at room temperature for 24 and 48 h for processed and unprocessed samples.

3. Results and discussion

Representative chromatograms of standards and experimental samples are shown in Fig. 2. Retention times for R123 and internal standard were approximately 1.4 and 4.3 min, respectively. Calibration curves were linear over the concentration range of 2.5–250 ng/mL with slope and *y*-intercept (mean ± S.D.) of 77.0 ± 3.8 and -41.7 ± 5.1, respectively, correlation coefficients >0.998. The intra- and inter-day precision and accuracy of standards and QC's are shown in Table 1. At the LOQ, the signal to noise ratio was greater than 5:1 and inter- and intra-day CV was less than 1.3%. Low, medium and high QCs in triplicate were unchanged with average difference $\leq$3.8% during freeze-thaw cycles, and were stable at room temperature for 24 and 48 h for processed and unprocessed samples. The percent coefficient of variation was $\leq$5.1% for all test conditions.

Limitations of previous methods for R123 detection include the need for fluorometric analysis, solid phase extraction, a multi-well plate reader, and dual wavelength for detection of internal standard [1–5,9–11]. These approaches often require technical expertise, expensive equipment, and time commitment. In contrast, the method presented involves simple sample preparation without extraction, includes an internal standard (R6G) that can be detected at the same wavelength as R123 (500 nm) with an advantage of a short run time of 6 min. The detection of rhodamine within the visible wavelength range indicates that it will have minimal interference with other drugs that typically absorb light within the UV range. From our in vitro studies, we have shown that the drugs sirolimus and tacrolimus do not interfere with the assay. The presence of rhodamine metabolites in the in vitro system is also unlikely due to the lack of substrates required for formation of rhodamine esters or amide conjugates.

This method is currently being used to support studies in HK-2, MDCK and MDR1-MDCK cell lines to evaluate P-GP activity following exposure to immunosuppressive agents such as tacrolimus and sirolimus. In summary, the rapid and simple method reported here can be utilized in drug transport studies investigating the functional activity of P-GP in different in vitro models.

References

[1] M. Fontaine, W.F. Elmquist, D.W. Miller, Life Sci. 59 (1996) 1521.
[2] R. Yumoto, T. Murakami, Y. Nakamoto, R. Hasegawa, J. Nagai, M. Takano, J. Pharmacol. Exp. Ther. 289 (1999) 149.
[3] G. Tramonti, N. Romiti, M. Norpoth, E. Chieli, Ren. Fail. 23 (2001) 331.
[4] N. Romiti, G. Tramonti, E. Chieli, Toxicol. Appl. Pharmacol. 183 (2002) 83.
[5] P. Pavek, F. Staud, Z. Fendrich, H. Sklenarova, A. Libra, M. Novotna, M. Kopecky, M. Nobilis, V. Semecky, J. Pharmacol. Exp. Ther. 305 (2003) 1239.
[6] C. Chen, D. Clark, K. Ueda, I. Pastan, M. Gottesman, I. Roninson, J. Biol. Chem. 265 (1990) 506.
[7] P.W. Wigler, F.K. Patterson, Biochim. Biophys. Acta 1154 (1993) 173.
[8] M. Raderer, W. Scheithauer, Cancer 72 (1993) 3553.
[9] M.D. Perloff, L.L. von Moltke, E. Stormer, R.I. Shader, D.J. Greenblatt, Br. J. Pharmacol. 134 (2001) 1601.
[10] D. Schwab, H. Fischer, A. Tabatabaei, S. Poli, J. Huwyler, J. Med. Chem. 46 (2003) 1716.
[11] N. Feller, C.M. Kuiper, J. Lankelma, J.K. Ruhdal, R.J. Scheper, H.M. Pinedo, H.J. Broxterman, Br. J. Cancer 72 (1995) 534.