Recombinant Human CYPs are Stable when Stored Frozen in a Bulk Assay Premix.

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Abstract

Most assays are set up using a premix containing enzyme, substrate and buffer which is then aliquotted across an assay plate. Preparing the premix during assay set up is one of the most time-consuming aspects of any experiment and performing this on a regular basis adds to variability of the data generated. Currently, premixes are prepared fresh from the individual components each time an assay is set up, here we show that assay premixes can be prepared in bulk and then frozen for use at a later date, decreasing the inter-day variability of the assay and significantly reducing the preparation time needed for assay set up.

Two trials were undertaken using 8 recombinant human CYPs in a fluorometric CYP inhibition assay, one with a complete premix and the other using a premix without phosphate buffer with both being tested for stability after storage at -20°C and -80°C. We found that the inclusion of KPO_4 in the pre-mix led to degradation of CYP activity with storage at -20°C and -80°C over time, so producing less reliable data. Whilst there was some loss of activity, the premix without phosphate (fresh stock phosphate buffer was added at the time of assay) was much more stable over time in storage and gave reproducible results in our fluorometric inhibition screen even after 9 months storage at -80 and -20°C.

In summary, we have shown that a premix containing CYP enzyme, substrate and magnesium chloride can be stored frozen and is sufficiently stable for use in fluorometric CYP inhibition screens. Using this system, assay set up has been shortened (a fixed volume of stock phosphate buffer just needs to be added to the premix before it is aliquotted across the assay plate) and day to day variations have been significantly reduced.

Introduction

As part of further improvements to existing fluorimetric CYP inhibition studies (presented at ISSX Atlanta, 2011), we investigated the use of frozen premixes to make the assay much easier to perform and less time consuming to set up.

We attempted the preparation of three separate components for the assay (excluding potential inhibitors) and confirmed that, with ready-made frozen premixes, the results are comparable to when freshly prepared components are used, with the advantage of being more reproducible.

Methods

The stability of frozen premixes was investigated by determining the IC_{50} values of 11 compounds against each of 8 CYP isoforms (enzyme / substrate pairs are shown in Table 1). All components were prepared from freshly prepared reagents in bulk, then aliquotted into appropriate tubes and immediately frozen on dry ice. The assay premix contained: substrate (final assay DMSO 1.5% [v/v]), enzyme (final protein conc. 0.1mg/ml in the assay) and magnesium chloride (final conc. 5mM in the assay). Separate tubes contained stock potassium phosphate buffer (50mM final conc. in the assay) and co-factor regenerating system. Aliquots of premixes and buffer were either frozen at -20°C or -80°C. The co-factor regenerating system was stored at -80°C.

The frozen premixes were checked for stability after 1 week and 1, 3, 6, 9 and 12 months post-freezing.

After defrosting the reagents rapidly at 37°C, the appropriate volume of phosphate buffer (either frozen or fresh), was added to the premix before applying to the incubation plate containing potential inhibitors. The reaction was initiated after a 10 min pre-incubation at 37°C by adding co-factor regenerating system and fluorescent readings were taken every 1.5 min for 30 min. IC_{50} values were determined using XL fit software.

Conclusions

- IC_{50} values were reproducible when determined with enzyme premixes that had been stored at -20°C or -80°C.
- Frozen bulk premixes are suitable for use in this assay for up to 6 months after preparation and storage at -20°C.

Results

- Enzyme premixes stored for up to 6 months at -20°C or -80°C retained sufficient activity to be used for inhibition screening.
- The premixes appear to perform better when stored at -20°C compared to -80°C with respect to uninhibited control rate (Fig. 1 for CYP3A4). The tendency of the uninhibited control rate to decrease in premixes stored at -80°C became more pronounced as storage time increased. This resulted in up a 50% decrease in rate after 6 months for -80°C premixes compared to -20°C equivalents.
- The quality of the data fit for slopes to the IC_{50} curve fitting model was lower as storage time increased (more data points needed to be excluded), probably as a result of the slower control rates. In an attempt to retain higher quality data and uninhibited control rates, the 6 month assays were performed in duplicate using buffer which had either been prepared fresh or stored frozen. Using fresh buffer did improve the data fit and reaction rates (Fig. 2 for CYP2D6) .
- IC_{50} values for all 11 compounds with all 8 CYP isoforms between fresh and frozen premixes were very comparable irrespective of storage period or temperature but tended to give slightly higher results when premixes were freshly prepared (e.g. Miconazole, Fig. 3, CYP2D6, Fig. 4). These differences were not statistically significant.
- Inhibition data were more reproducible when reagents were frozen although fewer experiments were conducted with frozen reagents. (Fresh assays [n=19-36], frozen components including buffer [n=10-12].

Fig. 1 CYP3A4 activity in frozen premixes stored at -20°C and -80°C.

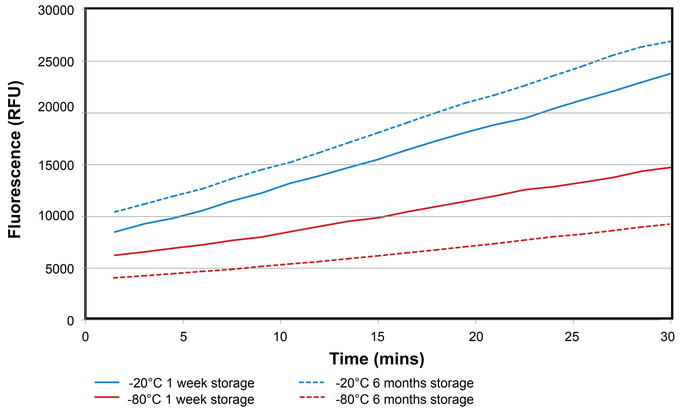

Fig. 2 CYP2D6 activity in premixes stored at -20°C with fresh and frozen phosphate.

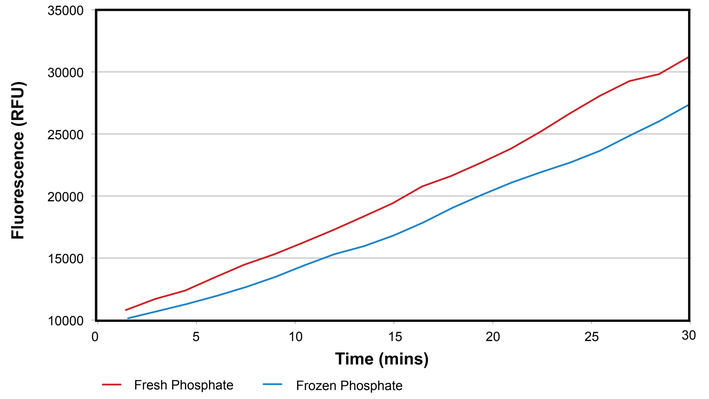

Fig. 3 Miconazole IC_{50} determined using fresh and frozen assay premixes.

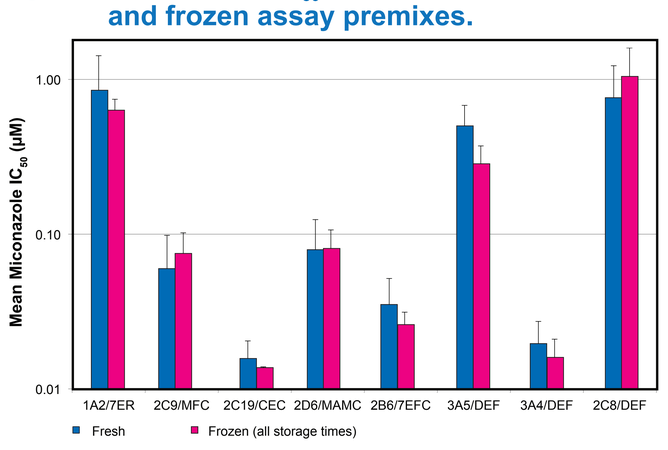

Fig. 4 IC_{50} values for 10 compounds using frozen (-80°C) CYP2D6 premixes.

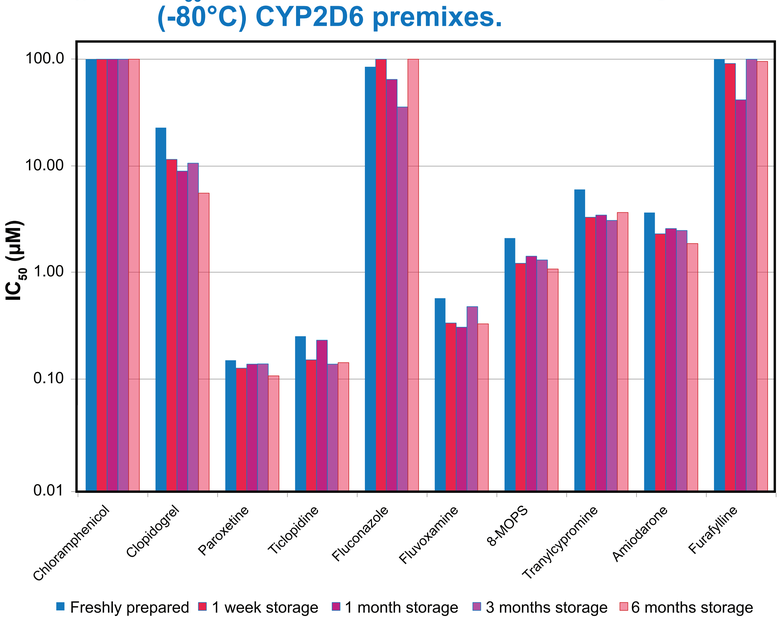

Table 1. Fluorescent IC_{50} Assay Details

CYP Isoform	Cypex Product code	Substrate	Final [S] (µM)	Detection wavelength (nm)	
				Excitation	Emission
1A2	CYP/EZ012	7-ER	1	572	604
2B6	CYP/EZ041	7-EFC	1.5	431	535
2C8	CYP/EZ049	DEF	0.5	485	530
2C9	CYP/EZ037	MFC	40	431	535
2C19	CYP/EZ008	CEC	25	410	460
2D6	CYP/EZ007	MAMC	6.4	429	470
3A4	CYP/EZ005	DEF	1	485	530
3A5	CYP/EZ048	DEF	2.5	485	530

7-ER = 7-ethoxyresorufin (Cypex CYP510) 7-EFC = 7-ethoxy-4-trifluormethylcoumarin (Invitrogen E2882)
DEF = Diethoxyfluorescein (Cypex CYP531) MFC = 7-methoxy-4-trifluoromethylcoumarin (Cypex CYP517)
CEC = 3-Cyano-7-ethoxycoumarin (Sigma UC455)
MAMC = 7-methoxy-4-aminomethylcoumarin (Cypex CYP515)