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Automation of metabolic stability studies in microsomes, cytosol and plasma using a 215 Gilson liquid handler

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Abstract

A 215 Gilson liquid handler was used to automate enzymatic incubations using microsomes, cytosol and plasma. The design of automated protocols are described. They were based on the use of 96 deep well plates and on HPLC-based methods for assaying the substrate. The assessment of those protocols was made with comparison between manual and automated incubations, reliability and reproducibility of automated incubations in microsomes and cytosol. Examples of the use of those programs in metabolic studies in drug research, i.e. metabolic screening in microsomes and plasma were shown. Even rapid processes (with disappearance half lives as low as 1 min) can be analysed. This work demonstrates how stability studies can be automated to save time, render experiments involving human biological media less hazardous and may be improve inter-laboratory reproducibility.

Keywords: Automated incubations; Metabolic screening; Microsomes; Cytosol; Plasma; Reversed phase liquid chromatography

1. Introduction

The assessment of stability in biological media is very important when studying the pharmacokinetic profile of a compound. For example, the accurate assessment of plasma stability is critical for ester compounds [1] and microsomal and cytosolic incubations are routinely used for the prediction of in vivo drug metabolism [2–4]. Such studies are becoming more important with the heightened awareness of the polymorphism of enzymes such as glutathione transferase [5], *N*-acetyltransferase [6] and particularly some cytochrome P_{450} isoenzymes [7,8]. The identification of the specific cytochrome P_{450} isoenzyme responsible for the metabolism of a candidate drug can significantly shorten the drug development time by allowing an intelligent anticipation of the factors which will influence the bioavailability of the drug and a better prediction of potential drug–drug interactions [9–11]. Moreover, the advent of combinatorial chemistry [12] has led to a vast increase in the number of candi-

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date drugs requiring screening. All those considerations are making the automation of biological stability studies a highly desirable option. This automation was approached from the following stand points:

- A program for performing kinetic measurements in biological media to achieve higher throughput, free up scientist time and improve within- and between- laboratories reproductibility.
- The use of 96 deep well plates, which is the standard format used in bioanalytical automation [13]. Working with plates allows centrifugation, which is a key step in sample preparation, and avoid to order tubes.
- The use of HPLC-based methods for assaying the substrate. Unfortunately, most compounds used in lead optimisation are unsuited to determination by means of direct fluorescence or UV/vis measurements. HPLC assays offer a choice between different detection methods and are also amenable to complete assay automation by interfacing HPLC instrumentation with other instruments such as liquid handlers, data acquisition systems and structure-resolving instruments such as mass spectrometers.

This paper described the automation of metabolic stability studies and was performed using a 215 Gilson liquid handler. This apparatus was selected for the flexibility and ease of use of the Microsoft™ Windows®-based 709 programming software (in Turbo pascal from Borland™) and for the vacutainer-piercing design of its arm and needle, which was useful for studies using human plasma. Many different racks can be used to accommodate all the required containers for the experiments (including thermostated racks using Peltier effect). Finally, Gilson liquid handlers permitted easy interfacing with HPLC apparatus [14,15] and Gilson 215 could be associated with a Rheodyne valve and a Gilson 819 injection valve actuator for HPLC or LC/MS analysis.

2. Experimental

2.1. Compounds

Ethoxy resorufin, resorufin, 1,2 epoxy-3-(*p*-nitrophenoxy) propane (ENPP), pure bovine serum albumin, sucrose, glutathione, NADPH, NADH, UDPGA, magnesium chloride, ammonium acetate were purchased from Sigma Aldrich Fluka (St Quentin Fallavier, France). Tris HCl was purchased from Prolabo (Gien, France), and acetonitrile from SDS (Peypin, France).

2.2. Animals

Male OFA rats weighing 250–300 g (Charles River, Cleon, France) or female DBA2 mice weighing 18–20 g (Iffa Credo, L'Arbresle, France) were used in the studies. The animals were housed in a temperature controlled (21°C), light cycled (12 h day^{-1} light cycle) room for at least 1 week prior to the study. The animals were allowed free access to both food and water, but fasted the night before their sacrifice.

2.3. Preparation of cytosol and microsomes

Livers were washed with saline, minced, chopped, and then homogenized (1:5 w/v) in ice-cold 0.2 M Tris 0.25 M sucrose buffer using a Potter Elvehjem homogenizer. The cell debris, nuclei and mitochondria were removed by centrifugation at $10\,000 \times g$ for 15 min in a Beckman (J2-21M/E) centrifuge. The pellet was homogenized in Tris/sucrose and centrifuged again at $10\,000 \times g$ as described by Erickson [16]. The supernatants were recentrifuged at $100\,000 \times g$ for 75 min in a Beckman L80 ultracentrifuge. The supernatants obtained from the first centrifugation were quickly frozen in nitrogen and used as cytosol samples. The microsomal fractions were resuspended in Tris/sucrose buffer and then frozen in liquid nitrogen. Protein concentrations were determined using a Pierce kit (Interchim, Montluçon, France).

2.4. Instrumentation for automated incubations

2.4.1. Instrumentation

A Gilson 215 liquid handler (Gilson Medical Electronics, Villiers le Bel, France) with an integrated dilutor (1-ml syringe), a 178 mm Z arm

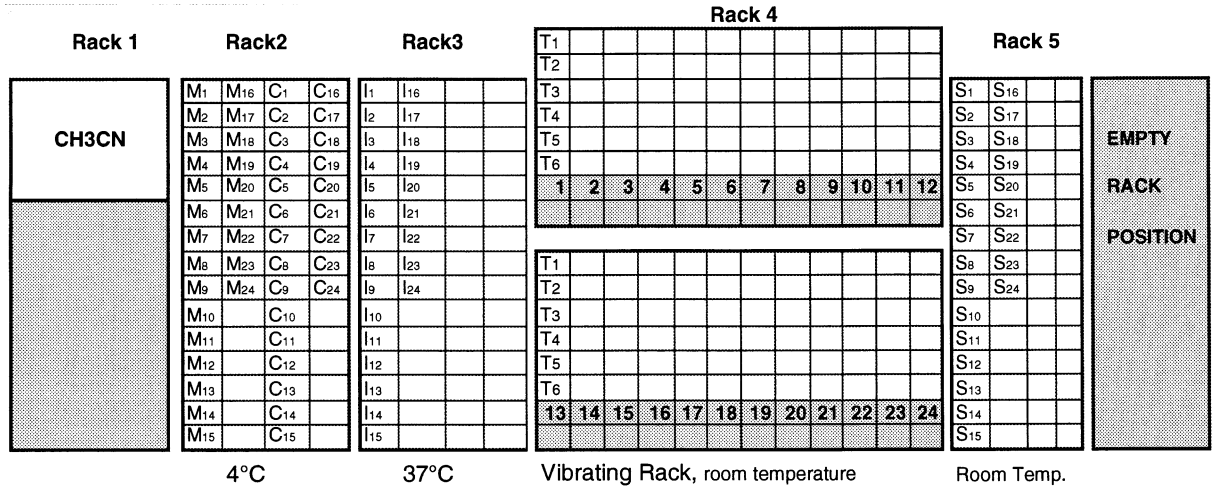


Fig. 1. Configuration of the Gilson 215 when used for automated incubations. Abbreviations: M, Biological media; C, Coenzymes; I, Incubation tube (empty at the beginning). For the vibrating rack 1–24 kinetic number, *T*, kinetic time point; S, test compound (substrate).

and a tray adapted for seven code 20–30 racks was used. The rack configuration used in the experiments (shown in Fig. 1, from left to right) was as follows. The Rack 1 was a code 61 rack, for a 250 ml bottle of acetonitrile. The Rack 2 and Rack 3 were two thermostated aluminium racks controlled by a Gilson 832 temperature control module. They were thermostated at two different temperatures using the Peltier effect. Rack 2 was used to keep coenzymes and biological media at 4°C. The Rack 3 contained empty tubes at the required incubation temperature (e.g. 37°C). The two Racks 2 and 3 were used with 75 × 10 mm glass tubes to reduce void volumes.

A customized vibrating rack (Rack 4) which could accommodate two 96 deep-well plate was designed in collaboration with Gilson Medical Electronics France. Vibration of the 96 deep-well plates was performed using an aquarium pump motor. The plates were covered by a septum sheet to reduce evaporation held in place by a polypropylene plate pierced with holes and clamped on the vibrating rack. Finally, a 2-ml rack (rack 5) 2-ml vials contained the solutions of test compounds (Rack 5).

Microplates (2-ml wells, polypropylene) were provided from Polytron (Tomtec, Les Clayes sous Bois, France). Septum sheets were purchased from Chromoptic, Auxerre, France.

2.4.2. Methodology

Setup of the Gilson 215 liquid handler tray is described in the Fig. 1. Each incubation run requires an empty tube in which the incubation is to be carried out (I). Sequentially, the biological fluid (from the tube M, maintained at 4°C) the substrate to be tested (S, kept at room temperature) and the coenzymes C were transferred to the incubation tube. The empty places on the tray allows the preincubation of the coenzymes at 37°C. Two 96 deep well plates were used which corresponds up to 24 kinetics of six points. The two empty rows in the plates were used as guides.

The duration of each step of a 5 min incubation in microsomes are shown in Table 1. Microsomes and substrate solution were dispensed sequentially to a thermostated tube (maintained at 37°C). During the pre-incubation time, acetonitrile was transferred to a 96 deep-well plate (six wells for one kinetic), one well for each kinetic time point.

The preincubation time was 3.5 min (between transfer of microsomes from 4 to 37°C and the start of the kinetic experiment). The kinetic run was started with the addition of the coenzymes to the incubation tube. A 30 s interval was necessary between the coenzyme addition and the dispensing of the mixture to the first well of the plate (time = 0). The content of the well was mixed with air by the Gilson 215 needle. The needle was rinsed and the apparatus waited for the delay before the next time point. At various times an aliquot of the incubation media was dropped into a well containing acetonitrile to quench the reaction by means of protein precipitation. A vibrating rack was designed to ensure better results. The vibrations were of sufficient magnitude to allow rapid and efficient mixing of solvent, and incubation media for a prompt quenching of the incubation. The motor was activated by an electrical contact closure signal from the 215 liquid handler. At the end of the last kinetic time point, the plates were centrifuged. Then, the supernatant was then either injected directly from the plate onto the HPLC system, or transferred to 200 μl Hewlett Packard vials by the Gilson 215 liquid handler using another program.

Table 1
Time table of 215 operations during a 5 min automated incubation

	Time (min)	
Dispensing microsomes in the incubation tube	00:00	
Rinsing needle	00:35	
Dispensing substrate in the incubation tube	00:49	
Mixing the incubation tube with air	01:09	
Rinsing needle	01:22	
Dispensing CH_3CN in the six wells	01:36	
Rinsing needle	02:46	
Dispensing coenzymes in the incubation tube	03:00	
Mixing the incubation tube with air, waiting	03:15	
Dispensing time 0 in the plate	03:27	00:00
Mixing time 0 well with air		00:10
Rinsing needle		00:15
Mixing the incubation tube with air, waiting		00:47
Dispensing time 1 in the plate (then three steps)		01:01
Dispensing time 2 in the plate (then three steps)		02:01
Dispensing time 3 in the plate (then three steps)		03:01
Dispensing time 4 in the plate (then three steps)		04:01
Dispensing time 5 in the plate (then three steps)		05:01
Mixing time 5 well with air		05:11
Rinsing needle	8:43	05:16

A program with various options was written. It allows the choice of many parameters such as for instance volumes, delays between points. For rapid kinetics, the program carried out kinetic experiments sequentially. For longer duration kinetics, the kinetic experiments were carried out in batch mode: the 215 liquid handler in a first step dispenses microsomes to all the incubation tubes of the batch, then dispenses the test compounds to the incubation tubes and acetonitrile to the wells. Kinetic runs are started sequentially with the same duration at each step. This permits a time saving of the pre-incubation processes.

2.5. Conditions for in vitro assays

2.5.1. Ethoxyresorufin deethylase assay

The concentrations in the incubation tube were 0.15 mg ml^{-1} microsomal protein, 0.5 mM NADPH and 2.5 μM of ethoxyresorufin in 0.1 M Tris HCl buffer containing 5 mM of magnesium chloride. The incubation tube contained 875 μl of biological media, 375 μl of substrate and 250 μl of coenzymes. The reaction is started with coenzymes. Aliquots (100 μl) of this mixture were transferred to the wells of the 96 wells plate designed for fluorescence-reading during the Gilson 215 program. Each well contained 100 μl of acetonitrile. The plates were read in a Fluoroscan II fluorimeter (Life Science International, Erigny, France). This allowed the comparison between manual (direct reading at various time intervals in the Fluoroscan) and automated incubations (reading at the end of the incubation period). The same dilutions were used for Fluoroscan kinetics but the wells contained 200 μl of incubation mixture. The plates were read with an emission wavelength of 540 nm (excitation wave-

length, 490 nm). Results were calculated from calibration curves of resorufin standards subjected to identical incubation conditions.

2.5.2. Comparison between automated and manual kinetics (murine and rat microsomes)

Automated and manual incubations were performed under the same conditions. The incubation concentrations were: 0.5 mM of NADPH, 0.5 mM NADH, 0.5 mM UDPGA, 2.3 mg ml^{-1} of microsomal proteins and 5 μg ml^{-1} of test compounds (final methanol content was 5%). The buffer used was 0.1 M Tris HCl buffer containing 5 mM of magnesium chloride and 3% BSA. The BSA was added to assist the dissolution of compounds with poor solubility. The incubation tube contained 875 μl of biological media, 375 μl of substrate and 250 μl of coenzymes. The volumes transferred to the well were 180 μl of acetonitrile and 180 μl of the kinetic mixture. Controls were prepared by omitting the coenzymes. The concentration of test compounds was measured by HPLC. Results were expressed as the remaining percentage of substrate against time. Linearization in logarithm allows the calculation of in vitro half life.

2.5.3. Metabolic screening in rat microsomes

A series of PDE V inhibitors (synthesized by B. Dumaitre, N. Dodic, Glaxo Wellcome France laboratory [17]) was used. All the conditions were identical to those for Section 2.5.2, except for the methanol content of the assays (0.5%). The coenzyme solution consisted of 0.5 mM NADPH and 0.5 mM UDPGA. The remaining concentrations of PDE V inhibitors were measured by HPLC.

2.5.4. Reproducibility of incubations in rat cytosol

Incubations were performed at 37°C using 2.5 μM ENPP and 1 mM glutathione in the assay. The cytosolic protein content was 9 mg ml^{-1}. Cytosol was diluted (v/v) in ratio of 1/2, 1/5, 1/10 and 1/20 with 0.1 M Tris/3% BSA buffer for the automated incubations. Controls were prepared from buffer without cytosol. Results were analysed by HPLC for metabolic screening.

2.5.5. Incubation in rat plasma

Rat plasma was purchased from Biotec (Orleans, France). Test compound (ethyl ester, MW 256) was incubated at 50 μM. The incubation tube contained 375 μl of a 200 μM test compound solution (in 0.1 M Tris with 3% BSA) and 1125 μl of rat plasma.

2.6. Instrumentation for HPLC analysis

The HPLC equipment consisted of the following components: an ABI 400 or Hewlett Packard 1050 pump, a Hewlett Packard 1050 or a Gilson ASPEC Xli automatic injector (loop volume of 100 μl), a 785A ABI UV detector, a Hewlett Packard ChemStation control/data acquisition system, a Kromasil C8 column (4.6 × 150 mm; 5 μm particle size, AIT Chromato, Saint Germain en Laye France) fitted with a 1 cm guard column containing the same stationary phase. The mobile phase consisted of mixtures of acetonitrile and ammonium acetate buffer (pH = 6.9, 5 g l^{-1}) ranging from 40:60 to 60:40 v/v (depending on the analysed substrate, but always under isocratic conditions). The concentration of ENPP was measured with a mixture of acetonitrile–ammonium acetate (50:50 v/v). Detection was by UV absorption at 290 nm, range was set at 0.01 AUFS and the time constant was set at 2 s. For analysis of incubations in rat plasma, the chromatographic eluant and other chromatographic conditions were the same as for the ENPP analysis, but the UV wavelength was 265 nm.

3. Results

3.1. Reproducibility

These is no doubt that automation can perform very reproducible work. The apparatus can perform reproducible tasks whatever the position of the tubes and the wells on the tray. The delay caused by the movement of the arm was measured to be at a maximum of 4 s. This is insignificant compared to the time between each sampling time (at least 1 min).

Table 2
Comparison between ethoxyresorufin deethylase (EROD) activities (pmoles min^{-1} mg^{-1}) in rat microsomes determined by the fluororescence reading 'on the fly' with Fluoroscan II or read after automated incubations with Gilson 215[a]

GILSON 215 (*end point readings*)			
Intra assay ($n=5$)			
Mean activity ($\pm$SD)	40.2 ± 6.9	39.0 ± 2.1	42.9 ± 1.2
r^2 ($\pm$SD)	0.98 ± 0.014	0.99 ± 0.003	0.98 ± 0.005
RSD (%)	2	5	3
Inter assay ($n=3$)	–	–	–
mean activity ($\pm$SD)	–	40.7 ± 2.0	–
RSD (%)	–	5	–
FLUOROSCAN II ('*on the fly*' *readings*)			
Intra assay ($n=5$)			
Mean activity ($\pm$SD)	42.4 ± 1.8	48.7 ± 1.5	49.4 ± 1.6
r^2 ($\pm$SD)	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
RSD (%)	3	3	3
Inter assay ($n=3$)			
mean activity ($\pm$SD)	–	46.8 ± 3.8	–
RSD (%)	–	8	–

[a] Intra-assay represented the variation of five incubations in the same run of Gilson 215 apparatus, inter-assay was three independent experiments in different days.

3.2. Appearance of resorufin

This reaction is catalysed by cytochrome P_{450} 1A2 [9,10]. The reaction kinetics for rat microsomes could either be monitored by fluorescence reading 'on the fly' or determined at the end of the incubation by reading the quenched incubations in each well. (endpoint reading kinetics). The results obtained using both techniques (Table 2) were very close, in terms of both actual values and reproducibility in spite of the differences between the two techniques.

3.3. Comparison between automated and manual incubations

Twenty test compounds from different research groups were incubated in rat or murine microsomes with manual and automated incubation. A good correlation was obtained between both the manual and automated incubations (Fig. 2). Intra-assays corresponds to one incubation in triplicate during one experiment with the Gilson 215 apparatus. Inter-assay was the result of three different experiments.

3.4. Intra-assay and inter-assay for cytosolic incubations

ENPP undergoes glutathione conjugation in rat cytosol [18]. Different dilutions of cytosol were used to simulate different half lives (from 1 to 6 min). The results are shown in Fig. 3 and Table 3. Intra-assays corresponds to one incubation in triplicate during one experiment with the Gilson 215 apparatus. Inter-assay was the result of three different experiments.

3.5. Assay for plasma incubations

Compound 7, an ethyl ester (MW 256) from our research group, was tested in rat plasma (Fig. 4). Plasma can be analysed only after only acetonitrile precipitation and centrifugation. The half life of compound 7 was determined to be ~ 1 min.

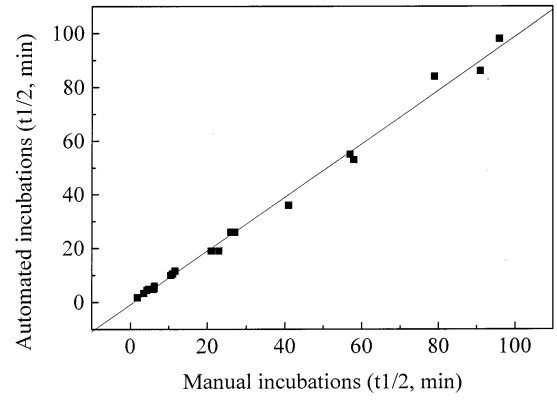


Fig. 2. Correlation between manual and automated incubations: stability of test compounds were analysed in microsomes and monitored with HPLC ($y = -0.78 + 0.99x$, $r = 0.9974$, $n = 20$).

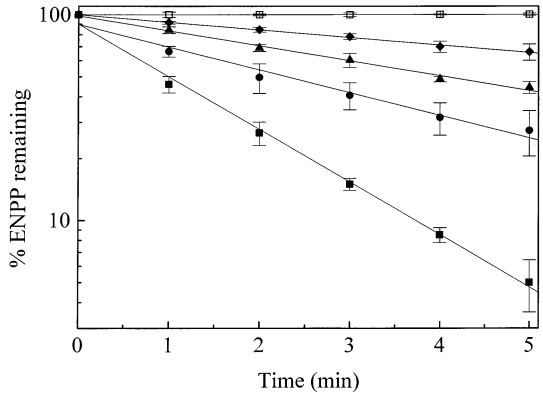


Fig. 3. Automated ENPP (1,2 epoxy-3-(*p*-nitrophenoxy)propane) incubations in rat cytosol (inter-assay, $n = 3$) with different dilutions of cytosol: (■) 2.6 mg ml^{-1}; (●) 1 mg ml^{-1}; (▲) 0.5 mg ml^{-1}; (◆) 0.26 mg ml^{-1} of protein in the assay, and (□) controls.

3.6. Assay for metabolic screening

The phosphodiesterases (PDE) which catalyse the hydrolysis of cyclic nucleotides (cAMP and cGMP), are a group of well characterized enzymes that have been subclassified into several isoenzyme families. PDE V is a particularly attractive target, as a potent and selective inhibitor that could be useful in the treatment of hypertension and congestive heart failure. In the pharmacokinetic support of this project [17], the stability in microsomes of some inhibitors were studied. Methyl and ethyl analogues were found to exhibit similar stability. Metabolic rates increased as the length of the chain (Table 4). The instability of the benzyl compound could be explained as a result of cytochrome P_{450} ring hydroxylations [19]

4. Discussion

4.1. Program design

Automated incubations in 96-well plates could be performed using two different strategies. In the first strategy the experiments could be carried out in the plate wells. The reaction could be quenched at various times. This first approach may have some drawbacks: all the components such as biological media, substrate, coenzymes had to be mixed and then quickly dispensed in six wells to obtain reproducible results. The plate had to be thermostated, but the temperature-controlled-microplate-holders for the robot are currently unavailable. Furthermore, in order to achieve microsomal incubations a vibrating and thermostated rack for microplates was needed. A second strategy could be adopted. The incubations could be carried out in a tube, with subsamples of the reaction mixture being transferred into the plate wells. The reactions were quenched because acetonitrile was transferred into the wells before the beginning of the experiment. This method required only 30 s between the mixture of the components and the first kinetic timepoint. Performing automated incubations using incubations in tubes (strategy 2) is therefore more readily achieved than automation by incubation of a plate (strategy 1).

4.2. Use of acetonitrile as a protein precipitant

The addition of acetonitrile is one of the most efficient and useful means of bringing about protein precipitation in biological fluids [20]. Acetonitrile allows a higher pH to be maintained than other precipitating agents such as trichloroacetic or perchloric acid, it also permit desorption of products from proteins, thereby avoiding the coprecipitation of compounds with low solubility. In addition, acetonitrile is a widely used solvent for HPLC mobile phases, hence it can be used both as a precipitant and for the preparation of samples for HPLC analysis in a single step. Nevertheless, programs can be easily adapted for use with other quenching agents such as perchloric acid or chloroacetic acid. Moreover, programs can be written where the incubation is stopped by direct HPLC injection. This has recently been described [21] for a HIV protease assay using a Gilson 232 sample injector. In this case, the biological media used was highly diluted (pure enzyme in buffer) and was injected onto reversed-phase columns without prior sample preparation [22].

Table 3
Disappearance of ENPP (1,2 epoxy-3-(*p*-nitrophenoxy)propane) by automated incubations in rat cytosol (detection by HPLC)[a]

		Cytosolic protein in the assay (mg ml^{-1})			
		2.63	1.10	0.53	0.26
Intra assay ($n=3$)					
Half life (min)	Mean ± SD	1.2 ± 0.04	2.9 ± 0.12	4.0 ± 0.11	6.9 ± 0.30
	RSD (%)	3	4	3	5
Rate (nmol min^{-1} mg^{-1})	Mean ± SD	4.2 ± 0.11	4.3 ± 0.20	6.1 ± 0.16	7.3 ± 0.26
Inter assay ($n=3$)					
Half life (min)	Mean ± SD	1.1 ± 0.05	2.6 ± 0.26	4.2 ± 0.20	7.2 ± 0.2
	RSD (%)	5	10	6	3
Rate (nmol min^{-1} mg^{-1})	Mean ± SD	4.5 ± 0.19	4.7 ± 0.47	6.1 ± 0.37	6.8 ± 0.2

[a] Intra-assay represented the variation of three incubations in the same run of Gilson 215, inter-assay was three independent experiments in different days.

4.3. Assessment of the programs

The programs described here are shown to be reliable for microsomal incubations, with a good comparison between 'on the fly' kinetic readings and the end point readings from kinetic experiments done with the Gilson apparatus (Table 2). Moreover, there is good correlation between manual and automated incubations (Fig. 2). These programs can also be used with other different biological fluids like cytosol (Fig. 3, Table 3) and plasma (Fig. 4). They can provide stability data even for very rapid kinetics, including compounds with half lives as low as 1 min (Table 3, Fig. 4). The 215 apparatus could be useful for metabolic screening, as described in Table 4. Reproducibility

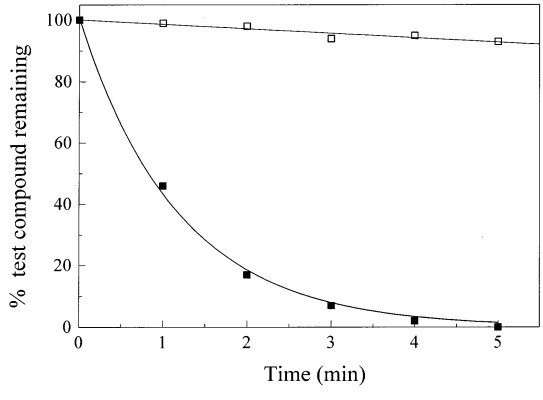


Fig. 4. Disappearance of compound 7 (ethyl ester) in rat plasma (■) plasma; (□), controls in buffer.

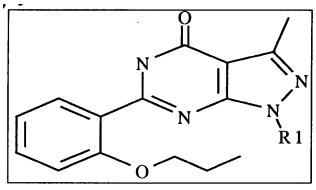
for the automated incubation was found to be acceptable (Table 2 and Table 3). Recent inter-laboratory studies with ethoxyresorufin assays showed a very high level of difference, even with standardized protocol [23,24]. The pre-incubation and the incubation are critical steps for good reproducibility. The use of an automated system could be used to reduce inter-laboratory discrepancies.

4.4. Evolution of such programs

In this type of program, the kinetic experiments required one tube of microsomes, one tube of coenzyme and one tube of substrate for each kinetic run. Thus, kinetic studies involving various biological media, coenzyme, substrates or protein concentrations on the same liquid handler surface are possible. Different programs could be easily written to use stock solutions of coenzymes or of biological media, to thaw biological media that was frozen in vials compatible with Gilson racks. In the latter case, the instrument could also prepare the solutions and use them during the programs.

Finally, complex programs could be used for microsome typing of different cytochromes P_{450} subtypes or other kind of isoenzymes [10]. In the same way, automated incubations in microsomes with selected inhibitors [7,9,10] or specific cytochromes P_{450} expressed in yeast or other vectors [25] could reveal the subtype involved in the

Table 4
PDE V inhibitors which were screened with rat microsomes (15 min incubation, HPLC detection)



	R1	Slope	T1/2 (min)
8	Methyl	0.05	14
9	Ethyl	0.06	12
10	Propyl	0.07	10
11	Butyl	0.14	5
12	Phenyl	0.41	2

metabolism of compounds of interest. Dilutions of the substrate or inhibitor could be incorporated to determine K_i or K_m.

The automation of metabolic stability studies is just the beginning. However, it can be time saving, reduce human contact with potentially hazardous biological media and it could give better inter-laboratory reproducibility.

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