An Increased Throughput Method for the Determination of Partition Coefficients

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Purpose. To present an increased throughput automated shake-flask method for the direct determination of the partition coefficients of solutes between octan-1-ol and buffer.

Method. The traditional shake-flask method has been transferred onto 96-well plate technology and a robotic liquid handler has been used for sample preparation. A custom programmed Gilson autosampler samples the organic and aqueous phases directly from the plate, circumventing the need for any manual separation. Analyses are performed by reverse phase high performance liquid chromatography (RP-HPLC). Generic fast gradient RP-HPLC conditions are used to eliminate chromatographic method development time and reduce analysis time.

Results. A full validation of the automated method is presented for a range of compounds with log D values between –2 and 4.

Conclusions. The advantages and limitations of this direct measurement method are discussed. The use of this methodology provides a means to rapidly assess log D values for large compound arrays.

KEY WORDS: partition coefficient; log P; log D; high throughput; physicochemical.

INTRODUCTION

Lipophilicity, a measure of the relative affinity of a molecule for a lipid environment, has been recognised for almost 100 years as having a profound significance relating to the biological effect of drug substances (1–4). In 1961, Pauling (5) described a series of anaesthetic compounds in which the biological potency could be related to lipophilicity and since then the determination of lipophilicity has become routine in the drug discovery and agrochemical fields. Numerically, lipophilicity is expressed either by log P, the logarithm of the partition coefficient P (6) between water and an immiscible non-polar solvent, such as octan-1-ol, or by log D, the distribution coefficient at a defined pH.

Partition coefficients (P) are usually determined by the shake-flask method (7). When performed manually, this method is time consuming, repetitive and tedious. Moreover, the number of compounds produced in drug discovery is increasing dramatically through rapid analogue synthesis and combinatorial chemistry (8). Thus, analytical chemistry faces the challenge to determine rapidly P on a larger number of compounds. The shake-flask method can also provide access to ionisation coefficients (pK_a) (9). By measuring pH dependence of partition coefficient pK_a and P may be determined simultaneously, hence, a higher throughput method to determine P would also have implications for pK_a determinations.

Several alternative methods have been developed to overcome the difficulty of the shake-flask method. One such direct, although time consuming method employed to measure P is counter-current chromatography (10) where water saturated with octan-1-ol is the mobile phase and octan-1-ol saturated with water is the stationary phase. Indirect chromatographic methods have also been investigated. A correlation between log P and the logarithm of the capacity factor (log k′) has been established on reverse phase high performance liquid chromatography (RP-HPLC) (11–14). Similarly micellar liquid chromatography (15), micellar electrokinetic chromatography (16) and potentiometric methods (17) have been used to estimate P. A number of computer assisted prediction algorithms have also been developed (18–21) but, in general, these are only as good as the datasets that have been used to develop the model.

In this paper, we describe the development of a modified shake-flask procedure designed for increased throughput measurement of P. Many higher throughput processes, particularly in biochemical screening and bioanalytical chemistry use 96-well plate technologies with robotic liquid handling to allow parallel and automated sample handling. We have transferred the partitioning process to a 96-well plate, and consequently realised automated sample preparation using a Beckman Biomek 2000. Each phase is injected directly from the plate using a customised autoinjector, minimising sample handling and post-partition transfer. Additionally, through the application of fast generic gradient RP-HPLC (21), analytical method development for each compound is circumvented and throughput increased as each injection may be performed with a cycle time of 5 minutes. 48 samples can be analysed in duplicate at two concentrations in 24 hours. Comparison of data obtained manually and with our new methodology, in terms of correlation and reproducibility, demonstrates the validity of the approach.

EXPERIMENTAL SECTION

Materials

The following standard compounds and drugs were purchased from Sigma (Poole, Dorset): 1-alprenolol tartrate salt, 1-benzylimidazole, 3-nitroaniline, amitriptyline, alprazolam, bromazepam, chlorpromazine hydrochloride, clofibric acid, diazepam, ephedrine hydrochloride, fluoxetine hydrochloride, hydroquinone, ibuprofen, lidocaine, naphthoic acid, p-nitrophenol, nortriptyline hydrochloride, paracetamol, phenacetin, phenobarbital sodium salt, procaine, quinidine hydrochloride, sulfaphenazole, temazepam, testosterone. The following were from Aldrich (Gillingham, Dorset): 4-chloroaniline, caffeine, naphthalene, phenolphthalein, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate and quinine. Octan-1-ol for analysis was purchased from Fisher Ltd. (Loughborough, UK). HPLC grade water was obtained from an Elga water system. Acetonitrile, HPLC grade, phosphoric acid and triethylamine were obtained from BDH (Poole, Dorset) and formic acid from Romil (Cambridge, Cambridgeshire).

Propylene deep round bottom 96-well plates (1.2 ml) and cap mats were purchased from Receptor Technologies

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(Adderbury, Oxon). The Biomek 2000 was purchased from Beckman Instruments Inc. (Fullerton, California, USA).

Instrumentation and columns

Reversed-phase HPLC was carried out on an HP1090 system consisting of a solvent delivery system and a diode array (operated at $\lambda = 260$ nm, $\delta = 4$ nm) connected to either the HP1090 autosampler or a Gilson 231 autosampler with a 100 μl injection loop. The Gilson 231 contains a custom rack (Figure 1) designed in-house to allow injection from a deep well microtitre plate. Data collection and integration were performed using an HP Chemstation (version 3.03) data acquisition system. The columns used were packed with 2 μm TSK gel Super-ODS (TosoHaas, Stuttgart, Germany) and were 50 or 100 mm long by 4.6 mm i.d.

Methods

Eppendorf Shake-Flask

For each compound, 3 determinations were performed in 1.8 ml Eppendorf vials. To 100, 300 and 400 μl of a 1 mg/ml solution of analyte in octan-1-ol (or 100 mM K_2HPO_4 at pH = 7.4) were added 400, 200 and 100 μl of octan-1-ol (or 100 mM K_2HPO_4 at pH = 7.4) and 500 μl of the other phase (or 100 mM K_2HPO_4 at pH = 7.4 or octan-1-ol respectively). All vials were shaken on a reciprocal shaker for 5 minutes. Vortex mixing or violent agitation was avoided to prevent emulsion formation. After centrifugation, both phases were manually separated and transferred into HPLC vials with a Pasteur pipette. Each phase was then analysed by HPLC and the log P derived from the peak area ratios for the analyte in each phase.

$$P = [X]_{\text{octan-1-ol}}/[X]_{\text{aqueous}}$$

Manual Plate Method

For each compound, 3 determinations were performed in different wells of a plate (as previously described). To 200 or 400 μl of a 1 mg/ml solution of analyte in octan-1-ol (or 100 mM K_2HPO_4 pH = 7.4) were added 200 or 0 μl respectively of octan-1-ol (or 100 mM K_2HPO_4 at pH = 7.4) and 400 μL of the other phase (100 mM K_2HPO_4 at pH = 7.4 or octan-1-ol respectively). On a separate plate, to 300 μl of stock solution were added 100 μl of octan-1-ol and 400 μl of the other phase. Plates were then sealed, placed on their sides and shaken on a reciprocal shaker for 30 minutes. After centrifugation, the plates were put onto a Gilson autosampler from where both phases were injected directly from the wells by sampling at different heights.

Biomek Method

A Beckman Biomek 2000 equipped with the following pipetting tools was used: a P1000L (single channel, 1 ml with liquid-level sensing), a P200L (single channel, 200 μl with liquid-level sensing), and an MP200 (eight-channel, 200 μl). The workstation was controlled by Bioworks version 3.0 for Windows NT.

The Biomek was supplied with a plate containing 1 mg/ml stock solutions of analytes in octan-1-ol. This plate is referred to as the mother plate. An empty 96-deep well round bottom plate (the analysis plate), was also supplied.

Automated pipetting on Beckman Biomek 2000: Using an 8-channel MP200 tool, 400 μl (2 × 200 μl) of 100 mM KH_2PO_4 at pH = 7.4 were pipetted into the analysis plate. Using a single channel P200L, 200 μl of octan-1-ol were added to every other well of the analysis plate. With the same tool, 200 μl of the analyte stock solution were pipetted from the mother plate into these same wells. Using a P1000L, 400 μl of

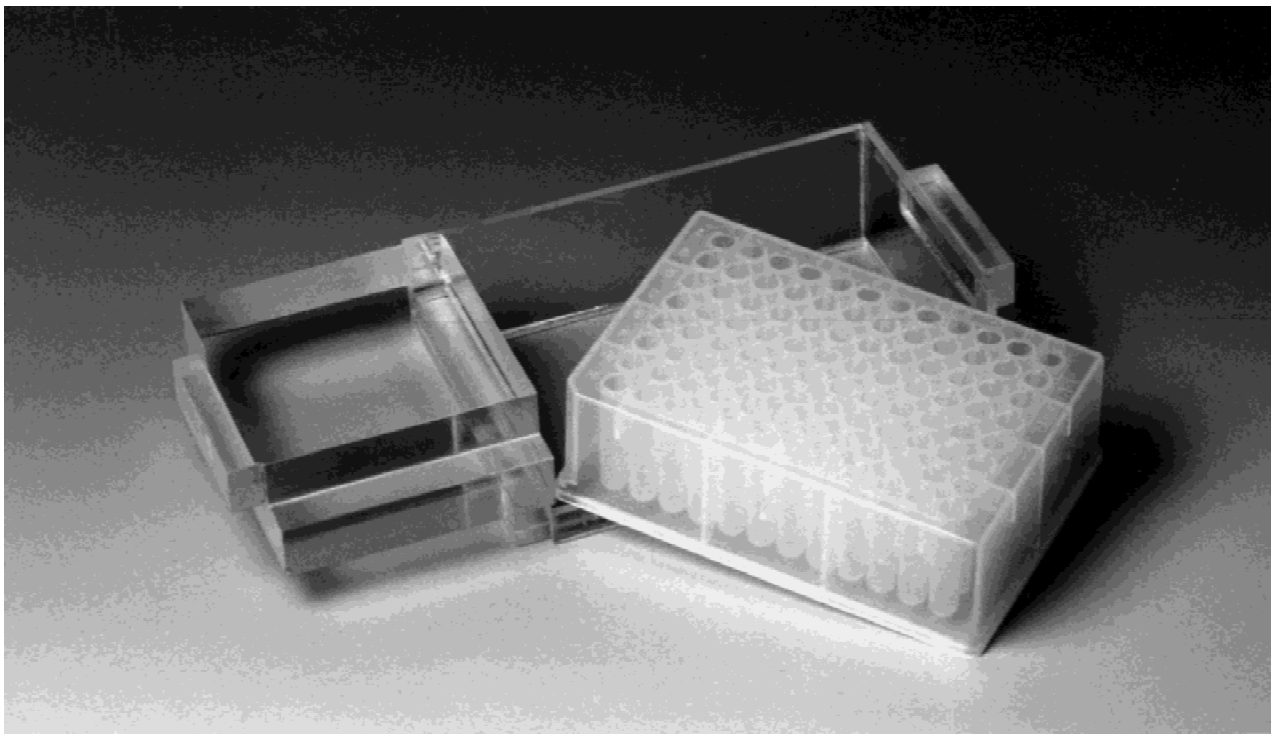

Fig. 1. Picture of the custom designed rack.

the 1 mg/ml stock solution from the mother plate were added to the wells containing buffer only. A second plate was prepared where the determination of the third log D was performed. As previously, 400 μl (2 × 200 μl) of 100 mM KH_2PO_4 at pH = 7.4 were pipetted into the analysis plate. Then, using the P200L, 100 μl of octan-1-ol were added to the analysis plate. Finally, the P1000L was used to pipette 300 μl of stock solution from the mother plate. The plate was then sealed, placed on its side in a reciprocal shaker and similarly shaken for 30 minutes. After centrifugation, the plate was put onto a Gilson autosampler from where both phases were injected directly from the wells by sampling at different heights.

Isocratic RP-HPLC

RP-HPLC, on a 100 mm long column, used acetonitrile as mobile phase A and 25 mM KH_2PO_4 with 0.2% triethylamine adjusted to pH = 3.0 with phosphoric acid as mobile phase B, at 1 ml/min. The percentage of A used was adjusted to obtain retention times of about 5 minutes.

Fast Gradient RP-HPLC

RP-HPLC, on a 50 mm long column, used 0.07% v/v formic acid in acetonitrile/water (95:5) as mobile phase A and 0.1% v/v formic acid in water as mobile phase B, at 3 ml/min. The gradient profile was such that the mobile phase ramped from 100% of B up to 100% A over 3.5 minutes and held for 1 minute. The system was then brought back to its original conditions giving a total cycle time of 5.5 minutes.

Aliquots of 10 μl of organic and 80 μl of aqueous for the Eppendorf and plate methods were injected.

RESULTS AND DISCUSSION

In the methods described, the partition coefficient was determined between octan-1-ol and pH = 7.4 buffer (i.e. physiological pH). Here, the partition coefficient is a distribution coefficient (D) and we report results as log Ds.

On analysing the steps required to perform a log P determination using the traditional shake-flask method, it was clear that a number of processes simply involved the transfer of liquids (such as transfer of samples and addition of octan-1-ol and aqueous phases). These procedures lend themselves to automation using robotic liquid handling. Manually, each partition is performed in a separate tube and each layer is then transferred to an individual vial. Such a multiplicity of tubes and vials imposed severe constraints on the assay throughput, hence, a more convenient format was sought. The use of microtitre plates was considered optimal from a number of standpoints: a number of experiments can be grouped together in a small area; once prepared, all the partitions can be performed simultaneously and modern robotic liquid handling systems are designed to work with multiple well plates. Two strategies were then considered: either to separate the layers post-partition to fresh microtitre plates or directly to inject each layer from the plate and these options are discussed below. A second rate limiting step in performing the anlaysis is the necessity to develop isocratic HPLC conditions for every compound, and rapid gradient analysis should provide a suitable generic alternative. To simplify the transfer of this method to an automated pipetting station, we standardised on the dissolution of all compounds in octan-1-ol. If subsequently any of them appear to have a negative log D, they can be repeated and dissolved in buffer (see discussion in the Eppendorf vs. plate methodology section).

Method Development

Initial attempts at transferring the shake-flask method to 96-well plate technology were confounded by cross-well leakage from the plates during shaking. This problem can be circumvented by judicious choice of microtitre plates that have cap mats specifically designed for the wells to ensure a gas and liquid-tight seal.

To streamline sample preparation and avoid the delicate and tedious task of manually separating the aqueous and octan-1-ol phases, two options were considered. Firstly, an automated liquid handling system could be used to transfer both phases into a clean plate. Although feasible through programming of the Biomek 2000 to yield two analysis plates, this adds an unnecessary separation step.

Alternatively, one could envisage a system in which an autosampler would be programmed so that the needle could be made to sample from both the bottom of the well (aqueous phase) and higher up in the well (organic phase) allowing both layers for a single sample to be injected consecutively.

Using a custom programmed Gilson 231 autosampler which allowed the needle depth to be varied, we investigated the feasibility of this latter approach. A customised rack (Figure 1) was designed to accommodate the deep-well microtitre plates being used. Then, by specifying the distance from the origin to the first well, the distance between wells and the number of rows and columns, a program was written for the rack which made the autosampler inject from each well sequentially. By looping the inject sequence, the injector returned to the same well and performed a second injection before moving on. By specifying a different value for the needle height for each of the injections, the same well could then be sampled in subsequent injections at two different depths. The sampling depths were optimised by filling the wells with 400 μl of a 1 mg/ml solution of resorufin and 400 μl of octan-1-ol to allow easy visual determination of both layers. The needle sampling depths were adjusted visually until the needle tip was approximately half way down each layer.

However, given that some drugs may be particularly lipophilic or hydrophilic, the concentrations in one layer relative to the other may be three or four orders of magnitude different, potentially leading to carry-over from one layer. In order to minimise carry-over, the autosampler can be set to wash the injection port and transfer line between injections with an external wash solvent (50/50 acetonitrile/water with 0.1% formic acid). In order to determine the optimum number of washes, 50 μl of a 1 mg/ml solution of caffeine in octan-1-ol was injected onto a RP-HPLC isocratic system followed by 50 μl of deionised water. Without washing, as expected, the carry-over of caffeine into the water injection was extensive, but it was found that by employing a 750 μl washes between injections no caffeine peak was detectable (i.e. carryover < 0.1%).

Validation

In order to validate the new approach, experiments were conducted to compare different aspects of this and the

manual methodology. Finally a direct comparison study was conducted where a series of 30 compounds were taken completely through both procedures.

The initial investigations concerned the comparison of isocratic and gradient analyses, the shaking time needed to obtain equilibrium, the reproducibility of different wells from the plate, comparison between the shake-flask and the plate methods and finally, the use of the Biomek 2000 for automated sample preparation.

Isocratic Versus Gradient Analysis

Partitioning between octan-1-ol and pH 7.4 buffer was performed on 19 compounds, using the traditional shake-flask method and the layers separated manually into 2 ml vials. These samples were then analysed by injection onto an HPLC system using both the isocratic and the fast gradient methods. The log D values were calculated as described above for each method and these results are summarized in Figure 2. An excellent correlation ($r^2 = 0.996$) between the two data sets is achieved indicating that generic fast gradient RP-HPLC conditions are suitable for this analysis. All subsequent work was carried out with generic fast gradient conditions.

Equilibration Time

To determine the optimum time required to shake the plate to ensure that equilibrium was reached, an experiment was conducted in which sampling of the octan-1-ol and aqueous phases for a partition of 3-nitroaniline was performed as a time-course. This was conducted using both a 96-well plate and Eppendorfs with sampling after 0, 10, 20, 30 and 60 minutes. The log D values were determined for each of the time points as described previously. Equilibrium was reached after just 10 minutes when the experiment was conducted in Eppendorfs. In contrast with the 96-well plate, 30 minutes were required to reach full equilibration.

Reproducibility

In changing the assay to a microtitre plate format, one concern was that the partitioning process may not be comparable across the entire plate. To demonstrate that all the wells are equivalent (i.e., the efficiency of shaking is similar), a plate was prepared according to the plate method containing 3-nitroaniline in all the wells. The plate was then injected using the Gilson autosampler and analysed using the fast gradient system.

Across rows, the standard deviation (s.d.) for the log D of 3-nitroaniline is less than 0.004, across the columns it is less than 0.005 and for the plate it is 0.001. Given the analytical error, we would generally allow for a difference of 0.2 log units between replicates, this data shows that no difference between wells is observed and that equilibration occurs in the same way across the plate.

Another 5 compounds having a spread of log D values were selected (phenolphthalein, 4-chloroaniline, ephedrine, chlorpromazine and 3-nitroaniline) and 12 replicates of each were prepared on a plate with one compound per row (Table I). Following analysis, the s.d. values are no greater than 0.02.

On the plate, both sets of data show good reproducibility indicating that transferring the shake-flask method to 96-well plate technology is feasible.

Eppendorf Versus Plate Methodology

As a final arbiter of the quality of the plate assay, the plate method was compared with the manual Eppendorf method. The log D values of 30 compounds were determined by both methods and analysed using the fast gradient system.

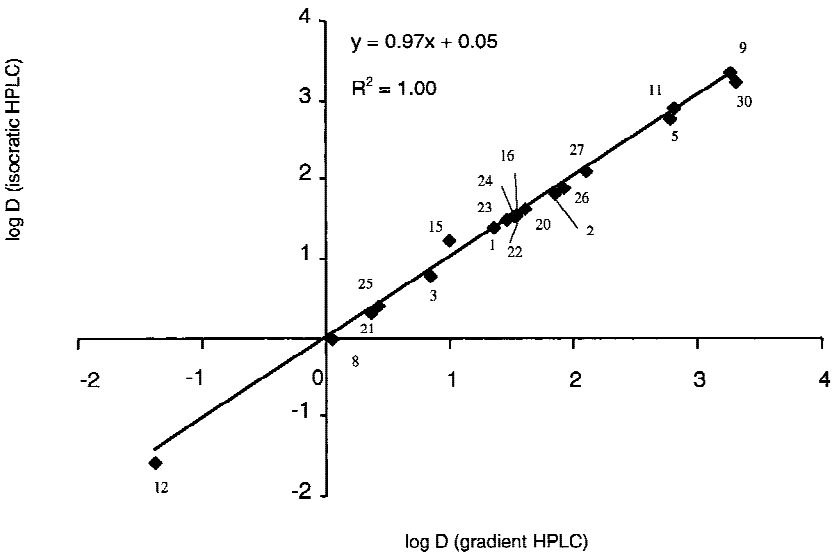

Fig. 2. Comparison between log D determined using an isocratic run and a gradient run. Compound numbers: 1: 3-nitroaniline; 2: 4-chloroaniline; 3: alprenolol; 4: alprazolam; 5: amitriptyline; 6: benzimidazole; 7: bromazepam; 8: caffeine; 9: chlorpromazine; 10: clofibric acid; 11: diazepam; 12: ephedrine; 13: fluoxetine; 14: hydroquinone; 15: ibuprofen; 16: lidocaine; 17: naphthalene; 18: naphthoic acid; 19: phenolphthalein; 20: nortriptyline; 21: paracetamol; 22: phenacetin; 23: phenobarbital; 24: 4-nitrophenol; 25: procaine; 26: quinidine; 27: quinine; 28: sulfaphenazole; 29: temazepam; 30: testosterone.

Table 1. Reproducibility of the Manual Plate Method

	Log D (pH = 7.4)	Standard Deviation (s.d.)
phenolphthalein	3.25	0.06
4-chloroaniline	1.84	0.02
ephedrine	–1.49	0.03
chlorpromazine	3.01	0.05
3-nitroaniline	1.32	0.04

The results are summarised in Figure 3. An excellent correlation between the two methdos was observed (r^2 = 0.985) demonstrating several key points. Firstly, once the shaking time is adjusted, the partitioning process is not affected by the different shape of the vessels in which the partitioning occurs. Secondly, the direct injection of the individual layers from the plate onto the HPLC yields comparable results to those obtained post-separating the layers indicating that this separation step has been rendered unnecessary. The high correlation observed between the two methods indicating that the aqueous phase is not significantly contaminated with the octan-1-ol phase even when the autosampler needle is lowered through it to reach the lower phase. Avoiding this separation step also removes errors due to phase mixing on separation. The methodology appears valid between measured log D values of +4 and –2, in agreement with the known limitations of the shake-flask method (22) for low solute concentrations. In fact, it was noted during this experiment that in order to obtain good reproducibility on compounds with negative log D values, it was better to dissolve them in buffer first rather than octan-1-ol. This maximises the concentration, although this may be limited by poor solubility in either phase. In this case, dilutions are changed accordingly. However, for the majority of pharmaceutical agents, especially where tissue penetration is important for pharmacological action, the log D values are likely to be positive making this less of an issue.

Automated Sample Preparation

The initial preparation of the sample involves adding fixed volumes of octan-1-ol and buffer. To further automate the method, a Beckman Biomek—programmed as described in the experimental section—was used to perform the repetitive pipetting steps of the sample preparation. The reproducibility and accuracy of pipetting was confirmed by preparing a plate containing only one analyte, (3-nitroaniline) and performing 48 duplicate log D determinations. Aspirating and dispensing speeds were adjusted until the data were reproducible. These gave an average log D = 1.39 (s.d. = 0.003) proving that the Biomek pipettes reproducibly. As a final validation of the method, the accuracy of the plate preparation on the Biomek, log D values for the same 30 compounds previously used before were re-determined (Figure 4). An excellent very good correlation (r^2 = 0.99) between the Biomek prepared plate and the manually prepared plate is observed.

General Discussion

The major advantage of adopting this approach is the time saving accrued through the integration of all of the assay components. By using the automated pipetting station to add the organic and aqueous phases into the 96 well plate, a complete plate of 48 samples in duplicate can be prepared in just under 2 hours. For comparison, manual preparation takes approximately twice as long for the same number of samples and by its nature consumes an analyst full-time. Furthermore, the risk of human error in pipetting a sample into the wrong well is eliminated. In analysing the samples, the ability to inject each layer directly from the plate, thus negating the layer separation step, reduces sample preparation time. Further improvements in sample preparation time could be gained by using a 96-tip pipetting system such as a Sagian Multimek to add the buffer to the plate in one step.

With a RP-HPLC cycle time of 11 minutes per log D determination (i.e. the injection of both phases), duplicate

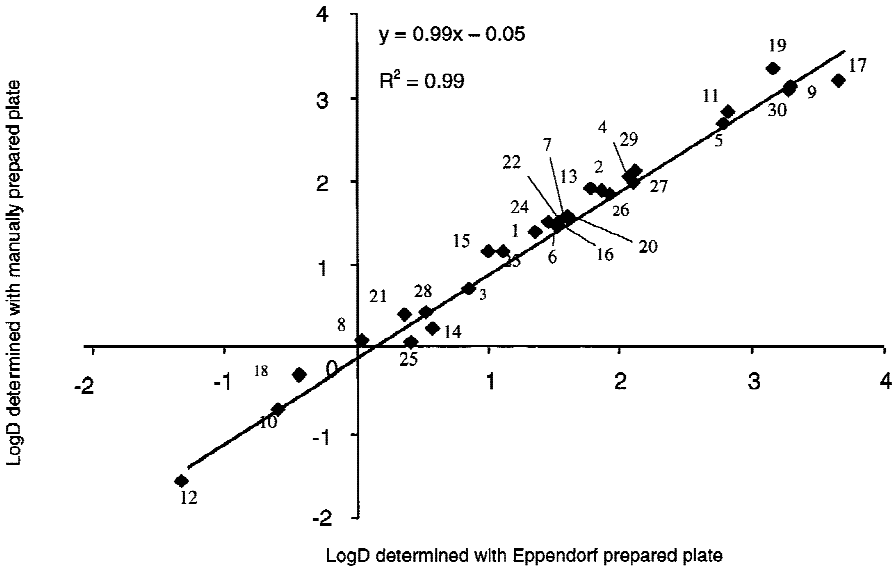

Fig. 3. Comparison between log D determined after preparing samples in Eppendorf vials and in a manually prepared plate. (Compound numbers are listed in Figure 2.)

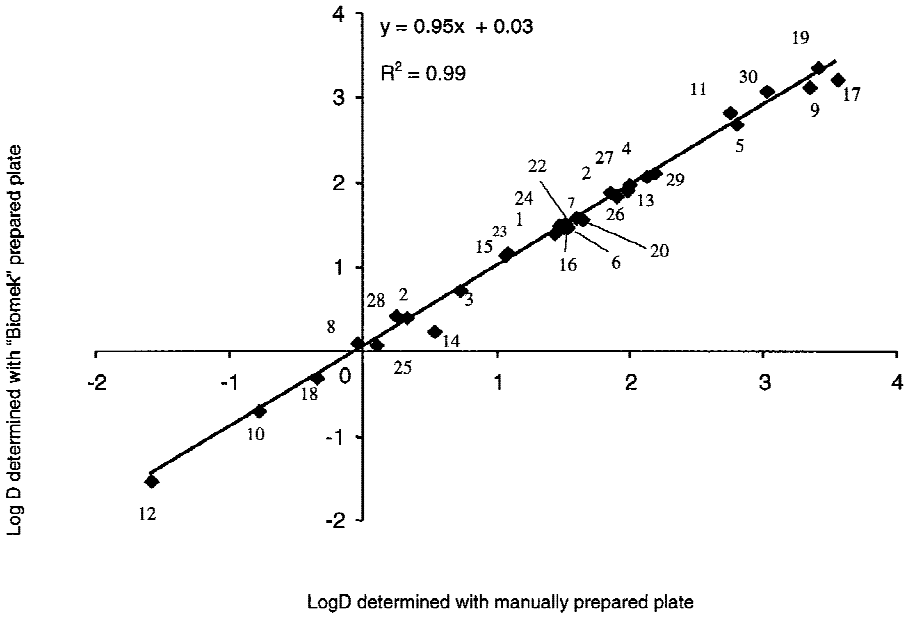

Fig. 4. Comparison between Log D determined after preparing the plate manually and on the robotic station. (Compound numbers are listed in Figure 2.)

measurements can be obtained for each sample in 22 minutes and analysis of a complete plate takes under 20 hours. This demonstrates a considerable advantage over isocratic conditions, where method development is time consuming with runs often exceeding the cycle times used for the fast gradient, and determining the log D of as few as 5 compounds per day represented a major effort. Moreover, extreme gradient conditions used for the generic fast gradient allow total flushing of the column thereby removing any trace of octan-1-ol, and avoiding perturbation of the stationary phase over time leading to variation in capacity factors.

CONCLUSION

We have demonstrated that, through application of automation technologies, the throughput of a traditional shake-flask method for determining log D may be substantially improved and manual intervention minimised. Sample preparation has been simplified and accelerated through the use of an automated liquid handler and a modified autosampler. By adoption of the 96 well plate format, preparation of 48 different compounds in duplicate is readily achieved and analysis of 192 samples (48 samples, in duplicate, each with two layers) by RP-HPLC can be achieved in less than 20 hours with the use of fast generic gradients. High throughput log D determination by direct measurement is now possible responding to the needs of a modern drug discovery effort.

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