COMPARISON OF AUTOMATED CONTINUOUS FLOW METHOD WITH SHAKE- FLASK METHOD IN DETERMINING PARTITION COEFFICIENTS OF BIDENTATE HYDROXYPYRIDINONE LIGANDS

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ABSTRACT

The partition coefficients (K_{part} , in octanol/water system) of a range of bidentate ligands containing the 3-hydroxypyridin-4-one moiety were determined using shake flask and automated continuous flow methods (filter probe method)). The shake flask method was used for extremely hydrophilic or hydrophobic compounds with a K_{part} values greater than 100 and less than 0.01. For other ligands which possess moderate lipophilicity (K_{part} values between 0.01-100) the filter probe method was used. Also the partition coefficient of four ligands with moderate lipophilicity was determined by shake flask method in order to check comparability of these two methods. While the shake flask method was able to determine either extremely hydrophilic or hydrophobic compounds efficiently, the filter probe method was unable to measure such K_{part} values. Although, determination of the K_{part} values of all compounds is possible with the classical shake-flask method, the procedure is time consuming. In contrast, the filter probe method offers many advantages over the traditional shake-flask method in terms of speed, efficiency of separation and degree of automation. The shake-flask method is the method of choice for determination of partition coefficients of extremely hydrophilic and hydrophobic ligands.

Key words: Partition coefficients, 3-Hydroxypyridin-4-one, Shake flask method, Filter probe method, Bidentate ligand.

INTRODUCTION

The lipophilicity of drugs plays an important role in their biological action. This property determines the fate of a drug in the body by governing the absorption, distribution, storage and elimination processes. The n-octanol/water partition coefficient (K_{part}) is a generally accepted physico-chemical parameter for characterization of lipophilicity.

A variety of methods have been used for determination of K_{part} values. Traditionally, the shake-flask technique has been the method of choice (1). The method involves mixing of an aqueous solution of the known concentration of a compound with a known volume of organic phase and to allow the system to attain equilibrium. When equilibrium is established, either the concentration remaining in the aqueous phase or the concentration in the organic phase is measured (usually spectro-photometrically) and the K_{part} values are calculated. There are a number of disadvantages with this technique (2,3,4) and skilled analysts are required in order to achieve reproducible results. Furthermore, the necessity to replicate measurements is tedious and time-consuming. The system is unsuited to volatile substances and the risk of emulsion formation can render the phase separation difficult. The technique also suffers from difficulties if the compound is poorly soluble in either of the solvent phases, a factor which leads to inaccurate and erratic results.

Attempts have been made to automate the shake-flask method by using a counter-current method (5). However, the method is complex and cleaning of the associated apparatus is tedious. An apparatus known as the AKUFVE (Swedish abbreviation for apparatus used for continuous measurement of partition coefficient in solvent extraction) was developed by Reinhardt and Rydberg (6) for determination of

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distribution and stability constants of metal complexes. The method was later adopted by Davis *et al* (7) for determination of the partition coefficient data. The system is based on a high-speed centrifuge separation of two well-mixed phases which are continuously fed into the centrifuge from a mixing chamber. Control of the input and output rates and the speed of centrifuge are critical for the optimum operation of the equipment. Measurement of the concentration in both phases was permitted by the use of online detection methods such as ultraviolet spectroscopy and radiometric techniques. The technique has been successfully used to obtain K_{part} data and to investigate the effect of factors such as temperature and pH on the partitioning process (7). However, there are disadvantages with this method. The apparatus is expensive and demands a skilled operator. After each experiment, the apparatus has to be dismantled and cleaned, and as a result it is not suitable for the routine determination of a large number of partition coefficient values. A further disadvantage is that large volumes (500 ml) of each phase are employed and hence large quantities of compounds are required. While Anderson *et al*. (8) developed a miniature version of the AKUFVE, difficulties were encountered with the complete separation of the n-octanol/water system.

Another method used for determination of the partition coefficient data is reversed-phase chromatography either in the form of thin-layer (9) or high performance liquid chromatography (10). While a major advantage of these techniques is the possibility of determination of K_{part} value of impure samples, they are indirect and are only applicable within limited range of pH and solute concentration (8).

In an attempt to develop an alternative automated method which is simple and reliable to operate, a filter probe method was introduced. This method was first described by Cantwell and Mohammed (11) for potentiometric acid-base titration in the presence of an immiscible solvent and subsequently was modified by Tomlinson (12) for the general determination of partition coefficient. This method has overcome many difficulties associated with the shake-flask method. In this study, a similar filter probe method with additional improvements has been employed for determination of partition coefficient values. A range of bidentate ligands which have potential for treatment of iron overload in thalassaemic patients have been investigated by this method.

MATERIALS AND METHODS

The 3-hydroxypyridin-4-ones used in the present study were synthesised as previously described (15). All other chemicals were obtained from Aldrich (Gillingham, UK).

Determination of partition coefficients of bidentate ligands

Partition coefficients were determined using two methods:

(a) automated continuous flow method (filter probe method);

(b) shake-flask method.

MOPS [3-(N-morpholino)propane sulphonic acid] buffer (50 mM, pH 7.4, prepared by the use of distilled water) and n-octanol were two phases which were employed in this study and each phase was pre-equilibrated with the other before use due to the limited solubility of water in n-octanol (2.3 M) (16). All solutions were stored and manipulated at 25 ± 0.5°C.

Determination of K_{part} of bidentate ligands by the filter probe method

Apparatus: The system consisted of an IBM compatible PC running, the "TOPCAT" program (13,14) (Fig. 1) which was controlled by both Metrohm 665 Dosimat autoburette and a Pye-Unicam Lambda 5 UV/V spectrophotometer, and performed all calculations of partition coefficients (Fig. 2). All K_{part} values were determined by the use a flat-based glass vessel (250 ml) equipped with a sealable lid at a constant temperature (25 ± 0.5°C). The buffer (100 µL) was circulated through a spectrophotometric flow-cell, which was returned to the mixing chamber by the aid of a peristaltic pump at a flow rate of 1 ml/min.

The filter probe consisted of a polytetrafluoroethylene (PTFE) plunger and a hydrophilic (Schleicher and Sehueu 589/3) blauband filter paper and it was ensured that none of the hydrophobic phase and even small droplet sizes generated by stirring permeated the filter device. To ensure effective partitioning of the sample, continuous rapid stirring were employed in a way that an emulsion of two immiscible phases was formed. In the case of the use the filter probe device, it was necessary to wet a filter paper (with water) prior to its attachment to the plunger. This ensures that the paper is not wettable by the organic solvent and thereby filters the small droplets of n-octanol

out of the aqueous phase successfully. A new filter paper was used for each measurement. The pumping rate was set at 1 ml min^{-1}, since higher rates tended to introduce gas bubbles into the system leading to inaccurate absorbance readings and also increase in the risk of drawing of droplets of n-octanol into the probe (11).

Method: Moderately hydrophilic compounds (n = 26) were measured by the following method. A known volume (normally 20-100 ml) of MOPS buffer was employed in the flat-based glass-mixing chamber. A baseline absorption value of the solution was used as a reference absorbance. A 10^{-4} M solution of the ligand was prepared in the aqueous phase (typically 40 ml) to give an absorbance between 1.5-2.0 at the preselected wavelength (~280 nm). Upon commencement of the computer program, absorbance measurements were automatically recorded at the preselected time intervals, usually 1 second. When the absorbance readings were stabilized as determined by the computer from equilibrium conditions selected by the operator (typically a constant absorbance is where the absorbance changes by less than 0.002 absorbance units over a minimum of 10 minutes), from the automatic dispenser a suitable volume of n-octanol was added to the aqueous phase. Absorbance readings were subsequently recorded until the system reached to the equilibrium again, at which point a further aliquot of n-octanol was added. This cycle was repeated at least for five times that n-octanol were added (Fig 3). At each stage of the addition of n-octanol, the corresponding partition coefficient was calculated using the following formula:

$$K_{part} = \frac{A_0 - A_1}{A_1} \times \frac{V_w}{V_0} \qquad \text{(Eq. 1)}$$

Where

A_0 = Initial absorbance

A_1 = Absorbance at equilibrium after addition of n-octanol

V_w = Volume of MOPS buffer

V_0 = Total volume of n-octanol which was added to glass vessel

Finally, a mean partition coefficient value and standard deviation were calculated

Determination of K_{part} values of bidentate ligands using the shake-flask method

In spite of disadvantages associated with shake-flask method which outlined in introduction, the method was adopted for the determination of K_{part} values of five compounds with extremely hydrophilic and hydrophobic properties (compounds **27-31**).

The partitioning of compounds with K_{part} values less than 0.01 was monitored by an aqueous layer/n-octanol volume ratio of 1:100 as previously reported (17). A solution of ligands with concentration of 10^{-4} M was prepared in MOPS buffer and the absorbance of the solution was measured in the ultraviolet region at a wavelength of approximately 280 nm using the buffer as a blank. A 10 ml sample of the solution was stirred vigorously with 1000 ml of n-octanol in a glass vessel for 1 h. The two layers were separated by centrifugation at 3000 rpm for 5 minutes. An aliquot of the aqueous layer was then carefully removed by a glass Pasteur pipette ensuring that the sample was not contaminated with n-octanol. The absorbance of the sample was measured as mentioned above and the partition coefficient, K_{part}, was then calculated using the following formula:

$$K_{part} = \frac{A_1 - A_2}{A_2} \times \frac{V_w}{V_o} = \frac{A_1 - A_2}{A_2} \times \frac{1}{100} \qquad \text{(Eq. 2)}$$

where

A_1=absorbance reading in the aqueous layer before partitioning

A_2=absorbance reading in the aqueous layer after partitioning

V_o=volume of 1-octanol layer used in partitioning

V_w=volume of aqueous layer used in partitioning

For each sample, the experiment was repeated at least four times for the calculation of a mean K_{part} value and standard deviation.

For highly hydrophobic compounds which possess K_{part} values greater than 100, an aqueous layer/n-octanol volume ratio of 100:1 was used. The partitioning and calculation of K_{part} were carried out as described above. The K_{part} values for compounds **19, 22, 13** and **2** which possess moderate hydrophilicity were also measured by this method.

RESULTS AND DISCUSSION

An example of results obtained by filter probe method is shown in table 1 for compound **17**. The K_{part} values for other bidentate ligands are shown in table 2. The ligands K_{part} values

Table 1. An example of the results obtained for compound **17** using the filter probe apparatus.

Reading	Volume (ml)	O.D.	Time (sec)	K_{part}
1	0.000	1.540	621.70	0.000
2	5.000	1.274	870.02	1.670
3	10.000	1.083	952.36	1.688
4	15.000	0.940	994.76	1.702
5	20.000	0.832	888.14	1.702
6	25.000	0.745	1299.3	1.707
7	30.000	0.674	737.92	1.713
8	35.000	0.616	855.13	1.714
9	40.000	0.567	909.18	1.716

Total time = 135.476 min, K_{part} = 1.702 ± 0.015 (n = 8), log K_{part} = 0.231 ± 0.004 (n = 8)

obtained by shake flask method are presented in table 3.

Titration parameters set on 665 Dosimat

Initial volume of MOPS buffer in titration vessel, 40 ml.

Total volume of 1-octanol for complete titration, 40 ml.

Aliquot size set on 665 Dosimat, 5ml.

Dispensing rate set on 665 Dosimat, 1 ml/min.

Refilling rate set on 665 Dosimat, 20 ml/min.

Equilibrium conditions currently set

Equilibrium is assumed if absorbance changes by less than 0.002 units per reading based on linear regression over 100 readings giving a slope of 0.00002. Time delay between readings is 5 seconds.

Special parameters set

Monitor wavelength = 279.4 nm

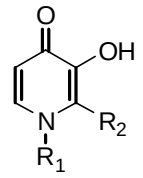
Background absorbance at 279.4 nm = 0 AU

Spectra were taken from 190 to 350 nm (1 nm interval), when the absorbance changes were by 0.01 AU increments.

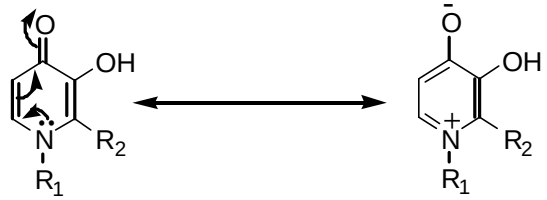
Table 4 shows the K_{part} values of four bidentate ligands, **19**, **22**, **13** and **2** which were determined by both methods. There are no significant differences between results obtained by two methods. In general, an increase in the length of the alkyl chain on the heterocyclic nitrogen resulted in an increase in the K_{part} value of ligands. As expected, the M series (which possess a 2-methyl substituent) have lower K_{part} values in comparison with those of the E series (which possess a 2-ethyl substituent). The charged molecules at pH 7.4 (**2**, **3**, **27** and **28**) had low values. Amongst the compounds **1** (K_{part} = 0.02) and **31** (K_{part} = 350) of the neutral; compounds were chelators with lowest and highest K_{part} values, respectively. Surprisingly, the unsubstituted pyridinones (**9** and **15)** had higher values than the corresponding N-methyl pyridinones (**6** and **13**).

R_2= CH_3 M series

R_2= C_2H_5 E series



A likely explanation for this observation is the change in the balance of the relative contribution of the canonical forms (**a** and **b**) for two classes: when R_1 is an alkyl group, the resonance form **b** is stabilized by electron donation of alkyl group. Such stabilization is not possible for non-alkylated pyridinone. As a result the dipoles of the N-alkyl pyridinones **6** and **13** are larger and therefore more hydrophilic than the non N-alkylated pyridinones **9** and **15**.



(a) (b)

In general the filter probe technique has several advantages over the shake-flask method. The automated addition of aliquots of n-octanol from the dosimat ensures greater accuracy and reproducibility of measurements. The use of a computer program TOPCAT (figure 1) to control and monitor the system means that the method is considerably less tedious and time consuming. However, difficulties arose in attempts to determine K_{part} values of compounds with a K_{part} value greater than 100. For this type of compound, the absorbance of the aqueous layer after partitioning was found to be low owing to the high solubility of the molecule in the n-octanol layer. As a consequence, the obtained results were unreliable. Also for compounds which are not sufficiently soluble in the aqueous phase, the above method is not useful because this technique is designed to process compounds which are soluble in the aqueous phase at the onset of the experiment.

Reproducible values for the partition coefficient values of highly hydrophilic compounds (K_{part} <0.01) are also difficult to obtain by the use of

Determination of partition coefficient

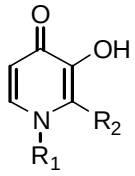


Table 2. Ligand K_{part} values obtained by filter probe method (n = number of determinations).

Ligand	R_1	R_2	K_{part}	n
1	$CH(CH_2OH)_2$	Me	0.020± 0.002	4
2	$CH_2CH_2NH_2$	Me	0.032± 0.001	5
3	$CH_2CH_2CH_2COOH$	Et	0.042± 0.001	5
4	CH_2CH_2OH	Me	0.080± 0.002	5
5	$CH_2CH_2CH_2OH$	Me	0.130± 0.001	5
6	Me	Me	0.170± 0.010	6
7	$(CH_2)_4OH$	Me	0.180± 0.001	7
8	CH_2CH_2OH	Et	0.220± 0.010	5
9	H	Me	0.320± 0.010	5
10	$CH_2CH_2OCH_3$	Me	0.390± 0.010	5
11	Et	Me	0.49± 0.01	5
12	$(CH_2)_4OH$	Et	0.52± 0.01	5
13	Me	Et	0.62± 0.01	5
14	$CH_3CH=CH_2$	Me	1.07± 0.02	5
15	H	Et	1.11± 0.03	8
16	Pr	Me	1.51± 0.04	10
17	Et	Et	1.70± 0.02	10
18	$CH_3CH=CH_2$	Et	2.95± 0.03	5
19	$CH_2CH_2CO_2C(Me)_3$	Me	4.70± 0.07	5
20	$CH_2CH_2CH_2CH_3$	Me	5.05± 0.02	8
21	$(CH_2)_3CO_2C(Me)_3$	Me	9.00± 0.10	8
22	C_6H_5	Me	10.20± 0.30	6
23	$(CH_2)_4CH_3$	Me	17.41± 0.20	10
24	$(CH_2)_3CO_2C(Me)_3$	Et	32.96± 0.30	5
25	$(CH_2)_5CH_3$	Me	79± 5	6
26	$(CH_2)_3CO_2C(Me)_3$	Me	84.5± 5*	10

*This value is the K_{part} value of iron (III) complex of compound **26** (15).

Table 3. Ligand K_{part} values obtained by shake-flask method (n = number of determinations).

Ligand	R_1	R_2	K_{part}	n
27	$CH_2CH_2CO_2H$	Me	0.0012± 0.0002	4
28	$CH_2CH_2CH_2CO_2H$	Me	0.0018± 0.0002	4
29	$(CH_2)_3CO_2C(Me)_3$	Et	102± 5*	5
30	$(CH_2)_5CH_3$	Et	149± 6	4
31	$(CH_2)_7CH_3$	Me	350± 10	4

*This value is the K_{part} value of iron (III) complex of compound **28** (15).

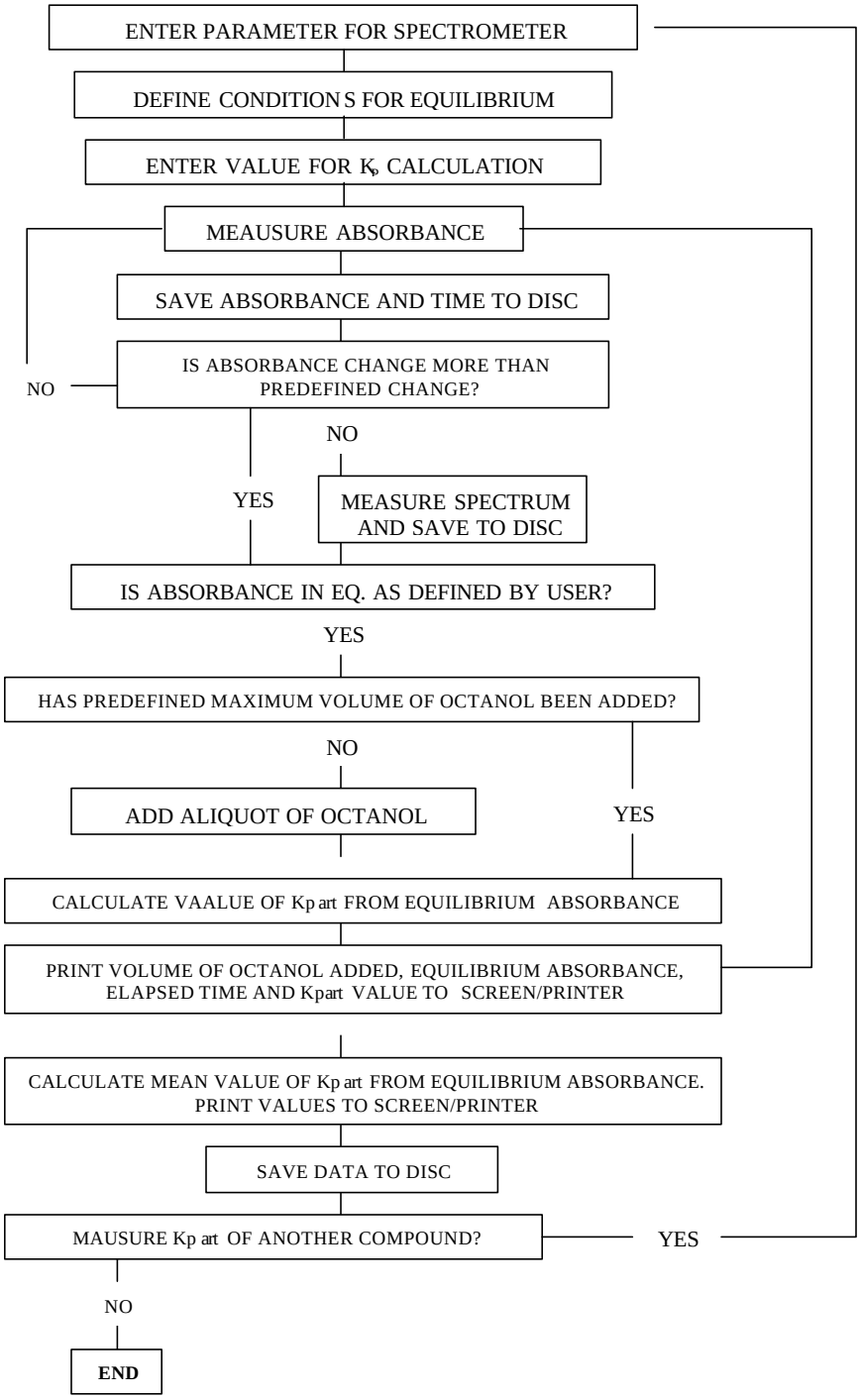


Figure 1. Flowchart of TOPCAT program.

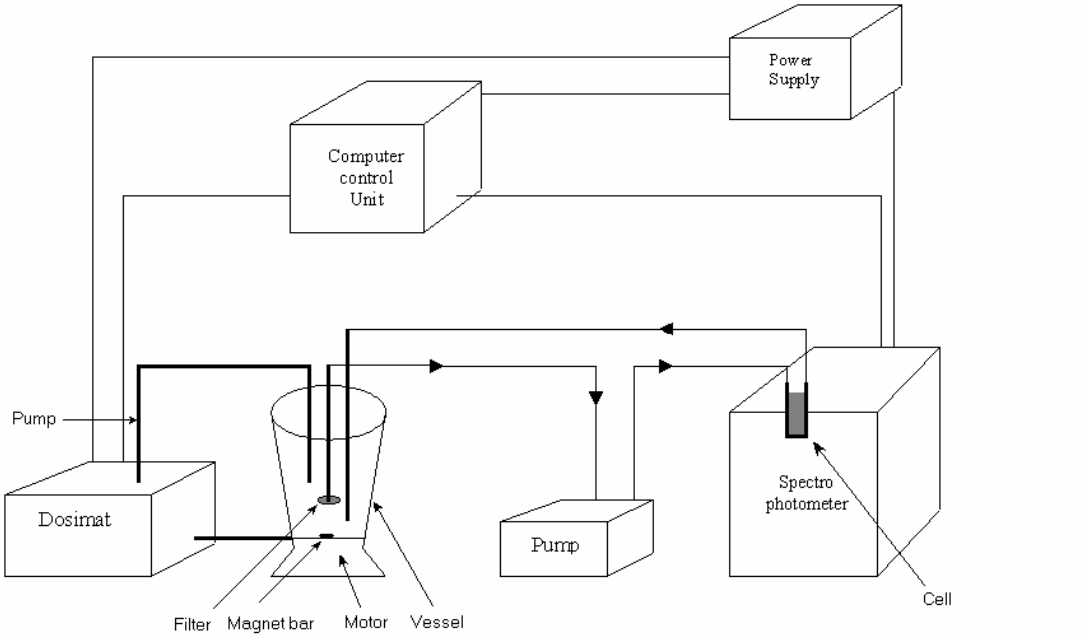


Figure 2. Diagram for auto-partition coefficient.

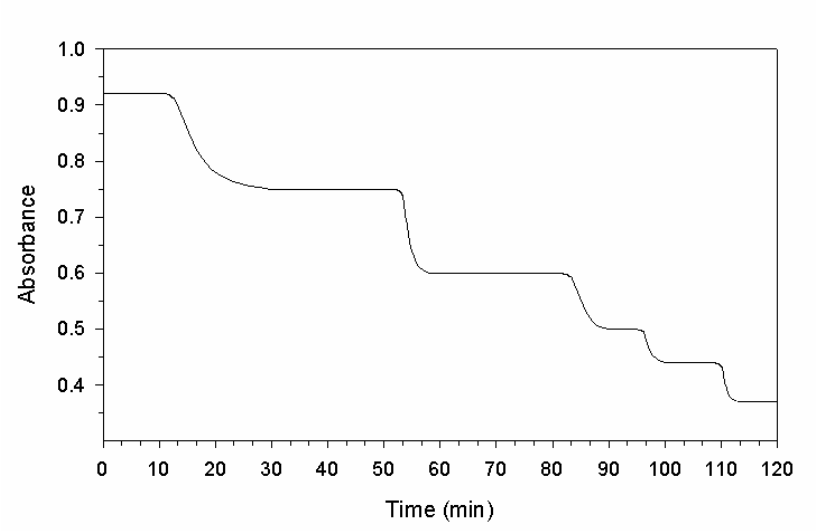


Figure 3. The decrease in absorbance of aqueous phase caused by the addition of aliquots of 1-octanol for 2-ethyl-3-hydroxy-6-methyl-4(1*H*)-pyranone

Table 4. The K_{part} values of compounds **19**,**22**,**13** and **2** obtained by both filter probe and shake-flask methods.

Readings	K_{part} (19)		K_{part} (22)	
	Filter probe method	Shake-flask method	Filter probe method	Shake-flask method
1	4.72	5.60	11.19	10.17
2	4.75	4.99	11.34	10.63
3	4.80	4.75	10.95	11.50
4	4.65	5.40	11.28	11.31
5	4.60	4.86	11.24	10.40
Mean	4.70± 0.07	5.10± 0.33	11.20± 0.13	10.80± 0.52
Reading	K_{part} (13)		K_{part} (2)	
	Filter probe method	Shake-flask method	Filter probe method	Shake-flask method
1	0.627	0.701	0.031	0.039
2	0.622	0.605	0.033	0.038
3	0.631	0.690	0.033	0.036
4	0.620	0.690	0.032	0.042
5	0.621	0.720	0.032	0.026
Mean	0.624± 0.004	0.690± 0.020	0.032± 0.001	0.036± 0.006

the filter probe method. The reason for this phenomenon is very low partitioning of compounds into n-octanol layers which leads to small changes in absorbance of the aqueous phase. With the shake-flask method this problem can be minimized by the change of the ratio of two phases, for instance 1:100. However, only a limited range of phase volume ratios is possible with the filter probe method, since separation of phases becomes increasingly difficult with large phase volume ratio due to the limitation of volume of the glass to 250 ml.

In conclusion, the filter probe method has been shown to be an efficient and rapid method for measurement of K_{part} values of these compounds. The method is simple and requires little capital investment and offers many advantages over traditional shake flask technique. Results obtained by this method showed excellent concordance with those obtained by shake flask method, which demonstrates the validity of the filter probe method.

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