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VALIDATION OF AN AUTOMATED SAMPLING SYSTEM WITH FRANZ DIFFUSION CELLS

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ABSTRACT

Transdermal penetration studies "in vitro" are tedious and time consuming, and sometimes require complex sampling schedules. If automatic equipment is used, full validation is mandatory. The aim of this study was to validate an automated "in vitro" sampling system using modified Franz diffusion cells. The reproductibility of sample and replace volumes was assessed. The dilution effect induced by the replaced volume was also studied and some equations in order to correct this factor were propossed. Ketorolac trometamol has been used as a model drug in this study.

INTRODUCTION

Transdermal delivery of drugs has several advantages in therapy compared with oral or parenteral administration[1]. The most important are: 1) plasma levels in steady state condition can be reached, avoiding peaks and troughs to

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be present, 2) there is no influence of gastrointestinal tract on absorption 3) first pass effect is avoided 4)improvement of compliance of the treatment[2]. However, this attractive route of administration is limited to potent drugs with special properties such as: low molecular weight, good hydrophilic and lipophilic balance, proper pKa, and high apparent partition coefficient.

Because of the great interest in this route of administration and the difficulties involved in the measuring of drug penetration across the skin, a variety of systems have been developed "in vitro" to mimic the transdermal penetration process "in vivo" [3]. The Franz cell is one of the most versatile systems as it can be used to test a variety of formulations, including those which incorporate volatile compounds. It closely simulates the situation"in vivo" since the membrane is exposed to ambient conditions. Because of these advantages, Franz cells remain a popular method for "in vitro" transdermal drug diffusion testing.

These cells consist of two chambers separated by a membrane whose specifications are dictated by the purpose of the assay and the physicochemical properties of the drug. The drug formulation is placed in the donor chamber. Samples are withdrawn from the receptor chamber at different times to measure the amount of the drug that crosses the membrane.

Typically a test may include 6 to 12 cells with up to 8-25 sampling intervals. However, manual sampling requires more than one person to achieve simultaneous withdrawal from the 6 or 12 cells and to perform 24h assay. In addition, because of the potential for human error in the extraction and reposition steps, variations may occur.

Automated operation of percutaneous absorption systems have been slow to develop. That developed by Higuchi et al.[3], which consists of a new diffusion cell connected to the micro flow cell of the spectrophotometer. In this type of system the fluid flows out of the lower chamber of the cell. Jamoulle et al. have reported a new automated system based on a flow-through diffusion cell[4]. Hanson et al. have launched another automated sampling system (named Microette), which has the advantage of being compatible with Franz cells.

This automated system withdraws samples from the Franz cells and replaces fresh medium automatically (see Fig 1). This mechanical device has

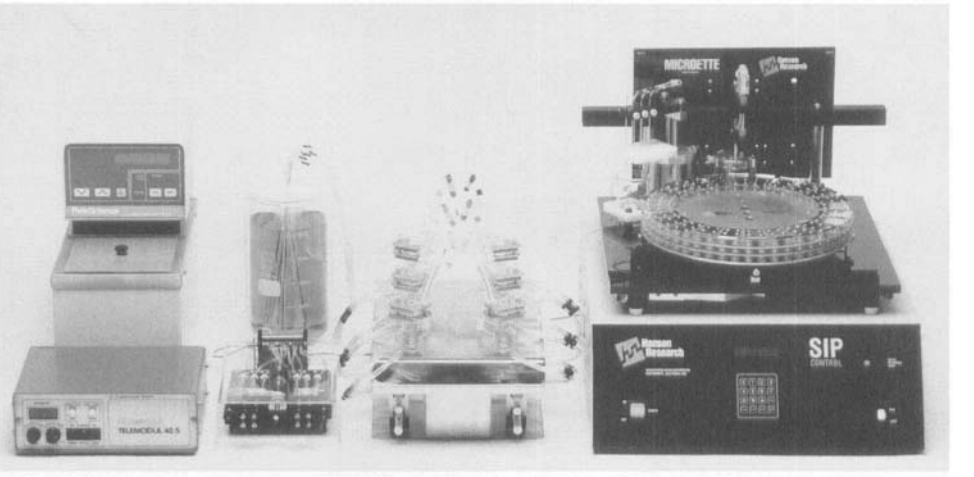

FIGURE 1

Transdermal Diffusion Cell Autosamplers

three main advantages:

1- Simultaneous sampling from 6 or 12 Franz cells.

2- Researchers are able to fit the sampling protocol to their own requirements and are not constrained by the working time table in the laboratory.

3- Precision and accuracy can be determined by measuring the sample volume and volume replaced.

The purpose of this study was to quantify the error associated with the process of extraction and replacement using the automated sampling device (Microette) in a diffusion test.

MATERIAL AND METHODS

Materials

Dialysis membrane Visking (Fungilab), milliQ water, ketorolac trometamol.

Apparatus

Metler Balance (AT100), HPLC (Hewlet Packard 1090), Microette Programme.(81-800-10B (Fig 1)).

Description of Microette 81-800-10B

1.- A set of six Franz cells are placed in a magnetic cell drive calibrated in rpm. The Franz cell modification (see Fig 2) allows for insertion of the automatic sample probe, and the input capillary port for the receptor.

The level probe allows maintence of a constant volume in the cell, wasting the extra-volume corresponding to the difference between withdrawn and replaced volume, after each sampling withdrawal.

The helix placed over the magnetic stirrer provides improved cell mixing. The cell is jacketed to maintain constant temperature (a) (see Fig 3).

Cells volume: 12.45 ± 0.05 ml, diffusional area 1.767 cm^2.

Calculated volume of the stirrer and helix: 0.8 ml.

Receptor volume = 11,65 ± 0,05 ml.

2.- The circulating bath (b) (see Fig 3) connected to the replace beaker and to the cell drive provides a constant temperature during the assay.

3.- The replacement medium is contained in the beaker (c) (Fig 3).

4.- The syringe pump (d) (see Fig 3) takes medium from the beaker and delivers it into the Franz cell through the receptor medium replace port (see Fig 2).

5.- The sampling process is carried out by means of a sampling probe which is inserted into the Franz cell at the sampling position (see Fig 2). Simultaneously the sample delivery end of the probe is inserted into the collector vial on the carrousel.

Vacuum withdraws the sample into the vial and the syringe pump introduces replacement media. Pressure then purges the sample line to avoid carry-over. Since replacement volume is always greater than sample volume, the level probe withdraws the difference between volumes after each sample withdrawal. The waste volumes are collected in the dilution flask (e). The delivery end of the sample probe is removed from the collection vial (f), then a cap is placed on the filled vial to avoid liquid evaporation and finally the carousel moves to the next sampling position.

Methods

A.- Gravimetric Method

Four assays were performing using distilled water. The sample volume withdrawn was determined by taring the receptor vials and reweighing them

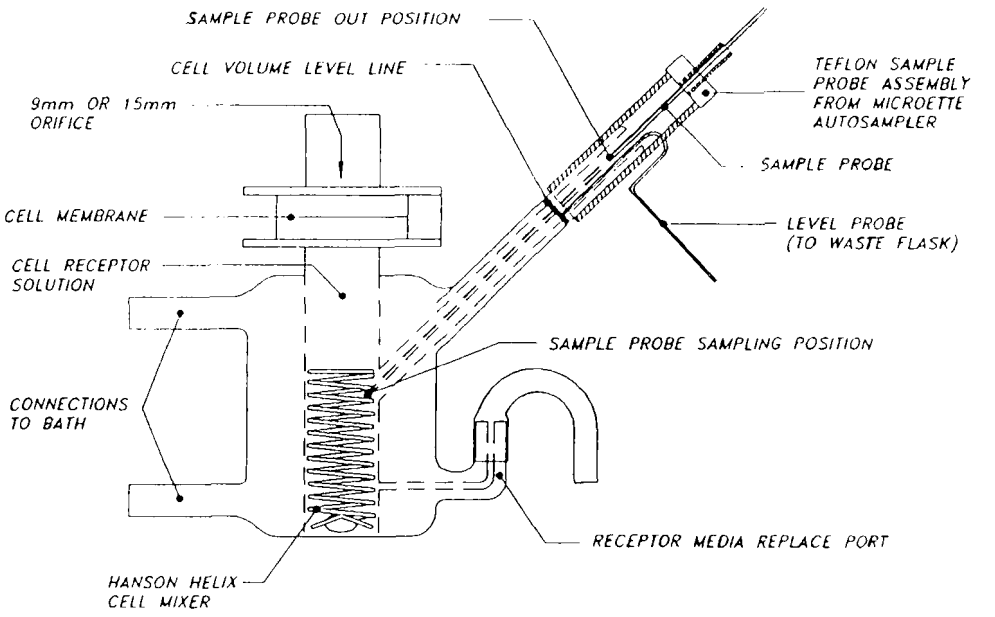

FIGURE 2

Modified Franz Diffusion Cell

again after the test. The difference between weights was equivalent to the volume withdrawn. The R.S.D obtained measured the accuracy of the volume sample recollected in the vials.

In the same way, the tubes that transport fluid from the syringes to the receptor media replacement port, were disconnected from the cells and introduced respectively in six tared vials. After each sampling withdrawal the vials were again weighed and the difference between them was established as the volume replaced. The results obtained indicate the accuracy of the syringe pump delivering the replacement volume into the cell Franz.

The test were performed using seven sampling withdrawal for each assay. The interval times between sampling points was 5 min in order to minimice posible evaporation. It is important to note, that despite using caps, some evaporation was noticed. We determined a decrease in weight of a approx. 5% in vials filled with water and stopered with the evaporation caps,in 24 hours.

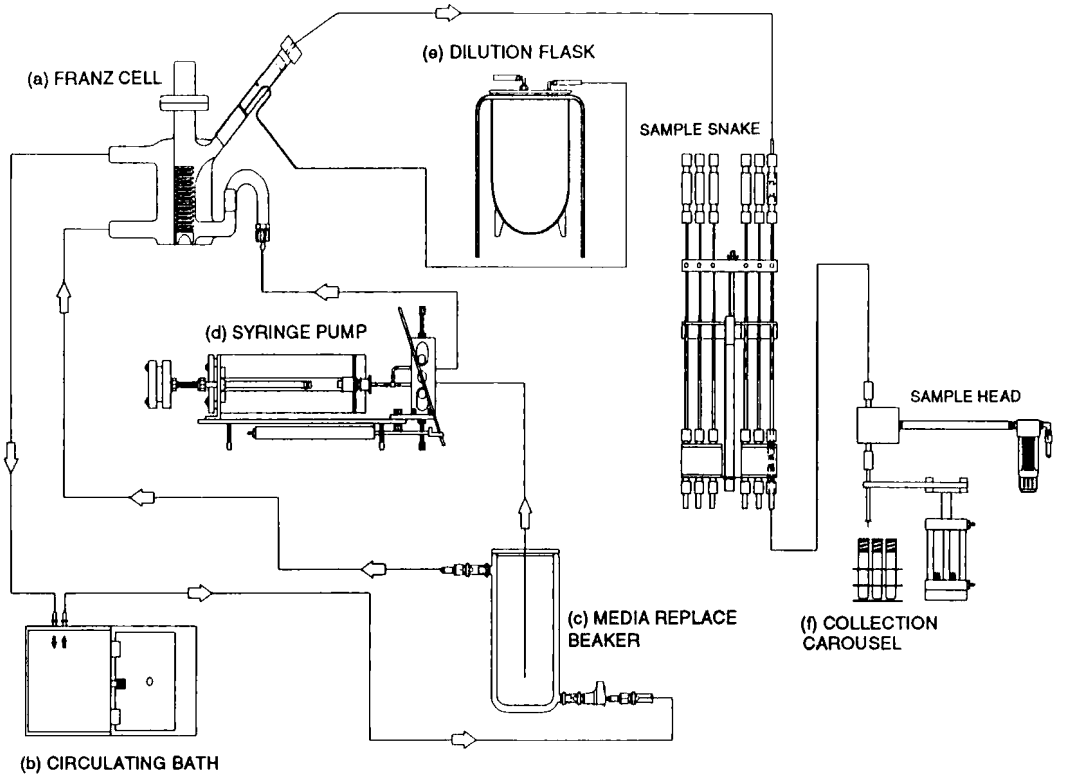

FIGURE 3

Fluid Line Installation

B.- Validation by Chemical Analysis

The cells were manually filled with a known concentration of ketorolac trometamol, and immediately connected to the system. MilliQ water was placed in the media replace beaker. The volume withdrawn at the different sampling times was analyzed by HPLC in order to confirm that a dilution process during by the automated sampling periode takes place.

Experimental results were compared with theoretical results obtained from a deduced equation (Eq. 11).

The sample volume recollected in the vials were controlled as was described in section A.

RESULTS

A.- Gravimetric Method

Four tests with 7 sampling withdrawal using 6 cells were performed. The withdrawn volume and replace volume were measured for each assay. The results shown in Table 1 are expressed as the mean and R.S.D of the 6 cells at each sampling point.

Two way analysis of variance (multifactor ANOVA) 95 percent confidence was applied to reveal significans differences. The results are shown in table 2.

The results indicated that there were statistically significant differences ($p < 0.05$) from the sample volume with respect to the sampling time and the test number, as well as the replacement volume with respect to the sampling point.

The statistically different sampling points are listed in table 3.

a) The difference in the first point is a consequence of the methodology used in the assay. To control the replacement volume, the tube from the receptor media replace port (see fig 2) was disconnected and introduced in a vial. This disconnection caused a drop of repostioning medium to be lost from the extreme of the tube and is the primary reason for the first low sample point. However, this is not important because during the normal running of the microette the tube is connected correctly with the receptor media replace port.

b) Although sample point # 7 showed a 1.1% deviation from the mean it represented only a 2 μl volume difference which was considered to be irrelevant.

The results which were statistically different are shown in table 4.

Both test numbers 2 and 3 were different from the mean by averages of 0.69 (1.3 μl) and 0.77 (1.5 μl), respectively. However, there differences are insignificant and be considered irrelevant.

B.- Validation by Chemical Analysis

The next table (Table 5) shows the mean of the sample volume of the 6 cells in each sampling point for each of the 3 tests performed. The RSD for each sampling point indicates the variation in the sample volume between the 6 cells.

The low RSD values are indicative of good automated sampling uniformity for the microette system

TABLE 1

	Sampling Point	Mean (ml) sample vol.	RSD %	Mean (ml) replace vol	RSD %
Test 1	1	0.192	3.22	0.190	4.06
	2	0.191	1.10	0.206	2.39
	3	0.188	2.24	0.202	1.31
	4	0.190	0.95	0.200	2.62
	5	0.190	1.37	0.203	1.53
	6	0.189	1.47	0.198	2.02
	7	0.193	0.95	0.200	1.09
Test 2	1	0.187	1.21	0.192	2.38
	2	0.188	1.51	0.200	1.01
	3	0.189	1.91	0.201	1.70
	4	0.189	0.58	0.200	0.81
	5	0.192	1.00	0.202	1.44
	6	0.188	1.01	0.200	1.54
	7	0.192	0.50	0.203	1.97
Test 3	1	0.189	0.96	0.192	3.63
	2	0.192	1.38	0.199	1.77
	3	0.191	2.26	0.201	0.82
	4	0.191	2.17	0.199	2.43
	5	0.193	1.48	0.200	0.82
	6	0.194	2.61	0.201	0.97
	7	0.194	0.47	0.203	1.39
Test 4	1	0.190	0.86	0.189	3.01
	2	0.191	0.43	0.201	1.14
	3	0.190	1.01	0.201	1.31
	4	0.191	1.53	0.202	0.53
	5	0.191	0.67	0.201	1.31
	6	0.191	1.13	0.202	0.82
	7	0.192	1.05	0.201	0.66

TABLE 2

	Source of variation	Sum of squares	d.f	Mean square	F ratio	Sig. level
Variance of sample volume	Sample point	1.9-E004	6	3.1-E005	3.99	0.0010
	Test nr.	1.6-E004	3	5.5-E005	6.93	0.0002
	Interaction*	2.0-E004	18	1.1-E005	1.39	0.1437
	Residual	0.00111	140	7.9-E006		
	Total	0.01676	167			
Variance of replace volume	Sample point	0.00247	6	4.1-E004	30.09	0.000
	Test nr.	0.00001	3	3.9-E004	0.29	0.8317
	Interaction*	3.9-E004	18	2.2-E006	1.612	0.064
	Residual	0.00192	140	1.37-005		
	Total	0.0048	167			

* Variance of volumes respect both sample point and test nr. were not statistically significative.

TABLE 3

Sample point	Sample vol. ± SEM (ml)	Replace vol. ± SEM (ml)
1	0.1894 ± 0.0007	0.1903 ± 0.0012[a]
2	0.1907 ± 0.0005	0.2018 ± 0.0008
3	0.1896 ± 0.0007	0.2012 ± 0.0005
4	0.1903 ± 0.0005	0.2003 ± 0.0007
5	0.1915 ± 0.0007	0.2017 ± 0.0005
6	0.1904 ± 0.0003	0.2003 ± 0.0006
7	0.1928 ± 0.0005[b]	0.2018 ± 0.0005

a) and b) are statistically different from the rest of sample volumes after ANOVA followed by Scheffé ($p < 0.05$).

TABLE 4

Test number	Sample vol. ± SEM (ml)	Replace vol. ± SEM (ml)
1	0.1905 ± 0.0005	0.2000 ± 0.0010
2	0.1893 ± 0.0004[a]	0.1997 ± 0.0007
3	0.1921 ± 0.0005[b]	0.1992 ± 0.0007
4	0.1907 ± 0.0002	0.1996 ± 0.0007

a) and b) are statistically different from the rest of sample volumes after ANOVA followed by Scheffé ($p < 0.05$).

TABLE 5

	Sampling Point	Mean (ml) sample vol	RSD %	Concent. mg/ml	RSD %
Test I	1	0.190	2.66	0.269	0.97
	2	0.189	1.16	0.271	0.08
	3	0.190	1.76	0.271	0.46
	4	0.190	1.69	0.267	1.32
	5	0.190	1.97	0.264	0.83
	6	0.189	2.33	0.260	0.72
	7	0.192	2.07	0.255	1.01
Test II	1	0.189	0.39	0.184	0.68
	2	0.192	1.36	0.186	0.62
	3	0.193	1.09	0.184	0.54
	4	0.190	1.40	0.183	0.44
	5	0.192	0.77	0.179	1.59
	6	0.192	0.91	0.175	1.07
	7	0.194	1.09	0.173	0.48
Test III	1	0.189	0.70	0.183	0.92
	2	0.190	1.88	0.183	1.42
	3	0.190	1.43	0.183	0.50
	4	0.190	1.61	0.179	2.20
	5	0.194	4.16	0.179	2.37
	6	0.190	1.43	0.178	0.53
	7	0.193	0.60	0.175	1.30

The initial concentration in the receptor chamber decreases at each sampling point, because water is replaced in the cell.

DISCUSSION

The dilution process observed experimentally, can be described using an equation based on the operating step sequence of the Microette. This equation establishes a correction factor that stand for the dilution process due to the difference between the withdrawn and replace volume.

An aliquot of volume (SC) is first withdrawn from the receptor chamber and collected into the vial. This step is followed by volume replacement (VR) in the receptor chamber. Because VR > VC, the difference between the replaced and withdrawn volume (SE) is wasted.

We can say that:

$$SC+SE=VR=ST \tag{1}$$

(ST = volume of total sample removed.)

C_1 = initial Ketorolac trometamol concentration in the receptor chamber.

$$C_1=\frac{mg_i\ (\ ketorolac\ trometamol)}{V}$$

(V = Receptor chamber volume (11,6 ml))

Following the operating step sequence:

The extraction of the SC volume from the receptor chamber can be expresed using the following equation:

$$C_2=\frac{mg_i-mg_i.\frac{SC}{V}}{V-SC} \tag{2}$$

Replacement of VR is then introduced:

$$C_2=\frac{mg_i-mg_i.\frac{SC}{V}}{V-SC+VR} \tag{3}$$

$$C_2=C_1.(1-\frac{SC}{V}).(1-\frac{SE}{V+SE}) \tag{4}$$

The cells are then levelled, and the sample excess is discarded to the dilution flask.

$$C_2=\frac{(mg_i-mg_i.\frac{SC}{V})-(mg_i-mg_i.\frac{SC}{V}).\frac{SE}{V+SE}}{V-SC+VR-SE}= \quad (5)$$

$$C_2=\frac{(mg_i-mg_i.\frac{SC}{V}).(1-\frac{SE}{V+SE})}{V}= \quad (6)$$

$$C_2=C_1.(1-\frac{SC}{V}).(1-\frac{SE}{V+SE}) \quad (7)$$

If we assume that no mixing occurs while the stirrers remain still ,during the sampling process, we can consider that the wasted volume has the same concentration as the withdrawn volume. This is because the replacement was performed through the bottom of the receptor chamber, and if no mixing occurs homogeneity of the receptor fluid cannot be achieved in a few seconds. (SE) is withdrawn from the cell volume level line (see Fig. 2) being negligible at this point the dilution effect produced by the replaced volume.

Then assuming that concentration of SE is the same of SC, we can simplify equation 7 as follows :

$$C_2=C_1-C_1.\frac{SC}{V}-C_1.\frac{SE}{V+SE} \quad (8)$$

$$C_2=C_1.(1-\frac{SC}{V}-\frac{SE}{V+SE}) \quad (9)$$

In addition, equation 9 can be further simplified if we assume $SE << V$, as follows:

$$C_2=C_1.(1-\frac{SC}{V}-\frac{SE}{V}) \quad (10)$$

$$C_2=C_1.(1-\frac{VR}{V}) \quad (11)$$

With the numerical values obtained from the gravimetric validation, we can compare the results obtained when using equations 7, 9 and 11.

SC = 0,190 ml : VR = 0,200 ml :SE = 0,010ml

Vol.cel.nr.1,2,3,4 = 11,6ml:Vol.cel.nr.5,6 = 11,7 ml

The correlation factor using equation 7 is 0,9827735

$$C_2 = C_1.(1 - \frac{SC}{V}).(1 - \frac{SE}{V+SE})$$

$$C_2 = C_1.(1 - \frac{0,190ml}{11,6ml}).(1 - \frac{0,010ml}{11,61ml}) = 0,9827735.C_1$$

The correlation factor using equation 9 is 0,9827594

$$C_2 = C_1.(1 - \frac{SC}{V} - \frac{SE}{V+SE})$$

$$C_2 = C_1.(1 - \frac{0,190}{11,6} - \frac{0,010}{11,61}) = 0.9827594.C_1$$

Using equation 11 the correlation factor obtained is 0,9827587

$$C_2 = C_1.(1 - \frac{VR}{V})$$

$$C_2 = C_1.(1 - \frac{0,200}{11,6}) = 0,9827587.C_1$$

As we can see, not significant difference was found among equations 7, 9 and 11. Then we assumed that simplifications performed were acceptable and we could apply the last equation 11. A main criticism could be made about that simmplifications,that is the narrow range in the experimental conditions we used, because difference between the withdrawn and replaced volume was very low (SE = 10 μl), and simplifications would be assumed easily. Let´s see the differences between the correlation factors if we consider greater volumes of work, and greater differences between them:

a.- Sample Volume (SC) : 0.50 ml Replace volume (VR) : 0.55 ml Waste volume: 0.05 ml

Correlation factor using equation 7 = C_1 0.9527

Correlation factor using equation 9 = C_1 0.9526

Correlation factor using equation 11 = $C_1$0.9526

b.- Sample Volume (SC) : 0.5 ml Replace volume (VR) : 0.6 ml Waste volume : 0.1 ml

Correlation factor using equation 7 = C_1 0.9487

Correlation factor using equation 9 = C_1 0.9483

Correlation factor using equation 11 = C_1 0.9482

The correlation factor is affected only in the fourth decimal number in both exemples. We consider acceptable the simplifications assumed. Nevertheless each one can assume freely the correlation factor level to work with.

The following expression was used to prove if the experimental results fit the theoretical ones:

The theoretical results were obtained from equation 11.

$$\frac{Concen._{experim.} - Concen._{theor.}}{Concen._{theor.}} * 100$$

As shown by results in table 6, the experimental concentration is greater than the theoretical concentration. This systematic error is a result of the experimental method designed to control the dilution sampling point.

As shown by Figure 2, the receptor chamber was not completely hermetic, because it is connected through the receptor media replace port with the syringe pump.(see Fig 3). When filling the cells with a known concentration of ketorolac trometamol, some of this liquid was also introduced in the replace port. The cells were closed using the dialisis membranes, followed by inmediate joining of the tubes from the replace port and the syringe pump filled with water. At the start of the dilution process, the volume incorporated into the cells as "fresh medium receptor" was the "dead "volume of the replace tube and not just "clean water" (0% ketorolac trometamol concentration). This would explain why experimental concentrations were larger than the theoretical ones.

Because of the lack of a hermetic receptor chamber on the Hanson cell Franz modification, There may be additional diffusion problems across the receptor media port of not easy quantification. Once the drug cross the membrane and is dissolved in the receptor medium, a small aliquot of this drug may diffuse through the replace port. This is an uncontrolled factor, indeed more important

TABLE 6

	Sampling point	Desviation between exper. and theor. results (%)
Test I	2	3,08
	3	4,84
	4	4,88
	5	5,86
	6	5,91
	7	5,60
Test II	2	2,74
	3	3,70
	4	4,72
	5	4,07
	6	3,70
	7	4,18
Test III	2	1,60
	3	3,38
	4	2,89
	5	4,89
	6	6,40
	7	6,42

that the correction factor occasionated by the difference between replace and withdrawn volume. The deviation shown in table 6, average between 4-5% and the error due the correlation factor is lower than 2%. We believe that this deviation between experimental and hypothetical values is due not just to the mechanical running of the Microette and depends on the Franz Cell modification (addition of the replace port (see fig 2).

CONCLUSIONS

The Hanson Microette is an autosampling system that directly samples from 6 or 12 Franz cells, and also replaces the removed media with fresh one as we have indicated this mechanism appears to be a reasonable and practical procedure for assuring batch to batch uniformity and an important aid in the development of new transdermal formulations. However, an initial validation trial

of the Microette using the receptor medium, membrane, and the stipulated drug, must be performed. The viscosity of the receptor medium is an important factor directly related to the withdrawn volume. After the volume is removed from the cell under vacuum, a correlation between the time of vacuum (fill time) and sample filled in the vial must be established.

The evaporation of the receptor medium from the replace beaker and vials covered with the evaporation caps depends on the receptor liquid composition and can be analytically solved using an internal standard if an autoinjector for HPLC is not connected with the Microette. Hydroalcoholic mediums may have additional evaporations difficulties.

In addition evaporation through membrane can influence the receptor media concentration over all the assay. Porosity and physicochemical properties of membrane must be controlled factors in a correct design.

Solubility and diffusion of drug in the receptor medium must also be controlled. If diffusion of drug is greater in the receptor medium, an aliquot of the drug may diffuse through the replace port and falsify the results obtained.

As previously described an initial validation, using the drug, receptor fluid, membrane, temperature and sample volume that would be assayed in the diffusion test should be performed to control the deviation between experimental and theoretical results. Correction of the dilution factor is also mandatory and directly depends on the size of the replace volume. With recommended replace volumes of approximately 0.5 ml, the correlation factor is around 0.95.

This would permit the detection of the error we assume using this automated sampling method.

Although the Microette system improves transdermal test performance, a previous and complete validation of all the conditions assayed must be completed.

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