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Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 1325–1333

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Keratin immobilized on silica as a new stationary phase for chromatographic modelling of skin permeation[1]

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Received 21 August 1996; accepted 11 November 1996

Abstract

Skin permeability of organic compounds depends on their lipophilicity but can also be affected by compounds interactions with specific skin components. A good chromatographic model of percutaneous penetration determined solely by lipophilicity is provided by the immobilized artificial membrane (IAM) columns. To complete the model a new high-performance liquid chromatographic (HPLC) stationary phase was prepared by physical immobilization of keratin on silica support. The keratin immobilized on silica has properties typical for the reversed-phase materials but it retains specifically acidic solutes. The keratin column can be used to conveniently compare keratolytic properties of xenobiotics. It was demonstrated that retention parameters determined on a keratin column can be combined with the retention parameters determined on the IAM column to predict differences in skin permeability within a class of drugs. It has been postulated that HPLC can model skin permeation thus reducing research time and costs as well as the use of laboratory animals. © 1997 Elsevier Science B.V.

Keywords: Immobilized artificial membrane stationary phase (IAM); Keratin immobilized on silica stationary phase; Keratolytic properties of drugs; Quantitative structure-activity relationships (QSAR); Quantitative structure-retention relationships (QSRR); Modelling of skin permeability

1. Introduction

The ability of drugs and toxins to penetrate skin and to absorb into general blood circulation is of pharmacological and toxicological importance. Recently, an even increased interest is observed in the predicting of systemic absorption of topically administered drugs. This is due to the introduction of the so-called transdermal therapeutic systems (TDS) into a wider practice.

The dependence of skin permeation properties of organic compounds on their lipophilicity (hydrophobicity) is well known. Lipophilicity determines the affinity of xenobiotics to the adipose (fat) cells in subcutaneous layer of the skin and to biomembranes of other cells. However, to reach the fat tissue, the agent must first cross the epider-

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[1] Presented at the Seventh International Symposium on Pharmaceutical and Biochemical Analysis, August 1996, Osaka, Japan.

PII S0731-7085(96)02009-2

Table 1
Elemental analysis of stationary phase materials comprising keratin physically immobilized on silica in relation to the silica support used and the method of keratin precipitation

Sample no.	Silica support	Precipitation of keratin by adding:	% Carbon	% Hydrogen
1	LiChrosorb 7 mm, 60 Å, irregular, Merck	Toluene slowly	12.6	2.8
2	LiChrosorb 7 mm, 60 Å, irregular, Merck	Water slowly	44.0	6.0
3	LiChrosorb 7 mm, 60 Å, irregular, Merck	Water rapidly	20.6	3.1
4	Nucleosil 7 mm, 300 Å, spherical, Macherey-Nagel	Water slowly	1.6	Not determined

mis. The outermost layers of epidermis comprise large amounts of keratin [1]. To model skin permeability one should take into account not only the lipophilicity of drugs but also their possible interactions with keratin.

In recent years Pidgeon and co-workers [2,3] introduced a new generation of HPLC stationary phase materials, the so-called immobilized artificial membranes (IAM). The IAM phases are synthesized by covalent binding of the biological membrane forming phospholipids to solid silica surfaces. Soon after introduction of the IAM columns the papers were published reporting their performance in chromatographic determination of biologically relevant measures of drug lipophilicity [4–6]. Good correlations were found between the HPLC retention parameters determined on IAM and human skin permeation coefficients of several classes of drugs [7]. However, not in every case the lipophilicity parameters sufficed to predict differences in skin permeation among drugs of a given class. To complete the chromatographic model of percutaneous penetration of drugs an attempt has been undertaken in our laboratory to produce HPLC columns comprising keratin.

Protein-bonded stationary phases for HPLC have normally been developed to separate enantiomers. Haginaka and Kanasugi [8] listed nine protein phases which called a wider analytical interest. From a pharmacological point of view, more interesting are the observations that chromatography in systems comprising biomacromolecules can serve to quantify drug-protein interactions [9–11].

Quantitative structure-activity relationships (QSAR) were studied for different classes of drugs considering human epidermis permeability data as activity parameters [7,12]. In the case of steroids, correlation between skin permeability and lipophilicity of the agents was good. On the other hand, it appeared difficult to predict permeability of phenolic compounds by means of lipophilicity parameters. There is a representative series of small molecule organic compounds for which reliable skin permeability data have been determined [7,13]. These compounds were collected and analyzed in this work.

2. Experimental

2.1. Immobilization of keratin [14]

2.0 g of keratin (ICN, Aurora, OH, USA) was placed in a round-bottom flask and 40 ml of dimethylsulfoxide (Fluka, Buchs, Switzerland) was added. The substances were refluxed on an oil bath at 160°C with vigorous stirring for 24 h. The resulting brown solution was cooled to room temperature and filtered through a glass fiber membrane filter. The filtrate was transferred to a round-bottom flask and 3.0 g of chromatographic silica was added. Two types of silica support materials were used: LiChrosorb (E. Merck,

Darmstadt, Germany) in case of Samples 1–3 and Nucleosil (Macherey-Nagel, Düren, Germany) in case of Sample 4 (see Table 1). The suspensions were mixed well with a magnetic stirrer and precipitating solvent (120 ml) was added dropwise. In case of Sample 1 the solvent used was toluene. Its volume of 120 ml was added within 60 min. To produce remaining three samples, water was used as the keratin precipitating solvent. In case of Sample 3 the total volume of water (120 ml) was added within 20 min. The resulting precipitate of silica covered with keratin was filtered off on a membrane filter. The solid was rinsed thoroughly with water, acetone and isopropanol in sequence till obtaining a clear filtrate. Next, the solid was dried at room temperature. The content of carbon and hydrogen was determined by elemental analysis.

2.2. Preparation of HPLC columns with immobilized keratin

The immobilized keratin silica stationary phase materials were slurry packed into Hibar columns 4 mm I.D., 12.5 cm length (E. Merck, Darmstadt, Germany). A pneumatic pump was used (Knauer, Berlin, Germany) at maximum pressure of 500 bar. Slurry solvent was isopropanol (POCh, Gliwice, Poland).

2.3. Apparatus

Waters LC I Module for HPLC was used (Waters, Milford, MA, USA) with a variable wavelength UV-VIS detector.

2.4. Chromatographic conditions

In case of keratin columns the eluent was an aqueous buffer modified with isopropanol added at proportions ranging from 0 to 10% (v/v). The buffer was a solution of NaH_2PO_4 or $(NH_4)H_2PO_4$ at concentration ranging from 0.05 to 0.1 M. Flow rate was 1 ml min^{-1}.

The capacity factors, k', were calculated assuming signal of $NaNO_2$ as dead time.

The capacity factors determined on an IAM column (IAM.PC.MG, Regis, Morton Grove, IL, USA) with phosphate buffer-acetonitrile 95:5% (v/v) were taken from an earlier publication [7].

2.5. Test solutes

Drugs and other test substances were used as purchased.

The structure of test compounds was subjected to molecular modelling employing the HyperChem 4.5 software with a ChemPlus 1.5 extension (Hypercube, Waterloo, ON, Canada). Structural descriptors resulting from calculation chemistry were employed in the quantitative structure-retention relationships (QSRR) analysis.

2.6. Statistical methods

QSAR and QSRR equations were derived by the multiple regression analysis method following the requirements for meaningful statistics [15]. A StatgraphicsPlus 6.2 software (Manugistics, Rockland, MA, USA) was used.

3. Results

The results of elemental analysis of four samples of silica covered with keratin are given in Table 1. The coverage highly depended on the preparation procedure applied. The highest keratin coverage was found for Sample 2. This sample was obtained when applying irregular LiChrosorb silica and a slow precipitation of keratin with water.

Dependence of $\log k'$ determined on a keratin column made of Sample 2 on volume fraction of isopropanol in eluent is illustrated in Fig. 1 for eight drug solutes.

In Fig. 2 the capacity factors are compared of seven test solutes determined at the same isocratic conditions on two columns: one packed with a bare silica support material and the other packed with Sample 2 of the keratin-covered silica stationary phase.

The 'best' QSRR equation describing logarithms of capacity factor of 17 test solutes determined on the Sample 2 keratin-silica column using 0.05 M NaH_2PO_4 (pH 4.20) buffer as the

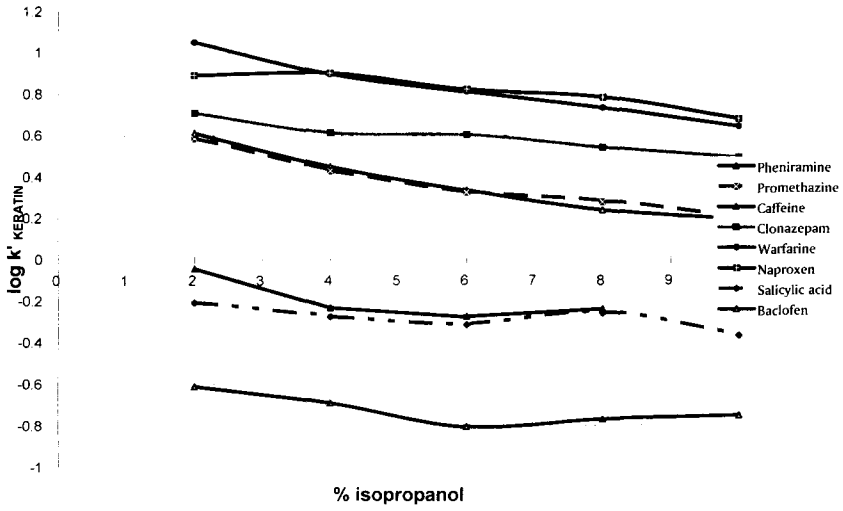

Fig. 1. Dependence of the logarithms of a capacity factors, $\log k'$, determined on a column packed with Sample 2 of keratin-silica, on volume fraction of isopropanol in the 0.05 M NaH_2PO_4 pH 4.20 buffer-isopropanol eluent.

eluent (Table 2) in terms of structural descriptors is:

$$\log k'_{KERATIN} = -0.563(\pm 0.153) + 0.006(\pm 0.001)Mass - 0.117(\pm 0.035)\mu$$

$$n = 17;\ R = 0.866;\ s = 0.18;\ p = 10^{-4} \quad (1)$$

where *Mass* denotes solute molecular mass, μ is its dipole moment from molecular modelling (Table 2), n is the number of solutes considered when deriving the regression, R is the multiple correlation coefficient, s is standard deviation from regression and p is significance level of the equation; numbers in parenthesis are standard deviations of regression coefficients.

It must be noted here that an equation analogous to Eq. (1) and derived for logarithms of capacity factor determined at the corresponding conditions on a bare silica column has no statistical significance at all.

In Fig. 3 the data observed experimentally are plotted against the values predicted by Eq. (1).

Correlation between logarithms of human skin permeability, $\log K_P$, and the measure of drug lipophilicity, $\log P$, (Table 3) is described by Eq. (2) and illustrated graphically in Fig. 4:

$$\log K_P = -6.892(\pm 0.307) + 0.612(\pm 0.141)\log P$$

$$n = 17;\ R = 0.745;\ s = 0.711;\ p = 6 \times 10^{-4} \quad (2)$$

Eq. (3) and Fig. 5 show correlation between $\log K_P$ and the logarithms of capacity factor determined [7] on an immobilized artificial membrane column, $\log k'_{IAM}$, for 17 agents from Table 3:

$$\log K_P = -6.420(\pm 0.139) + 1.458(\pm 0.138)\log k'_{IAM}$$

$$n = 17;\ R = 0.899;\ s = 0.47;\ p < 10^{-4} \quad (3)$$

The QSAR equation describing skin permeation, $\log K_P$, of the agents from Table 3 as a combination of $\log k'_{IAM}$ and $\log k'_{KERATIN}$ has the following form:

$$\log K_P = -6.558(\pm 0.130) + 1.920(\pm 0.242)\log k'_{IAM} - 1.039(\pm 0.413)\log k'_{KERATIN}$$

$$n = 17;\quad R = 0.932;\quad s = 0.40;\quad p < 10^{-4} \quad (4)$$

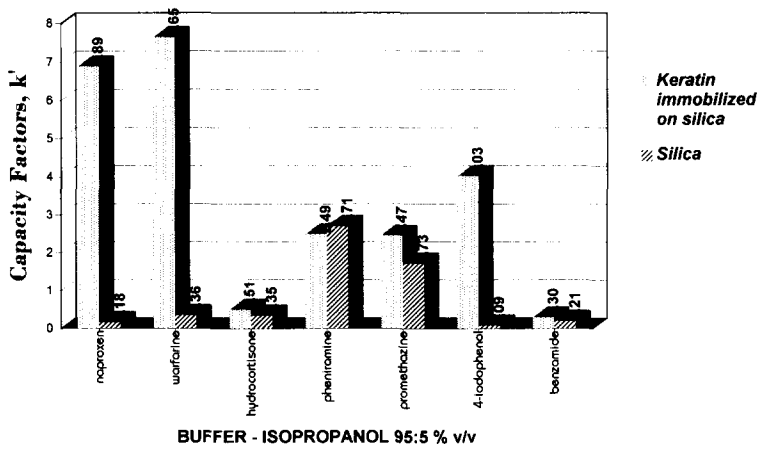

Fig. 2. Capacity factors, k', of seven basic, neutral and acidic solutes determined at the same conditions (0.05 M NaH_2PO_4 pH 4.20-isopropanol 95:5% v/v) on a silica and on a silica covered with keratin HPLC column.

The plot of the observed log K_P data against the ones calculated by Eq. (4) is given in Fig. 6. Intercorrelation between $\log k'_{IAM}$ and $\log k'_{KERATIN}$ is $R = 0.759$.

4. Discussion

Keratin is a very stable protein characterized by a high content of sulfur. It comprises large proportions of cysteine and glutamic acid. Keratin contains both hydrophobic and hydrophilic fragments as well as acidic and basic moieties. It is highly water insoluble. By using dimethylsulfoxide (DMSO) as the solvent, keratin was dissolved at elevated temperature. Next, it could have been precipitated on silica particles by water or other solvent mixing with DMSO. The coverage of silica matrix with keratin depended strongly on the starting silica material used and on the precipitation procedure. High coverage of silica with keratin can be obtained at appropriate reaction conditions as evidenced by elemental analysis. Further work is required to get reproducible coverage of keratin on the silica support.

The keratin-covered silica behaves as a reversed-phase material. In Fig. 1 a more or less linear decrease of logarithm of a capacity factors with increased content of organic modifier in aqueous eluent is observed for various solutes. On the other hand, marked deviation from linearity, especially in the case of salicylic acid, would suggest involvement of some specific keratin-solute interactions in the separation.

Specifity of the immobilized keratin stationary phase is best seen when compared to the starting silica as a stationary phase material (Fig. 2). For the basic and neutral solutes the capacity factors on a keratin column are similar to those obtained on a bare silica column. However, in the case of acidic solutes (4-iodophenol, warfarin and naproxen) the retention on the keratin column is markedly stronger than on the silica column. Keratolytic properties of organic acids have since long been known. The immobilized keratin HPLC column can be a convenient device to quantitatively compare such properties.

To get an insight into the molecular mechanism of separation on the newly prepared immobilized keratin column the quantitative structure-retention relationships (QSRR) were studied. The 'best' QSRR equation, Eq. (1), shows that retention of test solutes increases with their molecular mass but decreases with with their dipole moment. Such a type of QSRR is characteristic for the reversed-phase mechanism of separation [16]. The plot of the observed against the predicted retention data (Fig. 3) clearly shows the limited retention prediction ability of the QSRR derived. Most probably, Eq. (1) does not account for the

Table 2
Logarithms of HPLC capacity factors determined on the keratin column ($\log k'_{KERATIN}$) and on the IAM column ($\log k'_{IAM}$) [7], logarithm of octanol-water partition coefficient ($\log P$) [12], molecular mass (Mass) and dipole moment obtained by molecular modelling (μ) for a series of simple organic solutes

No.	Solute	$\log k'_{KERATIN}$	$\log k'_{IAM}$	$\log P$	Mass	μ [D]
1	1,2,3-*Tris*(1-methylethyl)benzene	0.754	2.428	—	204.36	0.015
2	1,4-Dinitrobenzene	0.453	0.157	1.46	168.11	2×10^{-4}
3	3-(Trifluoromethyl)phenol	0.190	1.234	2.95	162.11	2.731
4	4-Chlorophenol	0.273	0.728	2.39	128.56	0.947
5	4-Cyanophenol	−0.049	0.771	1.6	119.12	2.376
6	4-Iodophenol	0.797	1.593	2.91	220.01	0.687
7	4-Nitrobenzoic acid	−0.228	−0.228	1.89	167.12	3.096
8	Anizole	−0.090	0.310	2.11	108.14	0.890
9	Benzamide	−0.040	−0.099	0.64	121.14	3.019
10	Benzene	−0.265	0.093	2.13	78.11	4.43×10^{-6}
11	Benzoic acid	−0.205	−0.736	1.87	122.12	2.850
12	Benzonitrile	0.020	0.154	1.56	103.12	2.715
13	Caffeine	0.080	−0.396	−0.07	194.19	3.438
14	Chlorobenzene	0.126	0.655	2.84	112.56	1.343
15	Indazole	0.230	0.710	1.82	118.14	0.934
16	Phenol	−0.341	0.366	1.46	94.11	0.740
17	Toluene	−0.049	0.436	2.69	92.14	0.296

specific solute-keratin interactions contributing to retention: that is for other mechanism of separation than the solute partition between the mobile and the stationary phase.

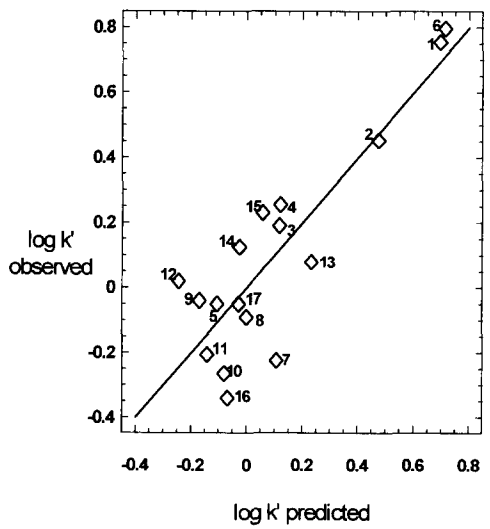

Fig. 3. Plot of capacity factors of solutes listed in Table 2 observed experimentally against the corresponding data theoretically calculated by Eq. (1).

To test the performance of chromatographic parameters determined on the keratin columns in predicting skin permeability a set of biological data was selected which could not be described by solute lipophilicity itself (Table 3). For a set of phenolic compounds supplemented by phenylalanine, salicylic acid, methylhydroxybenzoate and baclofen, the classical lipophilicity parameter, $\log P$, did not describe human skin permeation satisfactorily (Eq. (2), Fig. 4). For the same agents, the lipophilicity parameter determined on an immobilized artificial membrane column, $\log k'_{IAM}$, described the skin permeability better but still not precisely enough (Eq. (3), Fig. 5).

A significant improvement of prediction of skin permeability was obtained after combining $\log k'_{IAM}$ with the retention parameter determined on the keratin column, $\log k'_{KERATIN}$ (Eq. (4), Fig. 6). Now, the prediction of skin permeability of test agents is good and the regression equation, Eq. (4) can be of practical value for drug design.

Eq. (4) is not only significant statistically but it also makes a good physical sense. It shows that skin permeability increases with the lipophilicity of the agents but decreases with their affinity to keratin. It can be concluded from Eq. (4) that the

Table 3
Logarithms of capacity factor determined on the keratin column ($\log k'_{KERATIN}$) and on the IAM column ($\log k'_{IAM}$) [7], logarithm of octanol-water partition coefficient ($\log P$) [12] and logarithms of human skin permeation coefficient ($\log K_P$) for a series of phenols and other simple organic compounds

No.	Solute	$\log k'_{KERATIN}$	$\log k'_{IAM}$	$\log P$	$\log K_P$
1	3-Nitrophenol	0.241	0.598	2.0	−5.81
2	4-Bromophenol	0.338	0.995	2.59	−5.0
3	4-Chlorophenol	0.273	0.728	2.39	−5.0
4	4-Ethylphenol	−0.249	0.761	2.37	−5.01
5	4-Nitrophenol	0.189	0.595	1.91	−5.81
6	β-naftol	0.879	1.254	2.84	−5.11
7	Baclofen	−0.334	−0.725	−0.96	−6.77
8	Chlorocresole	0.678	1.183	3.1	−4.82
9	*m*-Cresole	−0.223	0.363	1.96	−5.37
10	Methylhydroxybenzoate	0.044	0.520	1.96	−5.6
11	*o*-Cresole	−0.178	0.363	1.95	−5.36
12	*p*-Cresole	−0.082	0.418	1.94	−5.31
13	Phenol	−0.273	0.366	1.46	−5.64
14	Phenylalanine	−0.198	−0.646	−1.35	−8.08
15	Resorcinol	−0.382	−0.141	0.78	−7.18
16	Salcylic acid	−0.058	−0.575	2.26	−7.82
17	Thymol	0.521	1.342	3.3	−4.83

keratolytic properties of phenolic and other acidic test compounds oppose their lipophilic properties as regards skin permeation. However, it should be noted at the same time that the coefficient at $\log k'_{IAM}$ is nearly two times larger than the coefficient at the $\log k'_{KERATIN}$ term. As the net effect, the phenol derivatives and the remaining test agents should be absorbed from skin which is experimentally observed.

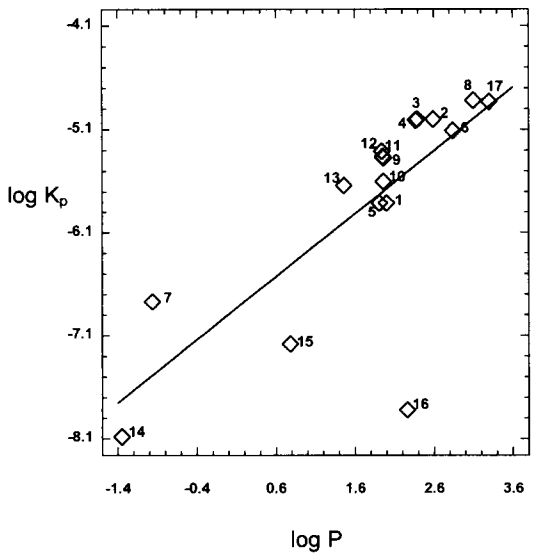

Fig. 4. Correlation between logarithms of human skin permeability, $\log K_P$, and of octanol-water partition coefficient, $\log P$, for a series of agents listed in Table 3.

Systematic studies on column stability has not been completed yet. Within a 3 month period of occasional use no deterioration of column performance was observed.

5. Conclusions

The results of this work can be summarized as follows:

(i) Keratin can be physically immobilized on silica support producing new HPLC stationary phase materials.

(ii) Retentive properties of the immobilized keratin stationary phase are specific and distinctive from the properties of the starting support material. The new phase strongly retains acidic solutes.

(iii) The keratin column can be used to quatify the differences in drug interactions with keratin, a basic skin protein. That way the

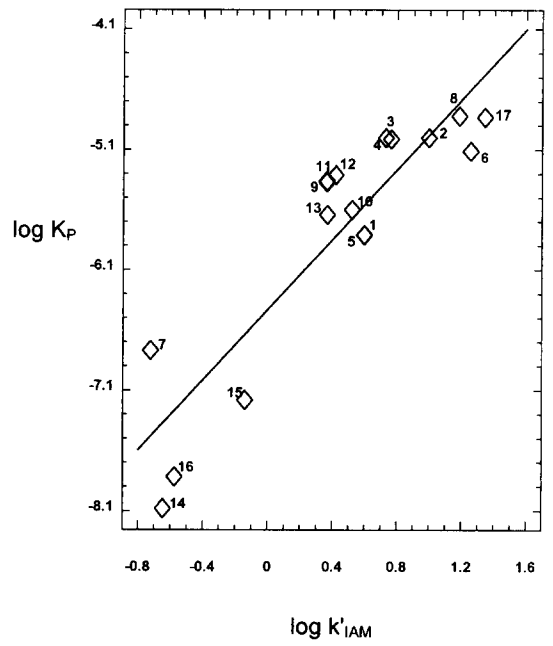

Fig. 5. Correlation between logarithms of human skin permeability, log K_P, and of capacity factors determined on an IAM column, log k'_{IAM}, for a series of solutes from Table 3.

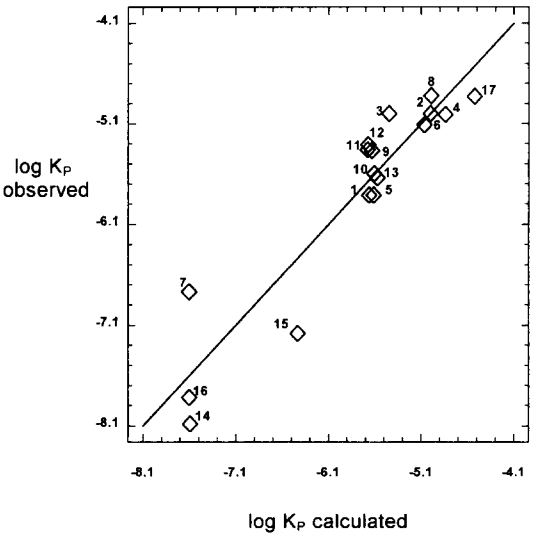

Fig. 6. Plot of logarithms of human skin permeability of agents listed in Table 3 observed experimentally against the corresponding data theoretically calculated by Eq. (4).

keratolytic properties of drugs can conveniently be compared in a quantitative manner.
(iv) Retention parameters determined on the keratin column along with the retention parameters determined on the immobilized artificial membrane (IAM) column can be combined chemometrically to predict skin permeability differences within individual classes of drugs.
(v) High-performance liquid chromatography (HPLC) can model skin permeation thus reducing research time and costs as well as the use of laboratory animals.

Acknowledgements

This work was supported by grants from the Komitet Badań Naukowych, Warsaw, Poland (4-PO5F-07-08) and from the II Polish-American Maria Skłodowska-Curie Fund (MZ/HHS-95-22 7). A support from Medical University of Gdańsk (project W-132) is also acknowledged.

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