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Immobilized artificial membrane chromatography coupled with atmospheric pressure ionization mass spectrometry

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Abstract

Liquid chromatographic separations on monolayers of cell membrane phospholipids covalently immobilized to silica particles at high molecular density is used for mimicking solute partitioning into biological membranes that generally correlates with membrane transport. This technique called immobilized artificial membrane chromatography usually employs ultraviolet (UV) detection where a single compound is analyzed in a chromatographic run limiting thereby its throughput for drug discovery applications. For coupling with atmospheric pressure ionization mass spectrometry, the phosphate-buffered saline mobile phase was replaced with one that used ammonium acetate as a volatile buffer. While atmospheric pressure chemical ionization accommodated a purely aqueous effluent, interfacing with electrospray ionization required effluent splitting and the addition of an organic modifier (5%, v/v, acetonitrile). Neuropeptide FF antagonists as early-phase drug candidates were used for the comparative evaluation of the methods. Whereas electrospray ionization produced essentially no fragment ions, several compounds involved in our study yielded low-abundance molecular ions with atmospheric pressure chemical ionization. The use of mass spectrometry yielded data that correlated well with those obtained by the method employing UV detection. Both atmospheric pressure ionization methods permitted the simultaneous determination of the k'_{IAM} capacity factors and, therefore, an increased-throughput ranking of potential new leads emerged from the drug discovery process based on affinity to artificial membranes.
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1. Introduction

A common biological process is the interaction of drug molecules with cell membranes. The ability to determine membrane interaction and diffusion of drug candidates through the membrane is essential in early-phase drug discovery. Membrane partitioning usually affects a drug's activity, toxicity, absorption, distribution, metabolism, and its pharmacokinetic properties. There are several in vitro models in which drug permeation through membranes can be estimated. Caco-2-cells [1,2] and intestinal tissue [3] are common methods employed, but they are both time-consuming and costly. Another commonly used method is *n*-octanol–water partitioning [4], which has limitations because it often reflects only the

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hydrophocity of a compound and is not suitable for highly polar and ionic compounds, nor is ideal in mimicking biological membranes. Chromatographic estimation of lipophilicity on ODS (octadecylsilica) reversed-phase high-performance liquid chromatography (HPLC) column [5] has essentially the same problems as the *n*-octanol–water partitioning method in this regard.

Diffusion through biological membranes, which is often a rate-limiting step in drug absorption, can be characterized by a K_m value that represents the fluid membrane partition coefficient. Immobilized artificial membranes (IAMs) are solid-phase membranes that are prepared by covalently immobilizing monolayers of cell membrane phospholipids to silica particles at high molecular densities. IAMs serve as models of biological membranes by mimicking the solute partitioning into fluid membranes that are similar to lipid bilayers [6] and this technique developed by Pidgeon and co-workers is an accepted chromatographic method for the prediction of membrane permeability of drugs [7,8]. The chromatographic capacity factor (k'_{IAM}) for a solute obtained by IAM chromatography (IAMC) correlates with its partition coefficient between the aqueous phase and the IAM bonded phase ($k'_{IAM} = \phi K_{IAM}$, where $\phi = V_s / V_m$ is the stationary phase to mobile phase ratio of the column) and, ultimately, with its K_m [9]. The greater the k'_{IAM} is, the greater the membrane permeability of the drug candidate. IAM chromatography has shown excellent correlation with transport of drugs across Caco-2-cells, intestinal tissue [9], skin [10], and into liposomes [11], and gives a better correlation to permeability data than ODS chromatography and *n*-octanol–water partitioning (log *P*). It has also been applied to HIV protease inhibitors [12], structurally diverse drugs [13] and steroids for assessing penetration into the blood–brain barrier [14], along with assessing the lipophilic properties of 1,3,5-triazines that have central nervous system activities [15]. In these studies, the assays were performed with the use of UV detection.

Coupling IAMC to mass spectrometry (MS) involving sensitive and selective atmospheric pressure ionization (API) methods allows for the analysis of compounds without UV chromophore and for a simultaneous analysis of several compounds in a mixture, as demonstrated by our preliminary report [16]. This method offers a clear advantage in throughput over UV detection where generally one compound is analyzed in a single run. Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are two variants of the API methods and they are both compatible with liquid chromatography and available on today's mass spectrometers. In this paper, we present methods developed for interfacing IAM chromatography with APCI and ESI mass spectrometry. For comparative evaluation of the techniques, neuropeptide FF (NPFF) antagonists emerged from early-phase drug discovery efforts [17] were used as test compounds (Fig. 1).

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile, acetic acid, citric acid, phosphoric acid and ammonium acetate were purchased from Fisher (Fair Lawn, NJ, USA). The thyrotropin-releasing hormone (TRH) analogue (**1**) [18] was kindly provided by C. Somlai (University of Szeged, Hungary). The NPFF antagonists 5-(dimethylamino)-1-naphthalenesulfonyl (dansyl)–Pro–Gln–Arg–NH_2 (**2**), Pro–Phe–Arg–L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl (Tic)–NH_2 (**3**), dansyl–Gln–Gln–Arg–NH_2 (**4**), dansyl–Gln–Ser–Arg–NH_2 (**5**) dansyl–Gly–Gln–Arg–NH_2 (**6**), dansyl–Gly–Ser–Arg–NH_2 (**7**), dansyl–Lys–Gln–Arg–NH_2 (**8**) and dansyl–Lys–Ser–Arg–NH_2 (**9**) were synthesized in our laboratory by procedures reported earlier [16].

2.2. IAM chromatography

Initial method development for determining capacity factors of analytes was done on 3 cm×4.6-mm I.D. IAM.PC.DD column, d_p=5 μm (Regis Technologies, Morton Grove, IL) protected with a 1 cm×3.0 mm I.D. guard cartridge. The IAM.PC.DD column, also known as $^{\delta G}$IAM.PC$^{C10/C3}$, indicates that the head group used in the stationary phase is phosphatidylcholine (PC), which is the major phospholipid found in cell membranes. The superscript "δG" denotes the deletion of the glycerol backbone from the PC molecule, and the superscript "C10/

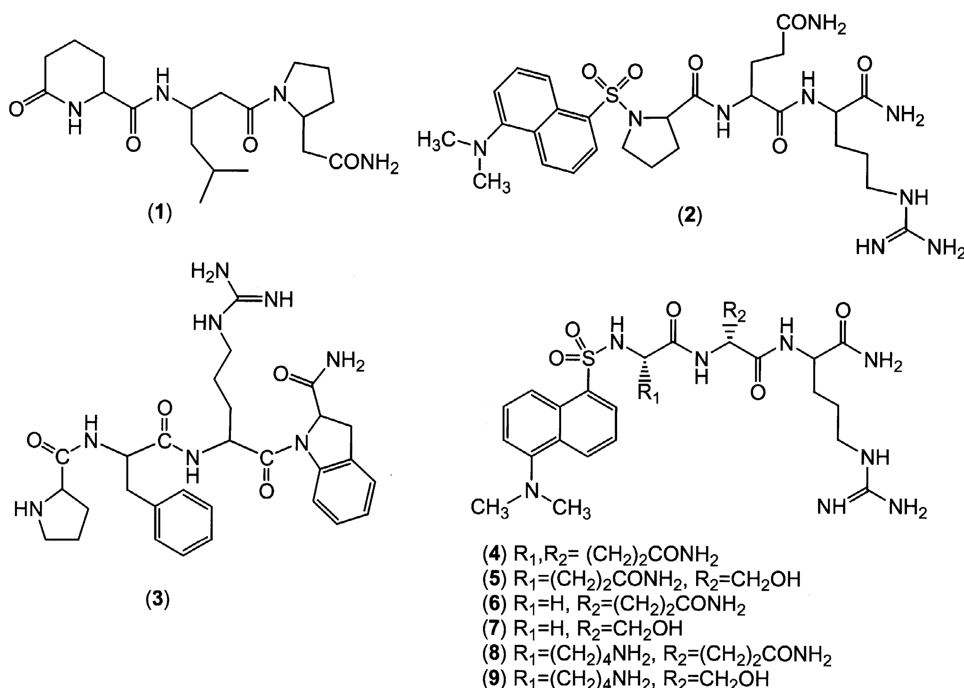

Fig. 1. Structure of compounds analyzed.

C3'' indicates that the unreacted propylamino moieties on the silica surface were end-capped with a mixture of C10 and C3 acyl groups [8]. (DD implies that the column was developed for drug discovery applications.) The following mobile phases were used in this study: Dulbecco's phosphate buffer saline (DPBS; 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 136.9 m*M* NaCl and 2.7 m*M* KCl) adjusted to pH 5.4 with phosphoric acid and containing 5% (v/v) acetonitrile; 50 or 10 m*M* ammonium acetate (NH_4OAc) adjusted to pH 5.4 with acetic acid; and 10 m*M* NH_4OAc (pH 5.4) containing 5% (v/v) acetonitrile. The flow-rate was 1.0 ml/min. UV chromatograms were obtained using a model SP8810 precision isocratic pump, an SP8880 autosampler with an injection loop of 20 μl, an SP8450 variable UV–Vis detector operated at 254 nm and SP4290 computing integrator (all from ThermoSeparation/Spectra-Physics). The void volume marker was citric acid and the k'_{IAM} capacity factor was calculated by using the following equation:

$$k'_{IAM} = (t_{R(x)} - t_{R(Citric\ acid)})/t_{R(citric\ acid)} \quad (1)$$

where $t_{R(X)}$ and $t_{R(Citric\ acid)}$ are the retention times for the analytes and the dead time/void volume marker, respectively. The capacity factor k'_{IAM} is linearly related to K_{IAM} [9].

2.3. Atmospheric pressure ionization mass spectrometry

Mass spectra were acquired on a quadrupole ion trap instrument (LCQ, ThermoFinnigian San Jose, CA, USA) operated with the manufacuters Xcalibur 1.2 software. An LKB (Bromma, Sweden) pump was used for delivering the mobile phase. IAMC–APCI-MS was conducted with 10 m*M* NH_4OAc (pH 5.4) at a flow-rate of 1.0 ml/min and 5 μl of sample injected. The vaporizer and capillary temperature were 450 °C and 150 °C, respectively, the discharge current was set to 5 μA, the sheath and auxiliary gas (nitrogen) flow were maintained at 40 and 10 arbitrary units, respectively.

IAMC–ESI-MS was performed with a mobile phase of 10 m*M* NH_4OAc (pH 5.4) containing 5% (v/v) acetonitrile. Effluent splitting was achieved

with a T-junction (Valco) [19] that provided a flow-rate of 2.5 μl/min to the ESI source of the instrument through a 25 cm×0.1 mm I.D.×0.19 mm O.D. fused-silica capillary tube. The capillary voltage and temperature were set to 4250 V and 200 °C, respectively, and the sheath gas flow was maintained at 80 units.

APCI and ESI were conducted in positive-ion mode. Mass spectra were obtained from mass to charge ratio (*m/z*) 300 to 700, and an automatic gain control (AGC) was applied to adjust the trapping of ions based on two 200 ms microscans. Selected-ion chromatograms were reconstructed from full-scan data.

3. Results and discussion

Early appraisal of blood–brain barrier (BBB) permeability has become important even in the earliest phase of drug discovery [20]. IAM chromatography appeared to be a rapid and reliable method for this purpose [13]. The first step in the method development was to find a mobile phase compatible with both IAM chromatography and with routine API interfaces. DBPS is not preferred for API mass spectrometry, because non-volatile salts precipitate during the introduction of the effluent contaminating the ion source and, ultimately, clogging the entrance orifice of the interface, which necessitates frequent cleaning. Volatile buffers, such as aqueous 50 m*M* NH_4OAc, have been suggested for IAM chromatography [21], and we confirmed that this purely aqueous mobile phase was indeed compatible with APCI mass spectrometric detection, as shown in Fig. 2. The TRH analogue (**1**) in the presented example had no UV chromophore.

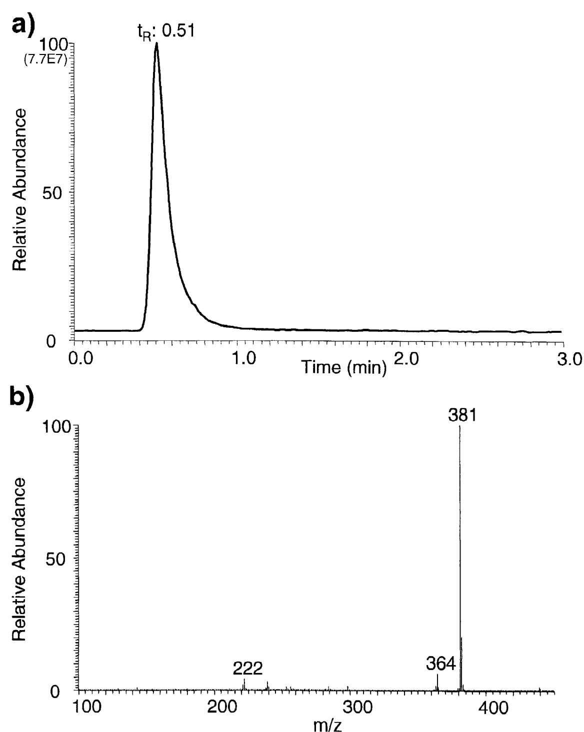

Fig. 2. IAMC–APCI-MS of **1**. (a) Total-ion chromatogram, (b) APCI mass spectrum of the peak at t_R =0.51 min (The base peak *m/z* 381 in the spectrum is the protonated molecule, MH^+).

For further development and evaluation of coupling IAM chromatography with API mass spectrometry, we involved another series of compounds. In chronological order, compounds **2** and **3** have emerged as early leads among NPFF antagonists with ability to penetrate the central nervous system after systemic administration [22–24]. In a recent effort involving a combinatorial study [17], we have identified several additional compounds (**4–9**) with higher affinity to the cognate receptor than the prototype lead compound **2**. Although receptor affinity is essential for a drug's ability to elicit desired pharmalogical effects, it must also be able to cross the BBB. Again, IAM chromatography has been our method of choice for the appraisal of our compounds in this regard. Initially, they were run individually with UV detection by using DPBS, pH 5.4, containing 5% acetonitrile as a mobile phase. With purely aqueous pH 7.4 DPBS, undesirable long retention times (>10 min) for the short IAM.PC.DD column were obtained. This could have been due to the strong binding of the Arg residue to the immobilized phospholipid in a purely aqueous medium at neutral pH, or other mechanisms that prolonged retention. However, the ''one compound—one chromatographic run'' approach with a cycle time of about 7 min has remained the bottleneck of our recent and future efforts to expand the scope of surveying the IAM interaction/retention for a large number of prospective lead compounds that can be

rapidly synthesized by today's combinatorial chemistry.

A method incorporating API mass spectrometric detection to increase throughput appeared to be very promising in our preliminary study [16]. Our aim was to preserve retention behavior we experienced for our eight test compounds upon using DPBS that is the general mobile phase used with IAM.PC.DD columns upon UV detection, while allowing the choice between APCI and ESI mass spectrometry as the API method without significant change in mobile phase composition. The aqueous 50 m*M* NH_4OAc found compatible with APCI may lead to signal suppression in ESI; therefore, we decided to lower the NH_4OAc concentration to 10 m*M*. This mobile phase was suitable for IAM chromatography even without the addition of an organic modifier, as shown in Fig. 3. However, inspection of the APCI mass spectra revealed that several of our test compounds exhibited very extensive decomposition, possibly due to thermal degradation of the analytes under the routine conditions employed for this method of ionization. Such high extent of decomposition may limit efforts (due to possible interferences from the ions from the decomposition products upon profiling the desired selected-ion chromatograms) to further increase the number of analyte components in the mixture during simultaneous estimation of the k'_{IAM} values. Therefore, we considered ESI as an alternative API method. Fig. 4 demonstrates that, while the relative abundance of the molecular ion (MH^+) for compound **5** at *m/z* 622 is merely 2% of the base peak in the APCI mass spectrum, ESI results in essentially no fragmentation.

ESI is usually unable to handle high flow-rate (>0.5 ml/min) aqueous liquid. Even upon interfacing with large (1–399) effluent splitting to reduce flow-rate for routine ESI, extremely weak analyte signals unsuitable for obtaining selected-ion chromatograms were obtained with a purely aqueous 10

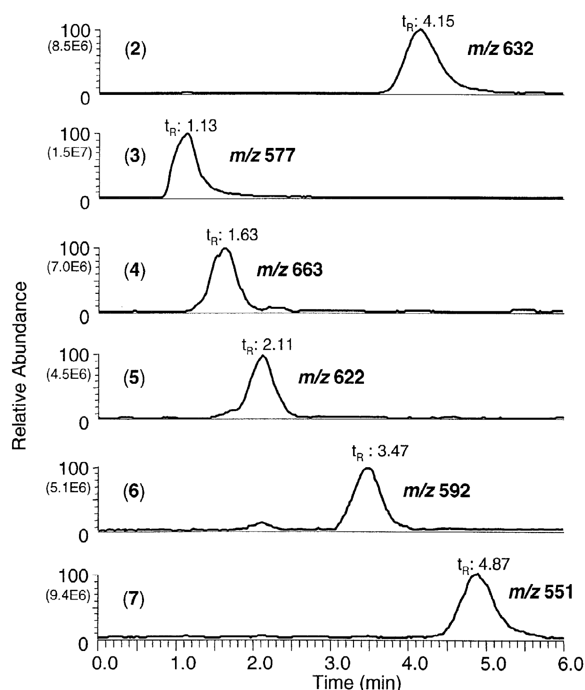

Fig. 3. Selected-ion chromatograms for the mixture of **2**–**7** by IAMC–APCI-MS (The traces were reconstructed for the *m/z* of the protonated molecules, MH^+, from consecutive full-scan acquisitions). Ion counts representing the normalization level (100) for the traces are given in parentheses on the relative abundance axes, respectively.

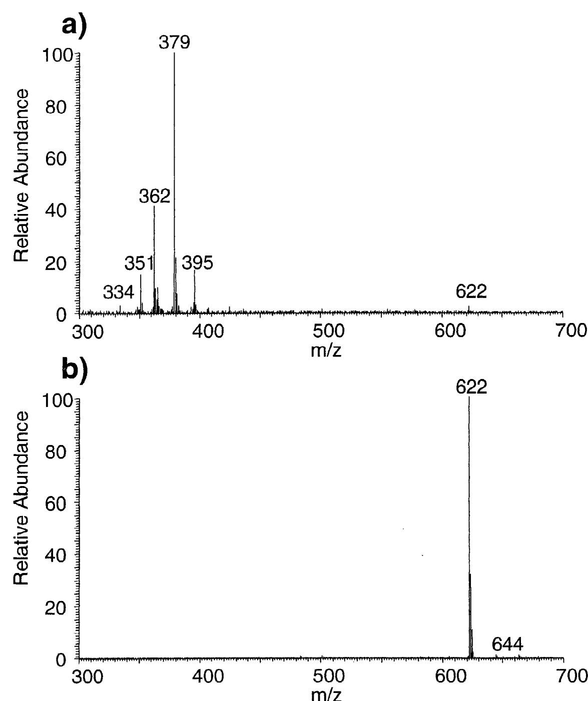

Fig. 4. Mass spectra of **5** by (a) APCI and (b) ESI.

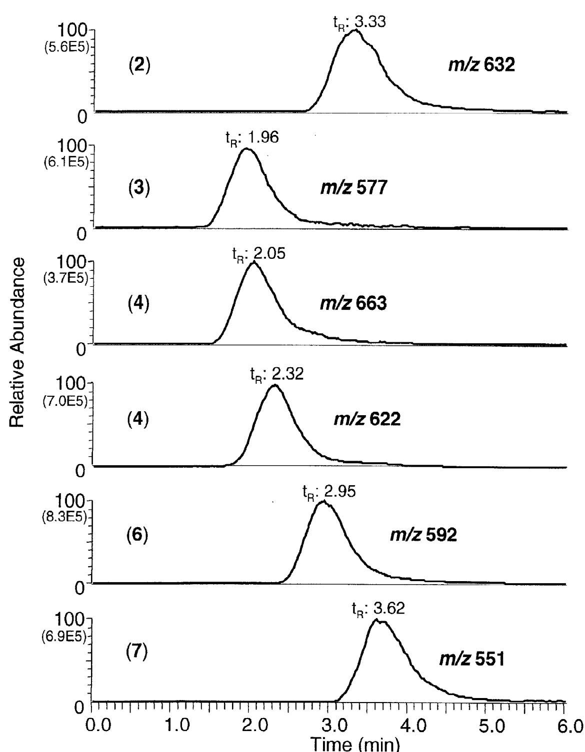

Fig. 5. Selected-ion chromatograms for the mixture of **2**–**7** by IAMC–ESI-MS (The traces were reconstructed for the m/z of the protonated molecules, MH^+, from consecutive full-scan acquisitions). Ion counts representing the normalization level (100) for the traces are given in parentheses on the relative abundance axes, respectively.

m*M* NH_4OAc mobile phase. However, the addition of a small percentage of the organic modifier yielded satisfactory signal intensities and selected-ion chromatograms, as shown in Fig. 5. Despite certain extracolumn peak broadening due to effluent splitting and the relatively long (25 cm) and large-bore (0.1 mm I.D.) fused-silica transfer line supplied by the manufacturer for interfacing a liquid flow to the ESI source, reasonably accurate estimations of the retention times and k'_{IAM} values were still obtained.

In Table 1, k'_{IAM} values determined in our study are shown. Compounds **2**–**9** followed a similar pattern of IAM partitioning with all three mobile phases (pH 5.4 DPBS with 5% acetonitrile, 10 m*M* aqueous NH_4OAc, and 10 m*M* aqueous NH_4OAc with 5% acetonitrile added). In comparison with DPBS as a mobile phase, the 10 m*M* NH_4OAc-based mobile phases gave k'_{IAM} values that had correlation coefficient (R) of 0.984 with the values determined without the addition of the organic modifier, and an R-value of 0.957 when 5% acetonitrile was added. However, the throughput of the method can be increased significantly upon using IAMC–API-MS due to simultaneous estimation of k'_{IAM} values. Theoretically, one may dissolve numerous compounds present in the solution to be injected, as long as the m/z values of the molecular/fragment ions do not coincide and the limit of reliable detection for the individual compounds is not reached. In this regard, ESI because of its low propensity to induce frag-

Table 1
The k'_{IAM} values obtained by IAM chromatography with UV detection, APCI and ESI mass spectrometry

Compound	DPBS, pH 5.4, 5% acetonitrile (UV, 254 nm)	10 m*M* NH_4OAc, pH 5.4 (APCI)	10 m*M* NH_4OAc, 5% acetonitrile, pH 5.4 (ESI)
2	5.88	6.94	4.27
3	0.35	1.06	1.63
4	1.81	2.10	1.81
5	2.73	2.98	2.33
6	4.96	5.79	3.54
7	7.96	8.40	4.83
8	1.77	1.35	1.42
9	2.69	2.27	1.62
Correlation with IAMC–UV, R	–	0.984	0.957

Column: 3 cm×4.6 mm I.D. IAM.PC.DD (d_p = 5 μm) protected by a 1 cm×3.0 mm I.D. guard cartridge; mobile phases as indicated in the table heading; flow-rate: 1.0 ml/min.

mentation of the molecular ions may be more advantageous than APCI. In practical terms, 10–20 compounds may be mixed together without any complications to arise during the simultaneous estimation of k'_{IAM} by using the methods reported here.

4. Conclusion

Modifications of the method generally employed to IAM chromatography with UV detection were needed to connect with API mass spectrometry. Both IAMC–APCI-MS and IAMC–ESI-MS are suitable for the analysis of compounds without UV chromophore and for the simultaneous determination of the k'_{IAM} capacity factors that were found to correlate with those determined by conventional IAM chromatography with UV detection. Thus, these LC–MS techniques can be used as high-throughput methods for early prediction of membrane permeability of potential drug candidates.

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References

[1] P. Artursson, J. Karlsson, Biochem. Biophys. Res. Commun. 175 (1991) 880.
[2] R. A Conradi, A.R. Hilgers, N.F.H. Ho, P.S. Burton, Pharm. Res. 8 (1991) 1453.
[3] L.S. Schanker, D.J. Tocco, B.B. Brodie, C.A.M. Hogben, Pharmacol. Exp. Ther. 123 (1958) 81.
[4] B. Testa, P. Crivori, M. Reist, P.A. Carrupt, Perspect. Drug Discov. Des. 19 (2000) 179.
[5] C. Pidgeon, S. Ong, H. Liu, X. Qiu, M. Pidgeon, A.H. Dantzig, J. Munroe, W.J. Hornback, J.S. Kasher, L. Glunz, T. Szczerba, J. Med. Chem. 28 (1995) 590.
[6] S. Ong, S.J. Cai, C. Bernal, D. Rhee, X. Qiu, C. Pidgeon, Anal. Chem. 66 (1994) 782.
[7] C. Pidgeon, U.V. Venkataran, Anal. Biochem. 176 (1989) 36.
[8] G.W. Caldwell, J.A. Masucci, M. Evangelisto, R. White, J. Chromatogr. A 800 (1998) 161.
[9] S. Ong, L. Hanlan, C. Pidgeon, J. Chromatogr. A 728 (1996) 113.
[10] A. Nasal, M. Sznitowska, B. Bucinski, R. Kaliszan, J. Chromtogr. A 692 (1995) 83.
[11] S. Ong, H. Liu, X. Qiu, G. Bhat, C. Pidgeon, Anal. Chem. 67 (1995) 755.
[12] B.H. Stewart, F.Y. Chung, B. Tait, C. John, O.H. Chan, Pharm. Res. 15 (1998) 1401.
[13] A. Reichel, D.J. Begley, Pharm. Res. 15 (1998) 1270.
[14] T. Salminen, A. Pulli, J. Taskinen, J. Pharm. Biomed. Anal. 15 (1997) 469.
[15] A. Ducarme, M. Neuwels, S. Goldstein, R. Massingham, Eur. J. Med. Chem. 32 (1998) 215.
[16] L. Prokai, A. Zharikova, T. Janaky, X. Li, A. Braddy, P. Perjesi, L. Matveeva, D.H. Powell, K. Prokai-Tatrai, J. Mass Spectrom. 36 (2001) 1211.
[17] L. Prokai, K. Prokai-Tatrai, A. Zharikova, X. Li, J.R. Rocca, J. Med. Chem. 44 (2001) 1623.
[18] C. Somlai, L. Balaspiri, J. Prak. Chem.-Chem. Ztg. 336 (1994) 525.
[19] L. Prokai, W.J. Simonsick Jr., Rapid Commun. Mass Spectrom. 7 (1993) 853.
[20] H. van de Waterbeemd, D.A. Smith, K. Beaumont, D.K. Walker, J. Med. Chem. 44 (2001) 1314.
[21] K. Valko, C.M. Du, C.D. Bevan, D.P. Reynolds, M.H. Abraham, J. Pharm. Sci. 89 (2000) 1085.
[22] D.H. Malin, J.R. Lake, D.A. Smith, J.A. Jones, J. Morel, A.E. Claunch, P.A. Stevens, K. Payza, K.K. Ho, J. Liu, I. Ham, K. Burgess, Drug Alcohol Depend. 40 (1995) 37.
[23] P.P.-C. Tan, J.C. Chen, J.Y. Li, K.W. Liang, C.H. Wong, E.Y.K. Huang, Peptides 20 (1999) 1211.
[24] L. Prokai, A.D. Zharikova, T. Janaky, K. Prokai-Tatrai, Rapid Commun. Mass Spectrom. 14 (2000) 2412.