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Use of Caco-2 cells and LC/MS/MS to screen a peptide combinatorial library for permeable structures

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Abstract

The transepithelial transport of a synthetic peptide combinatorial library containing 375 000 individual peptides was assessed using Caco-2 cell monolayers in order to screen for permeability and deliverability. A series of 150 pools, each containing 2500 tripeptide sequences, were applied to the apical side of Caco-2 monolayers. Basolateral side samples were collected after 4 h and screened by capillary high-pressure liquid chromatography. The majority of pools showed no permeable species, due to low solubility, limited permeability and extensive metabolism. Several pools contained permeable structures, and transport proved reproducible with passage number and time. Permeable structures were identified by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). To discriminate between isobaric structures, several tripeptides were resynthesized and tested as discrete compounds. For example, 1–2% D-Phe–D-Ala–D-Ser–OH was transported across the Caco-2 cell monolayer with a P_{app} value of $0.35–0.69 \times 10^{-6}$ cm/s, which is comparable with the permeability of amino acids (Leu, $P_{app} = 0.30 \times 10^{-6}$ cm/s) and dipeptides (L-Val–L-Val, $P_{app} = 0.18 \times 10^{-6}$ cm/s) (Lennernas, H., Palm, K., Fagerholm, U., Artursson, P., 1996. Comparison between active and passive drug transport in human intestinal epithelial (Caco-2) cells in vitro and human jejunum in vivo. Int. J. Pharm. 127, 103–107; Tamura, K., Bhatnagar, P.K., Takata, J.S., Lee, C.P., Smith, P.L., Borchardt, R.T., 1996. Metabolism, uptake, and transepithelial transport of the diastereomers of Val-Val in the human intestinal cell line Caco-2. Pharm. Res. 13, 1213–1218). These studies demonstrate the techniques used to screen combinatorial libraries for permeability across Caco-2 cells and structurally identify the resulting compounds. Such methodology can be of importance in the achievement of structure–permeability relationships, useful in the design of pharmaceuticallly bioavailable drugs.

Keywords: LC/MS/MS; Caco-2 cell monolayers; Oral absorption; Combinatorial peptide libraries

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1. Introduction

The enormous molecular diversity represented within combinatorial libraries is widely recognized as a valuable source of new leads for drug discovery programs. Compounds exhibiting a desired activity, such as enzyme inhibition or agonistic/antagonistic binding to specific receptors, are readily identified by automated activity screening of combinatorial libraries. For example, high affinity ligands for monoclonal antibodies, potent antimicrobial peptides, protein kinase substrates and peptide inhibitors for HIV protease have all been identified (Geysen et al., 1986; Houghten et al., 1991; Owens et al., 1991; Houghten et al., 1992; Coffen et al., 1994; Till et al., 1994). However, viable drug candidates must possess appropriate physico-chemical and metabolic properties, as well as biological activity. Ideally, drugs intended for oral administration should be reasonably soluble, readily absorbed and metabolically stable in order to reach their target. In vitro rates of drug transport across Caco-2 cell monolayers are frequently used to obtain an initial prediction of oral bioavailability for compounds known to possess a desired biological activity. If libraries or pools of compounds are applied to the apical donor compartment, those compounds capable of paracellular or transcellular transport will be detected in the basolateral receiver compartment. Failure to detect all membrane permeable compounds within a library is of little practical consequence to a drug discovery effort, provided that the subset of permeable species is sufficiently large to afford one or more bioavailable lead compounds. Therefore, we wish to implement screening methods to test membrane permeability during drug development.

As a class, peptides are generally believed to be poor drug candidates due to their low oral bioavailability and propensity to be rapidly metabolized. Nevertheless, synthetic strategies for limiting metabolism have been evolved and a number of peptide based drugs are marketed as nasal sprays (calcitonin, nafarelin), sustained release injectables (leuprolide, goserelin) and orally active formulations (cyclosporin) (Chan et al., 1988; Lindholm, 1991; Rock et al., 1993; Lee et al., 1994; Okada et al., 1994). In addition, specific tripeptides have been observed to permeate brush border membrane vesicles, rat lung tissue, and Caco-2 and alveolar cell monolayers (Amoss et al., 1972; Berteloot et al., 1981; Rajendran et al., 1985, 1986; Skopicki et al., 1988; Gan et al., 1993; Helliwell et al., 1994; Morimoto et al., 1994). Systematic peptide studies have also identified size, charge, conformation and hydrogen bonding potential as important factors governing the membrane permeability of peptides (Kim et al., 1993; Chikhale et al., 1994; Knipp et al., 1995; Okumu et al., 1997). To explore the feasibility of Caco-2 transport studies with combinatorial libraries, we tested a series of 150 peptide trimer pools, each containing 2500 tripeptides, and identified the permeable structures by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) instead of elaborate decoding techniques (Birkett et al., 1991; Metzger et al., 1994).

2. Materials and methods

2.1. Materials

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's phosphate buffered saline, Trypsin–EDTA, Hanks' balanced salt solution, glucose, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes), trifluoroacetic acid and non-essential amino acids were purchased from Sigma (St Louis, MO). Fetal bovine serum was purchased from Gibco (Gaithersburg, MD) and minimum essential medium was obtained from Fisher (Pittsburgh, PA). Transwell inserts were purchased from Costar (Cambridge, MA). Discrete tripeptides were synthesized on an ABI peptide synthesizer using Fmoc chemistry as previously described (Atherton and Sheppard, 1989).

2.2. Cell monolayers

Caco-2 cell subcultures were passaged in minimum essential medium with 10% fetal bovine serum and 1% non-essential amino acids in an atmosphere of 5% CO_2 and 90% relative humid-

ity. Caco-2 monolayers were seeded at a density of 80 000 cells per 12 mm transwell. Polycarbonate membrane (3 μm pore size) transwells had a surface area of 1 cm^2, with 400 μl media in the apical compartment and 1.5 ml media in the basolateral compartment. Cells monolayers were fed every other day for 21–26 days prior to use. All monolayers were from subculture passage numbers between 30 and 70.

2.3. Transport studies

Peptide library pools (Cambridge Research Biomedicals, MA) were reconstituted in 400 μl transport buffer, consisting of Hanks' balanced salt solution with 25 mM glucose, 25 mM Hepes buffer and 2% dimethyl sulfoxide. One milligram library samples contained 2500 tripeptides per pool, with a final concentration of >2 μM for each individual peptide. Transport studies on individual resynthesized peptides were performed at concentrations of 200 μM and 2 mM peptide in the apical compartment. Permeability coefficients (P_{app}) were calculated from the individual tripeptide data and not the library pools. Monolayers were preincubated for 30 min in transport buffer prior to measurement of transepithelial electrical resistance values (World Precision Instruments, New Haven, CT). Library pools were applied to the apical side of the wells and incubated at 37°C for 4 h. Both apical and basolateral samples were collected and quenched with 30 μl 10% trifluoroacetic acid (TFA), to minimize enzymatic digestion of intact transported tripeptides prior to analysis. Finally, transepithelial electrical resistance values were monitored to ensure monolayer integrity after 4 h, where levels were observed to drop slightly, but stayed well above 400 Ω.

2.4. Chromatography

Apical and basolateral samples were analyzed on a Hewlett–Packard 1090 (Avondale, PA) with an ABI UV detector (Pittsburgh, PA) equipped with an LC Packings Z-cell (San Francisco, CA) at 205 nm. An LC Packings Hypercarb S packed capillary column, 5 μm particle size, 15 cm × 320 μm ID was used at an on-column flow rate of 5 μl/min. Separations were obtained using a 1.66%/min gradient from 0 to 100B%, where mobile phase A was 0.1% TFA in water and mobile phase B was 0.05% TFA in acetonitrile:water (9:1).

2.5. Mass spectrometry

Electrospray ionization mass spectra were collected with a Sciex API-III mass spectrometer (Ontario, Canada) in conjunction with UV detection. Spectra were collected in the positive ion mode. The ion spray needle was maintained at 4800 V with an orifice potential of 70–90 V. A mass range of 100–600 was scanned continuously with a 0.2–0.5 Da step size and a dwell time of 2.0 ms. MS/MS spectra were conducted by operating MS-1 focused on the parent ion. Parent ions were collisionally activated by collision with argon:nitrogen (9:1) at a collision gas thickness of $(1–1.5) \times 10^{15}$ molecules/cm^2. Daughter ions were collected with unit mass resolution by scanning MS-2. The mass-to-charge ratio range 50–500 was scanned continuously with a 0.5 Da step size and a 2.0 ms dwell time.

3. Results and discussion

The synthetic combinatorial peptide library consisted of 375 000 C-terminally amidated tripeptides, ZXX-NH_2, where the N-terminal residue was held constant and the two remaining sites were variable and consisted of a combination of 50 D,L amino acids and analogs. Proteinogenic amino acids and non-proteinogenic derivatives such as 4-pyridylalanine, cyclohexylalanine, pyroglutamate, azetidine, β-alanine, sarcosine, pipecolic, nipectotic and isonipecotic acids were included. The library contained 150 pools, each consisting of 2500 compounds, and each pool defined by the N-terminal residue, Z. The 150 pools were further divided into three 50-pool categories possessing either (i) a free N-terminus, (ii) an *N*-acetylated N-terminus, or (iii) an N-terminal cap in place of Z, where N-cap consisted of an array of *N*-acyl groups (*N*-acetyl, *N*-propionyl, *N*-benzoyl, *N*-butyryl, *N*-pivaloyl) or *N*-car-

bamoyl groups (*N*-ethylcarbamoyl, *N*-benzylcarbamoyl, *N*-phenylcarbamoyl).

3.1. Permeability screen

Peptide library pools were reconstituted in 400 μl transport buffer, resulting in a final individual tripeptide concentration of >2 μM. Therefore, samples applied to the apical compartment contained 0.8–1.4 nmoles of each tripeptide. If 10% of a tripeptide were transported, ~ 80 pmoles would be detected in the basolateral media. Preliminary analytical method development on a series of six tripeptides with varying hydrophobicities indicated a limit of detection of 10–20 pmoles (data not shown).

Peptide pools were reconstituted in transport buffer with 2% dimethyl sulfoxide. Dimethyl sulfoxide allowed complete sample solubilization, with the exception of a few pools, and ensured a representative screen of the sample. Pools with visible precipitate were centrifuged prior to application of the supernatant to cells. Furthermore, 2% dimethyl sulfoxide was determined not to affect transepithelial electrical resistance values of cell monolayers.

Analysis of the pools applied to the apical compartments, by capillary LC, revealed numerous, poorly resolved peaks, where the number and intensity of peaks present in a given apical sample varied greatly (Fig. 1). This was expected for pools containing insoluble tripeptides and for tripeptides of similar elution time; however, it did not account for the large variation in peptide concentration between pools. Considering that library pools were supplied as lypophilized 1-mg samples, this suggests library pools were not equally soluble or contained an incomplete set of 2500 equimolar tripeptides. Thus, it was recognized that some permeable species may have been missed because they were either not synthesized, insoluble, competitively blocked from a transporter, or were present in too low a yield.

LC chromatograms of basolateral samples indicated that the majority of the pools contained no detectable permeable species, suggesting that Caco-2 monolayers provided a selective screening model. This was consistent with published transport values of 1–10% for individual peptides (Artursson and Karlsson, 1991; Conradi, et al., 1991; Burton et al., 1992). Furthermore, basolateral samples with no permeable species did not correlate with pools containing a low apical concentration, indicating that permeation was primarily a function of peptide structure and not peptide concentration. Several basolateral samples contained peaks, indicating permeable species (Fig. 2). Pools containing permeable species demonstrated good reproducibility with Caco-2 cells of different passage numbers and were selected for LC/MS analysis (Fig. 3).

3.2. LC/MS identification

Peaks detected by UV in LC chromatograms could be correlated to peaks in the total ion current trace by LC/MS (Fig. 4). This was useful for locating uncharged tripeptides that did not ionize well by MS and would have been missed without UV detection. The mass spectra corresponding to total ion current peak regions were baseline subtracted and analyzed for parent ions corresponding to the molecular weight range expected for tripeptides. Assuming the identity of the N-terminal residue was known, parent ions were searched for possible dipeptide combinations in positions 2 and 3. Mass spectral data was collected at a slightly higher orifice potential so that the parent ion mass and sequence ions derived from the parent ion were obtained (Roepstorff and Fohlman, 1984) (Fig. 5).

For example, when analyzing a pool identified as D-Phe-*X*-*X*-NH_2, where *X* can be any of 50 D,L-amino acids and analogs, LC/MS analysis showed that a peak at retention time 51.75 min had a MH^+ ion at m/z 324 (Fig. 3b). Possible peptide combinations were identified by searching all parent ions resulting from combinations of the N-terminal residue and all possible dipeptide combinations generated with both C-terminal amide and acid variation. Analysis of combinatorial library pools without elaborate decoding strategies required a complete database of molecular weights and fragment ions in order to deconvolute mass spectra in a logical manner. The range in possible molecular weights for the tripeptide li-

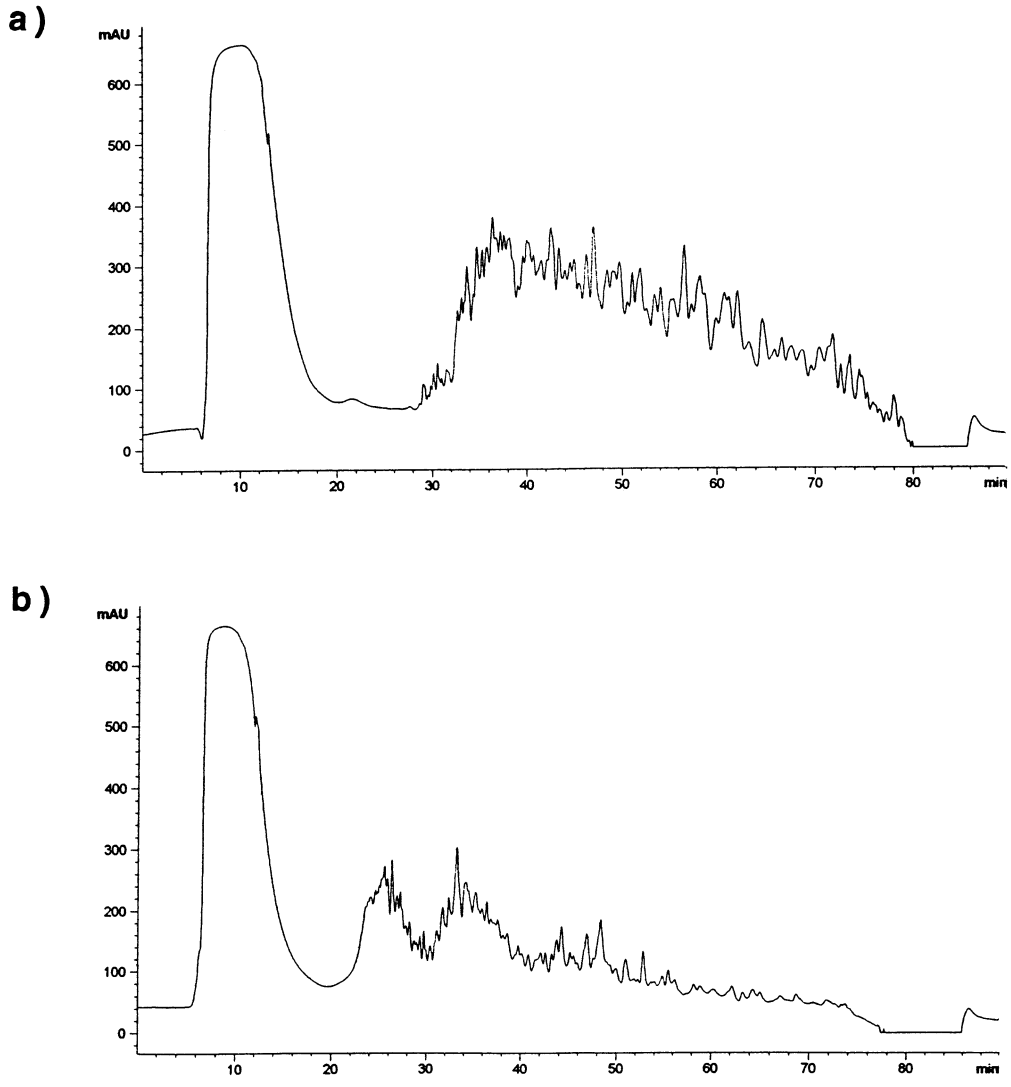

Fig. 1. Capillary LC chromatograms revealing variability in total peptide concentration of reconstituted library pool samples applied to the apical side of Caco-2 cell monolayers.

brary was 171–627, not including species that might have been synthesized by accident. No possible combination of amino acids would yield a tripeptide, D-Phe-*X*-*X*-NH_2, with MH^+ 324, suggesting that libraries may not necessarily contain the parent ion expected from the synthetic strategy. A secondary search strategy simply changed the C-terminal amide to a free carboxyl, allowing a single possible dipeptide combination from the parent ion. The mass indicated that the sequence was either D-Phe-Ala-Ser-OH or D-Phe-Ser-Ala-OH. However, a_1'', a_3, b_1, b_2, b_3, x_1, y_1'' and y_2'' ions from background subtracted mass spectra indicated that the more probable sequence was D-Phe-Ala-Ser-OH, where the chirality of Ala and Ser were not known (Fig. 5). It may be postulated that the C-terminal D-Phe-Ala-Ser-NH_2 serine residue was the L-enantiomer, and is more susceptible to proteolysis to the corresponding D-Phe-Ala-L-Ser-OH (Gan, et al., 1993).

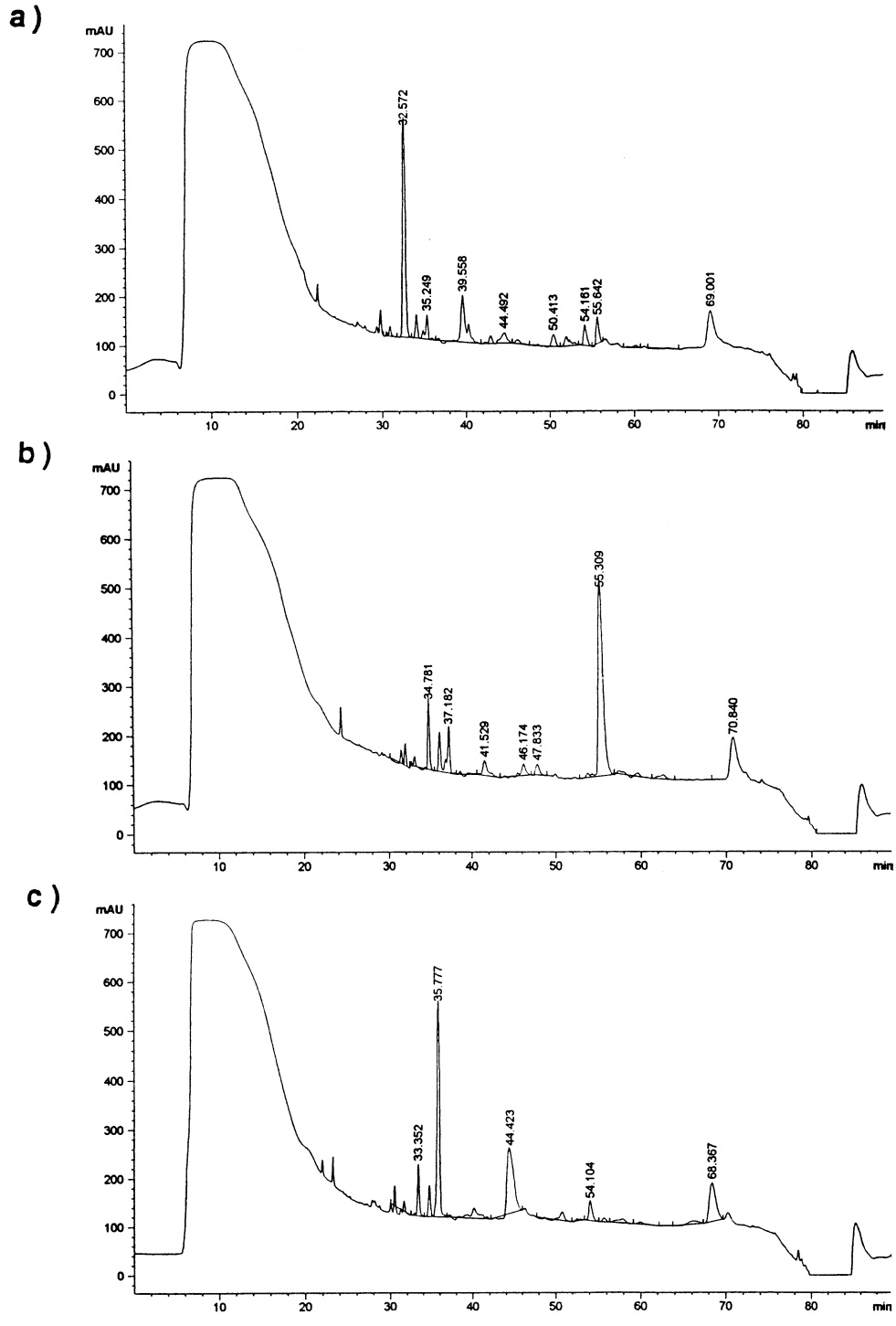

Fig. 2. Capillary LC chromatograms representing three basolateral library pool samples containing transported permeable species.

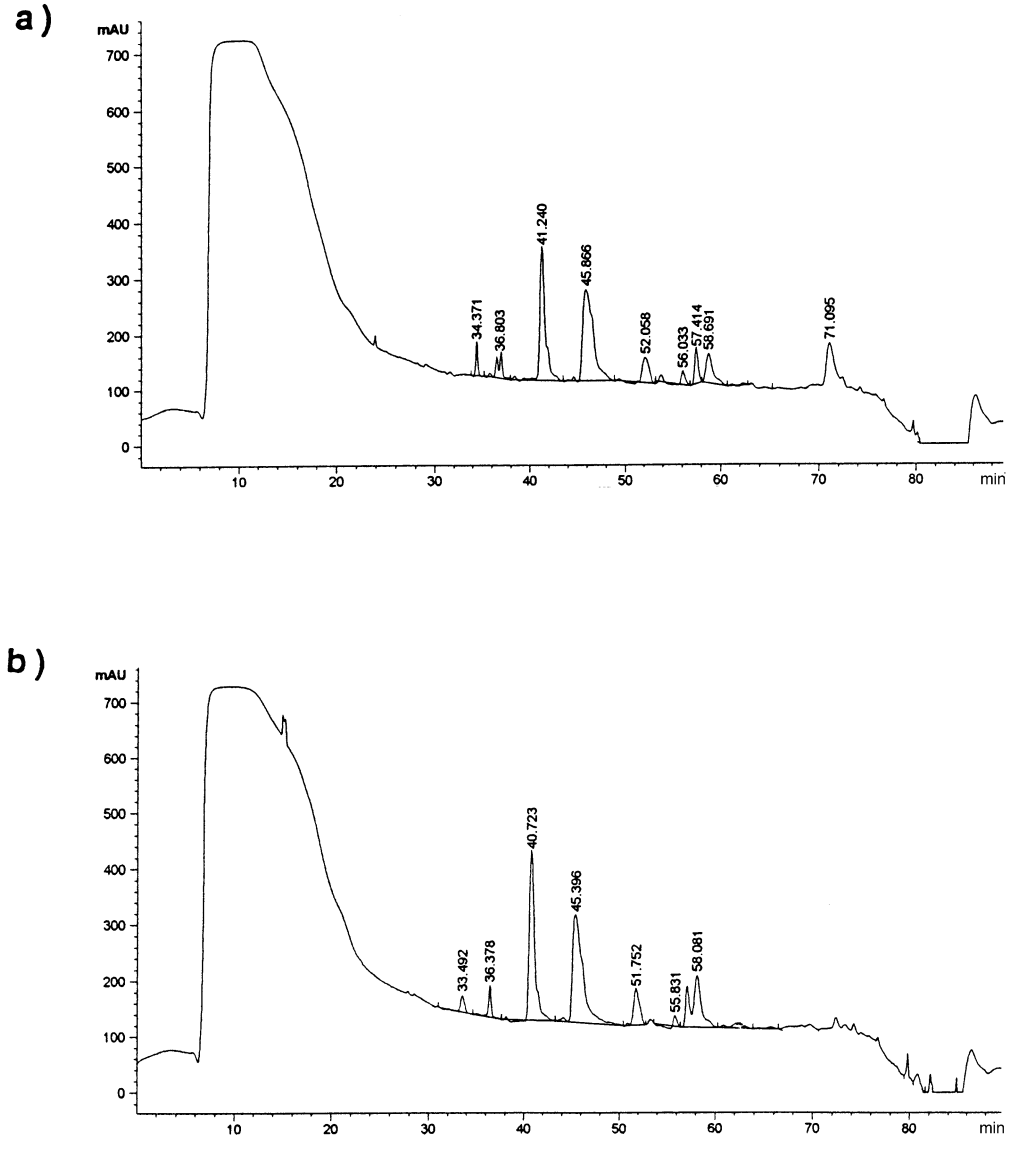

Fig. 3. Capillary LC chromatograms demonstrating reproducibility of tripeptide transport, where examples were performed by different workers with different subcultures.

Similarly, five additional tripeptides were identified using the same methodology (Fig. 6). These peptides were not the only permeable species detected, but were chosen as examples. In all cases, the N-terminal residue was known, and identification of the remaining dipeptide was accomplished by searching parent ions of all possible combinations of pool components. Parent as well as fragment ions were obtained to assist structure identification and are listed in parentheses. The tripeptides identified were Ac-D-Phe-Ala-Ser-OH (a_2, b_1, b_2, x_1, x_2), L-Trp-pyridylalanine-Gly-NH_2 (a_1, b_1, c_1'', x_1), L-Phe-Gly-Val-OH (a_1, a_2'', b_1, b_2, c_1'', c_2'', x_1, z_2), phenylcarbamate-Trp-His-NH_2 (b_2'', c_2'') and L-Tyr-Arg-azetidine carboxamide (a_1, b_1, c_1'', c_2'', y_1'', z_2) (Fig. 6). During the process of

sequencing peaks, dipeptides such as Phe-Asp-OH, Phe-Pro-OH, Phe-Arg-OH and Trp-Trp-OH were also identified. Detection of dipeptides was widespread throughout the basolateral samples, indicative of incomplete synthesis and/or peptide metabolism (Gan, et al., 1994). These dipeptide structures were not pursued because we were primarily interested in transportable tripeptides resistant to degradation.

3.3. LC/MS/MS identification

LC/MS/MS analysis was performed to confirm the tripeptide sequences identified by LC/MS. For example, the fragment ions of Phe-Ala-Ser-OH were generated from the parent ion at MH^+ 324 (Hunt et al., 1986) (Fig. 7). The a_1, a_3, b_3, x_1 and y_2'' ions indicated that the structure corresponded well with D-Phe-Ala-Ser-OH. MS/MS was also performed on Trp-pyridylalanine-Gly-NH_2 and yielded a_1, b_1, c_1'', c_3'', x_1 and several internal ions. Ions corresponding to the mass of glycine and pyridylalanine further supported the sequence. Additionally, MS/MS of Ac-Phe-Ala-Ser-OH confirmed the sequence with sequence ions a_1, a_2, b_1, b_2, b_3, x_1, x_2 and z_1''.

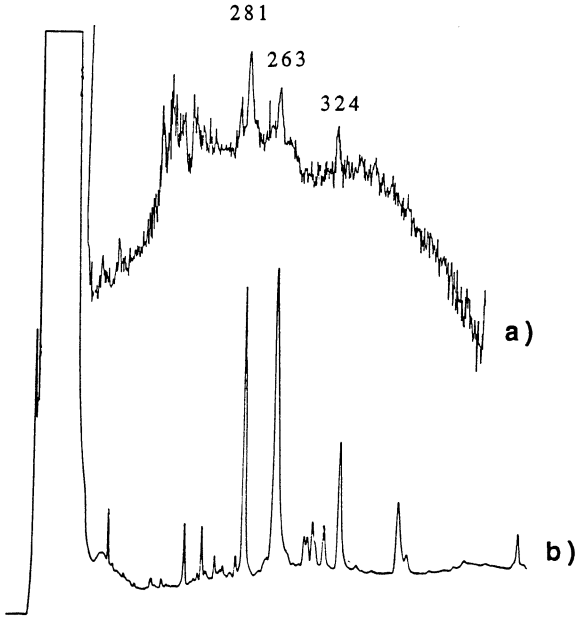

Fig. 4. On-line capillary LC/MS showing (a) TIC trace and (b) UV trace from the basolateral sample shown in Fig. 3.

Mass spectrometry also confirmed that the C-terminal amide was not present in Phe-Ala-Ser-OH. In half the structures identified, the C-terminal amide was present (Fig. 6). However, the library should have exclusively contained amidated C-termini, suggesting that either the library did not consistently contain C-terminal amides, contained additional compounds besides those originally planned, or were cleaved by proteases during transport. Regardless, the library still yielded structures that could be identified as permeable species, where species considered permeable to Caco-2 cells demonstrated at least 1% transport over 4 h.

Because mass spectrometry cannot differentiate between isobaric amino acids, it was not known whether the alanine was D,L-Ala, β-Ala or sarcosine, or whether the tripeptide contained a D- or L-serine in the sequence Phe-Ala-Ser-OH. Similarly, D-Ala-Phe-Pip-OH was identified, where the isomeric possibilities included D,L-phenylalanine and pipecolic acid, nipecotic acid or isonipecotic acid. However, it can be surmised that β- or D-amino acids and analogs would be less susceptible to cellular proteases and would be present in higher concentrations on the basolateral side. Therefore, discrete sequences were resynthesized and individually tested for permeability.

3.4. Transport of discrete tripeptides

Assuming transported species were not being metabolized, D-amino acids were used to synthesize tripeptides. The sequences had either free or capped termini in order to determine the preferred structure; specifically, D-Phe-D-Ala-D-Ser-NH_2, D-Phe-D-Ala-D-Ser-OH, Ac-D-Phe-D-Ala-D-Ser-OH. Individual discrete tripeptides were spiked into their corresponding peptide pools and assayed for transport. Only D-Phe-D-Ala-D-Ser-OH was determined to transport across Caco-2 cell monolayers, indicating that the terminal cap had an effect on overall peptide permeability, either by enhancing affinity to transporters or diminishing hydrolytic degradation. LC/MS of the basolateral peak co-eluting with D-Phe-D-Ala-D-Ser-OH produced a parent ion of 324, positively identifying the transported tripeptide. Approximately 1–2%

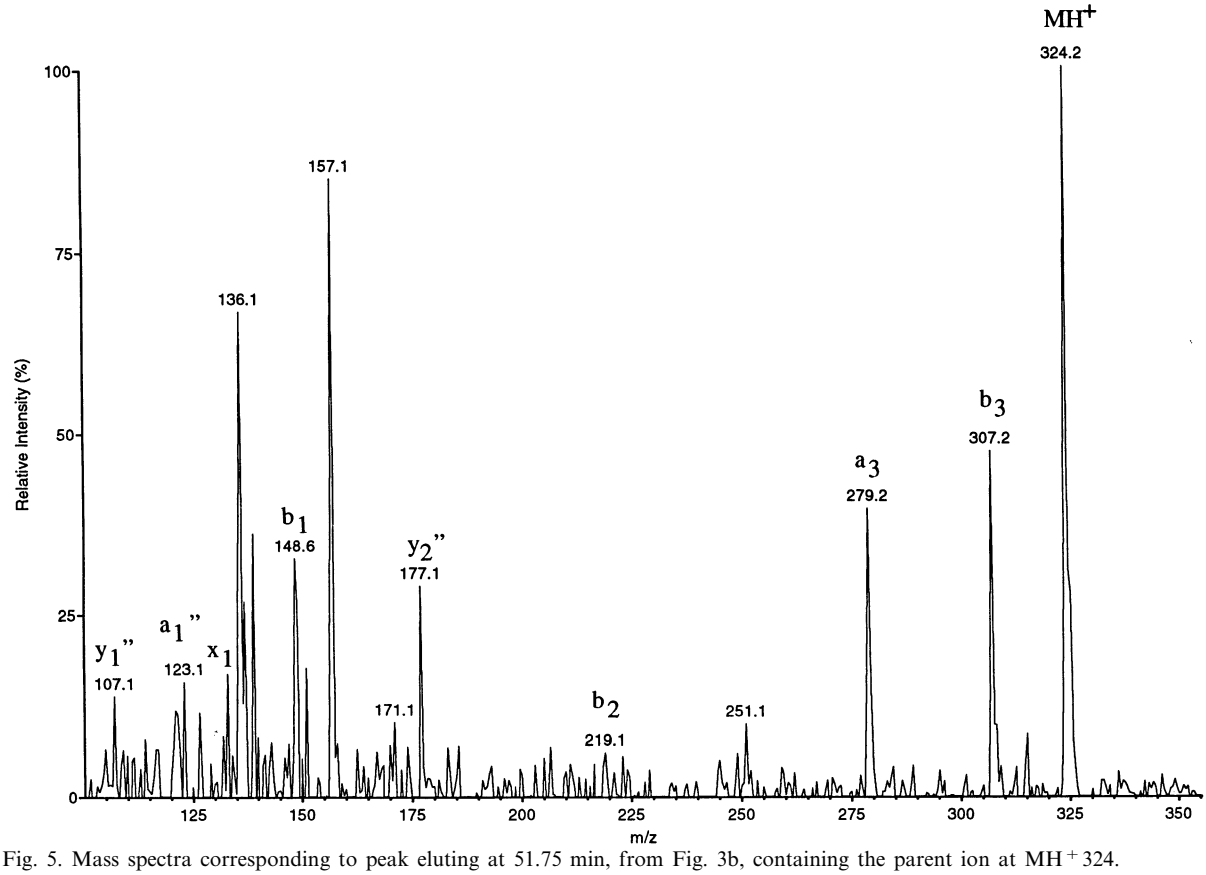

Fig. 5. Mass spectra corresponding to peak eluting at 51.75 min, from Fig. 3b, containing the parent ion at MH^+ 324.

of D-Phe-D-Ala-D-Ser-OH was transported over 4 h, with a P_{app} value of $0.35–0.69 \times 10^{-6}$ cm/s. P_{app} values between 0.1 and 1.0×10^{-6} cm/s indicate drug compounds transported between 1 and 100%, similar to those observed for pGlu-His-Pro-NH_2 ($P_{app} = 0.95 \times 10^{-6}$ cm/s) (Artursson and Karlsson, 1991; Gan et al., 1993). Similarly, other workers revealed P_{app} values of 0.55×10^{-6} and 1.05×10^{-6} cm/s for Phe-Phe-Gly-OH and Phe-Gly-OH, respectively (Conradi, et al., 1991). The mechanism of transport was not explored, as this was not the objective of the study.

4. Conclusion

We have demonstrated the feasibility of evaluating the transport of combinatorial peptide libraries across Caco-2 cell monolayers. The potential power of this technique, when coupled with capillary LC separation, low wavelength detection and structural determination by high-resolution MS, was evidenced by the successful identification of several transported peptides. Not surprisingly, little to no transport was observed for most of the library tested peptides. Nevertheless, significant rates of transport were measured for several peptide structures.

A major challenge to measuring transport rates of libraries is the need for sensitive quantitative analytical techniques. Sensitivity is dictated by the achievable concentration of transported compounds in the basolateral receiver compartment. Moreover, the concentration of discrete compounds transported will be limited by the size and concentration of the pool applied to the apical

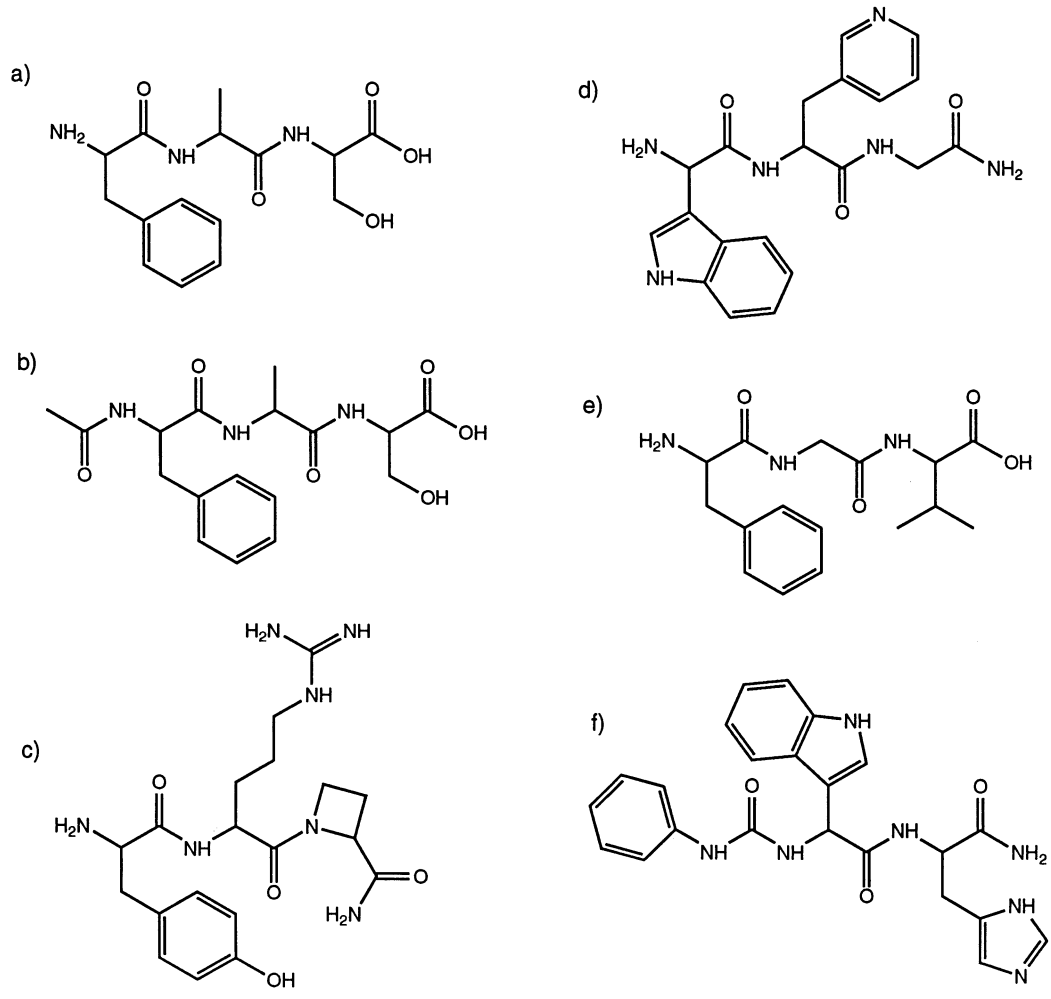

Fig. 6. Tripeptide structures for transported species: (a) Phe-Ala-Ser-OH, (b) Ac-Phe-Ala-Ser-OH, (c) Tyr-Arg-azetidinecarboxamide, (d) Trp-pyridylalanine-Gly-NH_2, (e) Phe-Gly-Val-OH and (f) phenylcarbamate-Trp-His-NH_2.

side, the solubility in the transport medium and the transport rate. The transport medium must be relatively non-toxic and must not compromise the tight junctions in the Caco-2 cell monolayer, as demonstrated by the 2% dimethyl sulfoxide used in these studies. Further development will be necessary to define biological compatible solvents, perhaps based on combination of detergents, co-solvents and/or molecular chaperones, which will increase the solubility of combinatorial libraries and thereby increase the detection limits for transport.

Although we screened for transported species by capillary LC/MS/MS, a much more powerful approach would be to couple permeability and enzyme activity or receptor-binding assays into one high through-put screening method. The target receptor or enzyme can be incorporated in the basolateral receiver compartment so that active species are detected within the subset of compounds transported across Caco-2 monolayers. Freely permeable, but inactive compounds will not be identified, nor will impermeable active compounds, when transported compounds are detected in an assay for their biological activity.

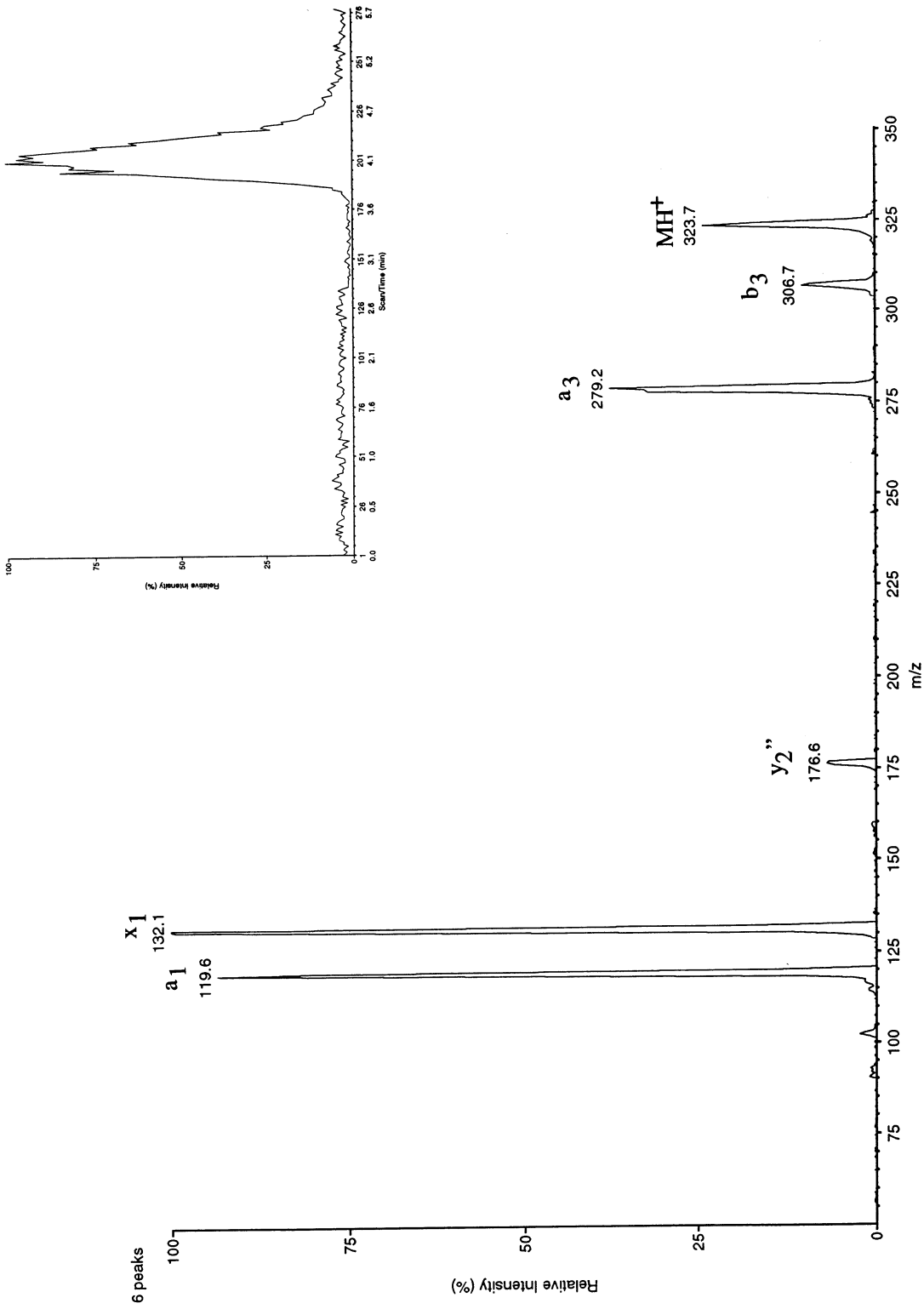

Fig. 7. LC/MS/MS for Phe-Ala-Ser-OH depicting sequence ions.

The ultimate goal of predicting membrane permeability and bioavailability purely on the basis of structure will be achieved much sooner if structure–permeability relationships are determined for entire combinatorial libraries, instead of compound subsets selected for specific biological activities. Clearly, more extensive analyses of a broader range of peptide structures will be needed if a structure–permeability algorithm of any general predictive value is to be devised. Eventually, permeability-conferring structures identified by screening combinatorial libraries for membrane transport characteristics independent of pharmacological activity might be combined with biologically active structures in an effort to rationally design bioavailable active drugs.

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