JOURNAL OF MASS SPECTROMETRY, VOL. 33, 264–273 (1998)

Rapid Methods for Screening Low Molecular Mass Compounds Non-covalently Bound to Proteins Using Size Exclusion and Mass Spectrometry Applied to Inhibitors of Human Cytomegalovirus Protease†

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General and rapid methods were developed for determining the extent of non-covalent binding between small molecules and proteins, using the model system of human cytomegalovirus protease and several drug candidates which inhibit the protease by non-covalently binding to it. The assay was performed by off-line coupling of size-exclusion methods with mass spectrometry in the following manner. The protease and inhibitor were incubated together under native conditions and then subjected to separation based on size, by use of a spin column (gel permeation chromatography) and/or a microconcentrator (ultrafiltration). The spin column selectively passed the high molecular mass (M_r) protease and trapped low M_r molecules. Alternatively, the microconcentrator passed low M_r molecules and retained the protease. If the inhibitor bound non-covalently to the protease, both the inhibitor and protease passed through the spin column (or were retained by the microconcentrator). Electrospray ionization mass spectrometry was used to assay the spin column eluate (or the microconcentrator retentate) and to characterize the amounts of protease and inhibitor based on known standards. An advantage of these techniques is that a mixture containing inhibitors can be analyzed in the presence of the protease, and inhibitors with the greatest binding affinity can be identified. Non-covalent binding specificity was demonstrated using spin columns by comparing the binding affinity of inhibitors using several mutants of cytomegalovirus protease. The techniques described are applicable to the rapid screening of compound libraries for selecting substances which bind non-covalently to a known protein. © **1998 John Wiley & Sons, Ltd.**

J. Mass Spectrom. **33**, 264–273 (1998)

KEYWORDS: electrospray ionization mass spectrometry; human cytomegalovirus protease; drug screening; non-covalent complexes; proteins; inhibitors; size-exclusion chromatography; gel permeation chromatography; ultrafiltration; microconcentration

INTRODUCTION

A property of a useful drug candidate is the ability to form a tightly bound non-covalent complex with its target protein. Using the model system of human cytomegalovirus protease (CMVP), a simple, reliable and rapid method was developed for identifying low molecular mass (M_r) inhibitors of CMVP which bind non-covalently to the enzyme. Separation techniques alone, such as affinity chromatography, gel permeation chromatography and dialysis, were not considered sufficiently rapid and specific to characterize fully the non-covalently bound complexes. However, a spectrometric method, such as mass spectrometry (MS), alone or coupled to a modified separation technique, was considered as a possible rapid method for determining the relative abundances of protease and inhibitor in the non-covalently bound complexes.

Mass spectrometry is a useful technique for analyzing non-covalent complexes formed between small molecules and proteins. Two possible approaches utilizing mass spectrometry, direct or indirect analysis, can be taken for characterizing protease–inhibitor complexes. Direct methods utilize exclusively mass spectrometry to analyze the nature of the non-covalent complexes formed under native conditions. Under appropriate experimental conditions the binding stoichiometry (distribution) of the non-covalently bound drug to the protein may be determined. In contrast, indirect methods utilize biochemical and chromatographic methods for preparing and separating the complexes prior to mass spectrometric analysis, which can be performed under denaturing conditions. In the latter case, mass spectrometry serves as an ancillary and selective detector for the individual components of the non-covalent complex, namely the small molecules and the

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† Preliminary reports of this work were presented at the 44th ASMS Conference on Mass Spectrometry and Allied Topics, May 12–16, 1996, Portland, OR, and at the 45th ASMS Conference on Mass Spectrometry and Allied Topics, June 1–5, 1997, Palm Springs, CA, USA.

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CCC 1076–5174/98/030264–10 $17.50

Received 16 October 1997
Accepted 3 December 1997

protein. Since the individual components are measured separately, the indirect method measures only the molar ratio (average loading) of the drug and protein in the non-covalent complex and not the binding stoichiometry (distribution) between the drug and protein in the non-covalent complex.

Electrospray ionization (ESI) MS has been used extensively for the direct analysis of non-covalent complexes[1–18] and matrix-assisted laser desorption/ionization (MALDI) MS to a much lesser extent.[19–23] In the electrospray studies, the underlying principle is that the mass spectrometer directly samples the complexes present in the condensed phase, thereby allowing the direct measurement of the binding stoichiometry (distribution) of the members of the non-covalent complex. A number of types of non-covalent protein complexes have been studied in this way, including heme–protein complexes, drug–protein complexes, nuclei acid–protein complexes and even protein–protein complexes.[24] In these studies, the proteins were generally prepared in the native state utilizing a solvent system consisting of water buffered with ammonium acetate at pH 7. Under these native conditions, the response of the ESI mass spectrometer is not optimum. To acquire data with a high signal-to-noise ratio, extensive signal averaging is necessary, which is very time consuming especially when nanospray methods are used. Higher sensitivities could be achieved if data for the complexes were obtained under denaturing conditions, such as acidic pH and volatile denaturing solvents (e.g. acetonitrile or methanol), allowing more rapid data acquisition.

Affinity chromatography coupled with mass spectrometry has been used extensively to characterize small molecules which bind selectively to proteins or antibodies.[25–28] In these cases, a mass spectrometer is used as the detector for the components eluted from the affinity columns. A unique application of affinity chromatography coupled to mass spectrometry is illustrated by the use of probe tips prepared with bound antibodies or proteins to select specific components from biological fluids.[29–32] Similar methodologies for the mass spectrometric analysis of non-covalently bound molecules on chips and optical fibers coated with bound proteins used for surface plasmon resonance-based biomolecular interaction analysis (SPR-BIA) have recently been reported.[33,34] The probe tips, chips and fibers, when analyzed under MALDI/MS conditions, can reveal unique analytes which specifically bind under non-covalent conditions. These affinity chromatographic techniques may not be very rapid or sensitive, and the preparation of columns, tips, chips and fibers may not be trivial.

Size-exclusion gel permeation chromatography (GPC) coupled off-line with mass spectrometry has been used extensively for polymer analysis[35–45] and recently for protein characterization[46–48] and the analysis of combinatorial libraries.[49,50] The generation of the gel permeation chromatogram is very time consuming and often cannot resolve the complex from the starting protein since the mass differences are relatively very small. However, the centrifugation of a miniature GPC column, referred to as a GPC spin column,[51] which retains low M_r components but excludes high M_r components, is a rapid and efficient method for resolving the protease and protease–inhibitor complex from non-complexed inhibitor. A preliminary study using GPC spin columns for screening a variety of ligands non-covalently bound to human serum albumin protein was recently reported by Vouros and co-workers.[52] In a similar manner, dialysis is an excellent method for separating low M_r from high M_r components but it can be very time consuming and can increase sample volumes, requiring additional concentration steps. A number of these disadvantages have recently been reduced by using the technique of pulsed ultrafiltration.[53] However, the technique of microconcentration, which combines centrifugation with ultrafiltration, utilizes membranes which retain molecules above a specific M_r cut-off, and can be used to rapidly and efficiently separate low M_r inhibitors from retained high M_r protease–inhibitor complexes.[54–56] An example of the use of microconcentration coupled with mass spectrometry is the isolation of active benzodiazepine analogs in the presence of polyclonal antibodies raised against specific benzodiazapines, as reported by Henion and co-workers.[56]

We have been studying the protease of human cytomegalovirus, in an effort to identify low M_r inhibitors useful for the treatment of viral infection. Previously, we described an inhibitor (compound CL13933, a thiophile) which forms disulfide bonds with CMVP[57,58] and also a number of additional inhibitors which promote intramolecular disulfide bond formation within CMVP.[59,60] In addition, we identified several compounds which bind non-covalently to CMVP and needed to develop a rapid, sensitive and specific method to understand better the nature of the interactions of CMVP with these inhibitors.[61] In this paper, we describe in detail the methodology developed and the results obtained using size-exclusion techniques and ESIMS for screening and characterizing this type of interaction: non-covalent binding of the inhibitor to CMVP via a reversible enzyme–inhibitor complex. The technique utilizes size-exclusion GPC spin columns and/or ultrafiltration devices (microconcentrators) for isolating non-covalently bound inhibitor–protease complexes prepared under native conditions, which are then introduced under denaturing conditions into an ESI mass spectrometer, for monitoring and quantitating the individual components of inhibitor and protease. The sample preparation, isolation and detection steps are performed and optimized individually. The methodology is simple to apply and rapid to implement, and allows the characterization of specific and non-specific binding of low M_r molecules to protease and the molar ratio (average loading) of inhibitor to protease in the complex.

EXPERIMENTAL

Proteases

Wild-type CMVP (M_r 28 040.6) contains 256 amino acid residues and readily autodigests between amino

acid (AA) residues 143 and 144, generating 12 kDa (AAs 1–143) and 15 kDa (AAs 144–256) CMVP fragments.[62,63] A number of mutant forms of CMVP have been prepared by site-directed mutagenesis and purified from solubilized inclusion bodies, for studying the biochemical properties of the protease.[57,59] Mutants A144L (M_r 28 082.8) and A144D/C87A/C138A/C161A (M_r 27 956.7) do not undergo autodigestion but otherwise retain full proteolytic activity. Mutants S132A (M_r 28 024.6) and E122V/A144G (M_r 27 996.6) are enzymatically inactive; serine 132 is an active site residue.[64–67]

Inhibitors

The inhibitors of CMVP used in these studies are listed in Table 1 and consist of a peptidic difluoromethylene ketone, DFMK (M_r 988.5) (**1**), a peptidic trifluoromethyl ketone, TFMK (M_r 545) (**2**), and a dibromoquinazoline, DBQ (M_r 489) (**3**). The IC_{50}s (the concentration at which 50% inhibition of protease activity is achieved) for these compounds are also listed in Table 1. CMVP (60 μM) was incubated with a known molar excess of inhibitors in 10 mM ammonium acetate (pH 7.5) for 1 h at 25 °C. The samples were then subjected to the separation methods described below.

Gel permeation chromatography (GPC) (spin column)

GPC spin columns[51] were prepared by filling 1 ml disposable polypropylene syringes (3 mm i.d. × 450 mm length), plugged with silanized glass-wool at the exit port, with Sephadex G-25 resin (Pharmacia), a dextran crossed-linked with epichlorohydrin, which was washed and swollen with water. The resin retains molecules with M_r < 3000 Da but elutes protein. The syringe was centrifuged without sample to pack down the resin to 0.8 ml and then loaded with about 100 μl of an incubated CMVP–inhibitor sample and subjected to centrifugation. The eluate was collected in a polypropylene microcentrifuge tube for further analysis. Each centrifugation step was performed at 900 *g* (Beckman Model GPKR centrifuge) for 2 min at 4 °C.

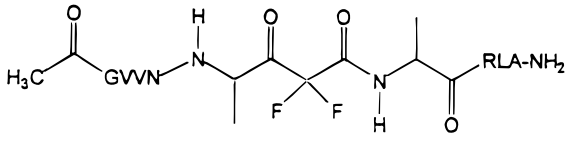
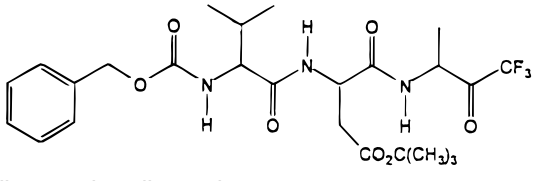
Ultrafiltration (microconcentrator)

Ultrafiltration microconcentrators (3000 Da cut-off; Microcon-3, Amicon, Beverly, MA, USA) were used as directed.[68] Generally, the microconcentrator was centrifuged at 14 000 *g* (Eppendorf Model 5415C centrifuge) for 10 min. After centrifugation, the filtrate contained material of <3000 Da and the retentate contained material of >3000 Da, such as CMVP or CMVP bound to inhibitor. To examine the retentate, the material above the ultrafiltration membrane was washed once with ~400 μl of water or buffer and centrifuged (14 000 *g* for 10 min) to remove free excess inhibitor. (The number and volume of the retentate washes should be kept to the minimum because each wash re-establishes equilibrium between the protein–inhibitor complex, the free protein and the free inhibitor, possibly permitting excessive amounts of inhibitor to be removed from the retentate.) The microconcentrator was then inverted and centrifuged at 1000 *g* for 3 min to collect the retentate. To examine only the low M_r components of the retentate which were bound to CMVP, the material above the ultrafiltration membrane (retentate) was washed once more with ~50–100 μl of 3% acetic acid in acetonitrile–water (1:1) (to denature the complex and release bound inhibitor) and centrifuged at 14 000 *g* for 10 min to collect the filtrate containing only the low M_r components.

Mass spectrometry

Electrospray ionization mass spectra were obtained with a Micromass Quattro I triple-quadrupole mass spectrometer equipped with a Micromass electrospray source, r.f. hexapole lens and Megaflow gas nebulizer probe. The capillary sprayer voltage was set to ~3.0 kV, the high voltage lens was set to 250 V, the sampling cone voltage was maintained at ~20 V, the nozzle–skimmer offset was 5 V and lens 3 was set to −20 V. The source temperature was maintained at ~75 °C. The nebulizer and bath gases were nitrogen delivered at flow rates of 10 and 300 l h^{-1}, respectively. The CMVP samples were prepared at ~10 pmol μl^{-1} in a solvent

Table 1. Structures and IC_{50}s of CMVP inhibitors

Inhibitor name	Nominal M_r	Inhibitor structure	IC_{50} for CMVP A144L (μM)
(**1**) DFMK	988		20
(**2**) TFMK	545		29
(**3**) DBQ	489	Dibromoquinazoline analog	41

consisting of 3% acetic acid in acetonitrile–water (1:1). About 10 μl of the CMVP sample were infused into the source of the mass spectrometer at a flow rate of ~4 μl min^{-1} utilizing a carrier solvent of acetonitrile–water (1:1) with a dual syringe pump (ABI Model 140B). Data were acquired at 16 data points Da^{-1} over the mass range ~300–1500 Da with scan times of 15–30 s. Ten to twenty spectra were averaged, smoothed and baseline subtracted. The mass spectrometer was calibrated with sodium iodide or polyethylene glycol mixtures.

Assay for non-covalent binding of low molecular mass compounds to CMVP

The two general methods developed for quantitating the extent of non-covalent binding between a protein (CMVP) and inhibitor using ESIMS are as follows. (i) The incubated CMVP–inhibitor sample, under native conditions, is passed through the GPC spin column which selectively passes the high M_r CMVP and retains the low M_r inhibitors. If the inhibitor binds non-covalently to CMVP, both the inhibitor and CMVP pass through the spin column, and ESIMS is used under denaturing conditions [3% acetic acid in acetonitrile–water (1:1)] to assay the eluate and to quantitate the amount of inhibitor based on known standards. If the CMVP and inhibitor signals overlap, the sample is transferred to a microconcentrator and washed with denaturing solvent and the low M_r filtrate is analyzed by ESIMS. (ii) Alternatively, the incubated CMVP–inhibitor sample, under native conditions, is placed in a microconcentrator and washed with solvents which do not denature the native state of the protein, generally water or buffer. The retentate is then analyzed by ESIMS under denaturing conditions [3% acetic acid in acetonitrile–water (1:1)]. If the CMVP and inhibitor signals overlap, the sample is transferred to a microconcentrator and washed with denaturing solvent, and the filtrate containing low M_r components is analyzed by ESIMS. These two procedures are illustrated in flow diagrams (Fig. 1), with the individual and total times needed to complete the processing of the complexes and ESIMS detection. The most rapid analysis utilizes a GPC spin column and ESIMS (7 min per assay), and the more time-consuming analyses utilize a microconcentrator (in one or two steps) and ESIMS (28 and 35 min per assay, respectively). An intermediate method utilizing a GPC spin column, microconcentrator and ESIMS takes 17 min per assay. The time per assay is considerably shortened when multiple numbers of samples are prepared in parallel and analyzed serially by ESIMS. Under these conditions, the main throughput limitation is the ESIMS analysis, which takes about 5 min per sample.

Background ESI mass spectra were obtained using method (i) and/or (ii) from the spin column eluates and/or the microconcentrator filtrates for the low M_r inhibitors alone and CMVP alone. These control experiments demonstrated that inhibitors, when not complexed with CMVP, do not pass through the spin column and are not retained by the microconcentrator, and that the background peaks of CMVP can be distinguished from the inhibitor peaks.

Quantitation of the inhibitors was performed by comparison of the integrated peak areas of the molecular ions in the ESI mass spectra for the known standards with that of the unknown concentrations. The CMVPs were quantitated prior to the spin column or microconcentration steps utilizing the Bio-Rad Bradford protein assay (Bio-Rad, Cat. No. 500-0006). Following the spin column or microconcentrator steps, the samples were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Equal volumes of protein samples, taken before and after the spin column analysis, were loaded on to a Phastgel (10–15% gradient acrylamide gel) and run on a Phastsystem (Pharmacia, Cat. No. 18-1018-23), then stained with Coomassie Brilliant Blue. Protein bands were compared visually for recovery. The protein recoveries were found generally to be nearly complete.

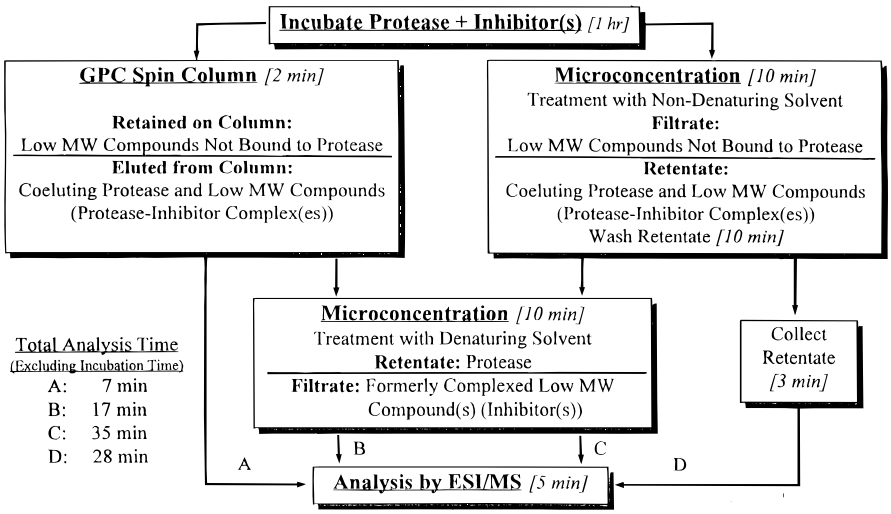


Figure 1. Flow diagram for preparing and isolating non-covalently bound CMVP–inhibitor complexes by the size-exclusion techniques of GPC spin column and ultrafiltration (microconcentration) prior to analysis by ESIMS. Also indicated are the times taken for each of the processing steps and the total time of each of the four possible analyses. Note that the GPC spin column steps are more rapid than the microconcentrator steps.

RESULTS AND DISCUSSION

Rapid screening size-exclusion–mass spectrometric assay for non-covalently bound complexes

Several inhibitors of CMVP were identified by screening using a scintillation proximity assay.[69] The DFMK (**1**) and TFMK (**2**) compounds are substrate analogs of CMVP containing, respectively, difluoromethylene ketone[70–73] and trifluoromethyl ketone moieties,[70–74] predicted to interact with the serine nucleophile S132 of CMVP.[64–67] The IC_{50}s for these compounds are given in Table 1.

An impure sample of DFMK (M_r 988.5) (**1**) [see ESI mass spectrum in Fig. 2(A)] was incubated with CMVP A144D/C87A/C138A/C161A in a molar ratio of CMVP to DFMK of 1: ~ 10 for 1 h at 25 °C. The resulting mixture was transferred to a GPC spin column and the eluate was analyzed by ESIMS. As illustrated in Fig. 2(B), the ESI mass spectrum of the eluate consists of a series of multiply charged peaks related to CMVP in the *m/z* region 700–1200 and a series of peaks related to DFMK at *m/z* 1007.4, 495.3 and 486.7, corresponding to $[M + H_2O + H]^+$, $[M + 2H]^{2+}$ and $[M + 2H - H_2O]^{2+}$, respectively. Note that components corresponding to **1** and the hydrated form of **1** eluted from the spin column together with CMVP, demonstrating non-covalent binding of the compounds to CMVP, otherwise only CMVP would have eluted from the spin column. As a control, the impure sample of DFMK, at the same concentration as used in the incubation experiment, was passed through the spin column, and all peaks, including those corresponding to DFMK, were absent.

The spin column eluate (the sample used to obtain Fig. 2(B) was next placed in a microconcentrator with a denaturing solution of 3% acetic acid in acetonitrile–water (1:1). This treatment will dissociate the DFMK from CMVP, and the DFMK (but not CMVP) should pass through the microconcentrator filter. The filtrate was collected and analyzed by ESIMS [Fig. 2(C)]. Note the absence in the mass spectrum of the ion distribution

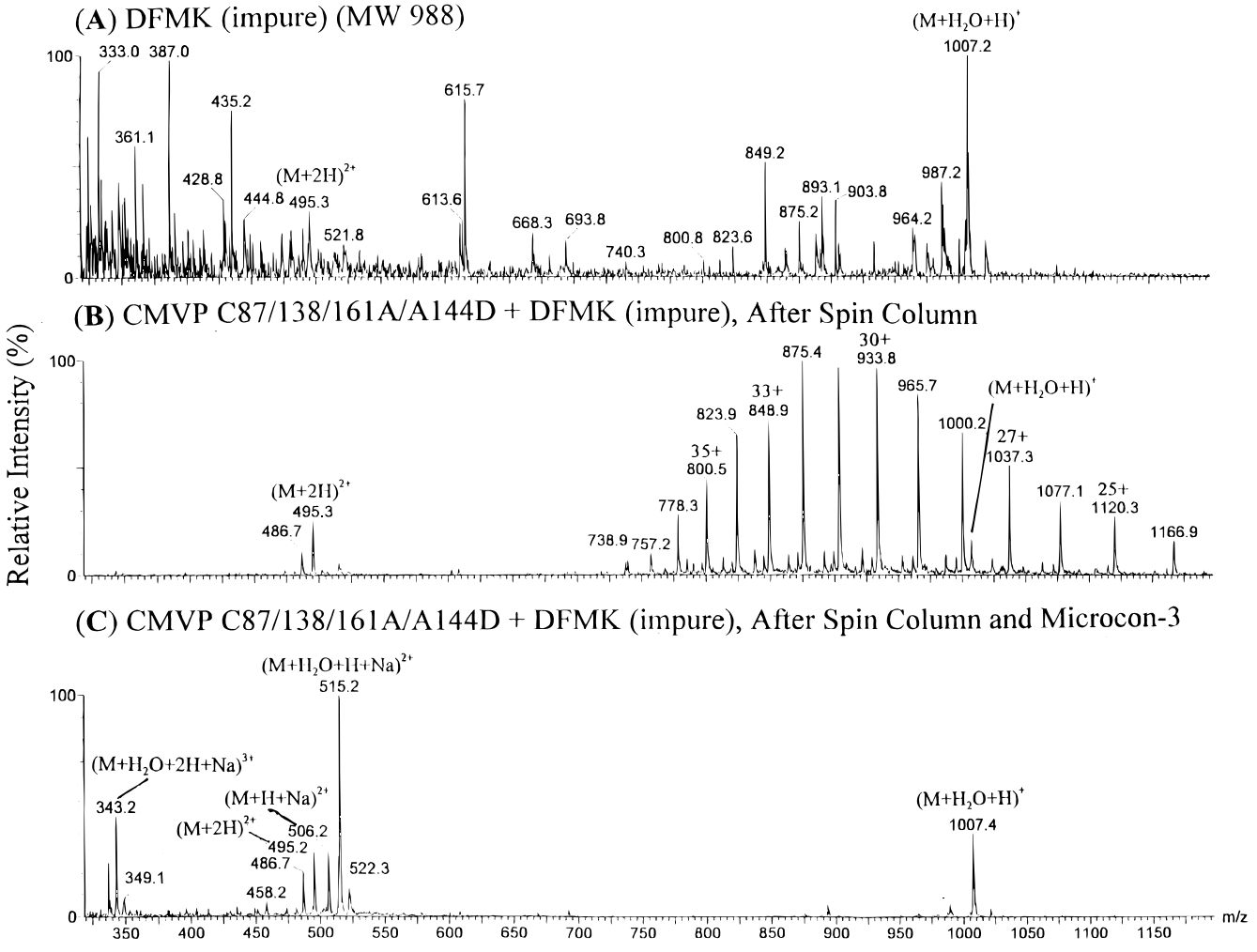


Figure 2. ESI mass spectra obtained from the rapid screening assay of non-covalently bound protease–inhibitor complexes. Enzymatically active CMVP A144D/C87A/C138A/C161A was used in this experiment. (A) ESI mass spectrum of impure inhibitor DFMK (M_r 988.5) (**1**), no spin column or microconcentrator used. (B) ESI mass spectrum of the spin column eluate of CMVP A144D/C87A/C138A/C161A and DFMK, incubated at a molar ratio of 1 : ~10. (C) ESI mass spectrum of the microconcentrator filtrate (3 kDa cut-off ultrafiltration membrane used) obtained under denaturing conditions (3% acetic acid in water–acetonitrile (1 : 1) from the non-covalently bound complex of CMVP A144D/C87A/C138A/C161A and DFMK produced from the spin column eluate.

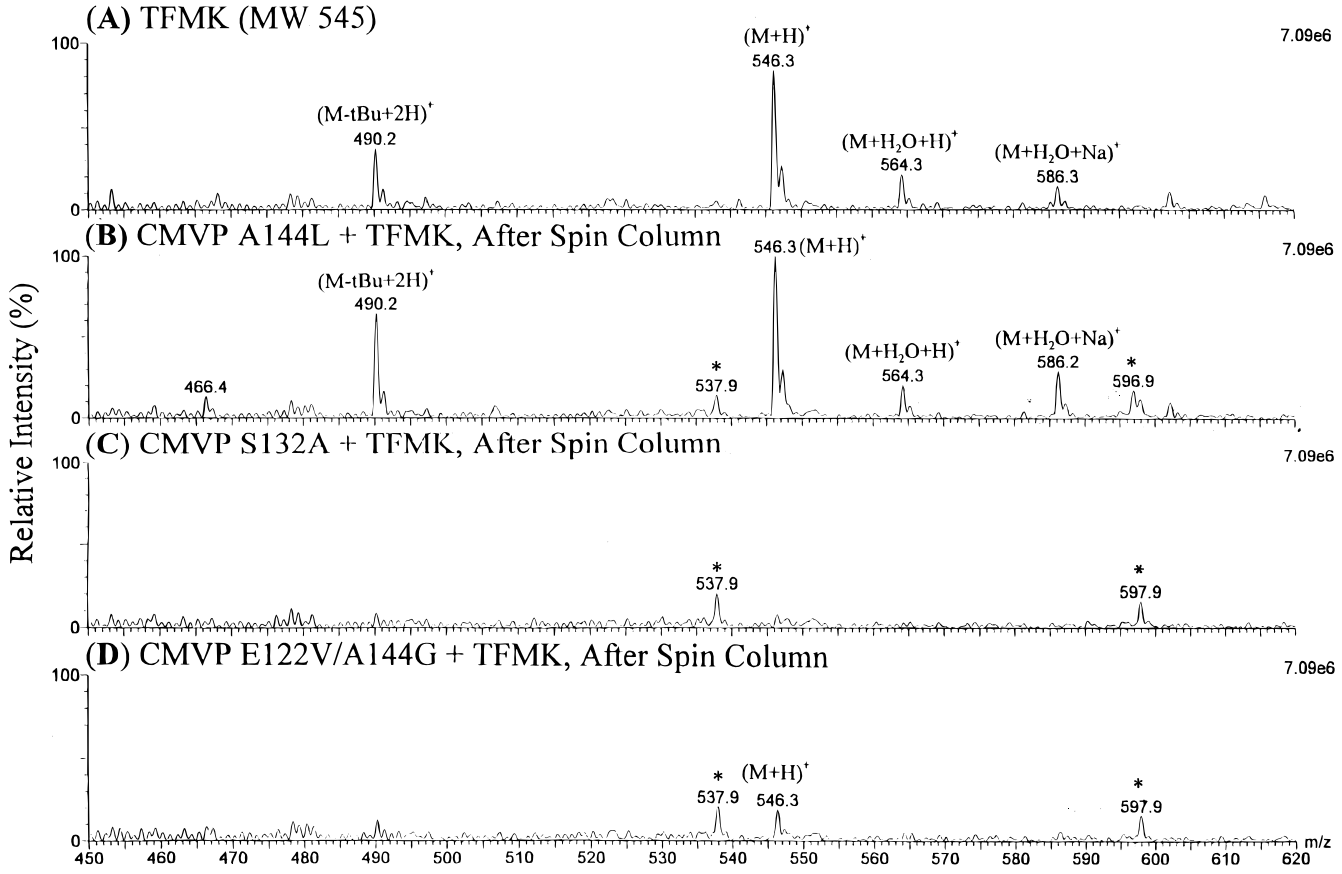


Figure 3. ESI mass spectra obtained from the rapid screening assay for a variety of CMVP mutants incubated with TFMK (**2**) illustrating specificity of the protease–inhibitor complex. (A) ESI mass spectrum of inhibitor TFMK (M_r 545), no spin column or microconcentrator used. (B) ESI mass spectrum of the spin column eluate of CMVP A144L and TFMK, originally incubated at a molar ratio of 1 : 40. The measured molar ratio for the eluate is 1 : 1. (C) ESI mass spectrum of the spin column eluate of CMVP S132A and TFMK, originally incubated at a molar ratio of 1 : 40. TFMK does not co-elute. (D) ESI mass spectrum of the spin column eluate of CMVP E122V/A144G and TFMK, originally incubated at a molar ratio of 1 : 40. The measured molar ratio for the eluate is 1 : <0.05. The mass range illustrated only covers the inhibitor region and not the higher mass range for CMVP. The peaks labeled with asterisks at m/z 538 and 598 are background peaks produced from the solvent (3% acetic acid in water–acetonitrile (1 : 1)). Note that all spectra are normalized to the same scale of 7.0×10^6 ion counts (indicated in the upper right hand corner of each spectrum).

corresponding to the CMVP and the presence of singly, doubly and triply charged peaks corresponding to **1** and the hydrated form of **1**. This additional ultrafiltration step is necessary only if the peaks corresponding to the inhibitor and CMVP overlap. For inhibitors with M_r lower than the lowest m/z ion observed in the CMVP distribution (<700 Da), this step is not necessary. The molar ratio of CMVP non-covalently bound to DFMK could not be quantitated since a pure standard of DFMK was not available.

It should be noted that when the above incubated sample of CMVP and DFMK was transferred to a microconcentrator and centrifuged, the ESI mass spectrum generated from the retentate was similar to that obtained from the eluate of the spin column [data not shown and Fig. 2(B)]. Likewise, when the microconcentrator retentate was washed with a denaturing solution of 3% acetic acid in acetonitrile–water (1 : 1), the ESI mass spectrum generated from the filtrate was similar to that obtained from the microconcentrator filtrate produced originally from the spin column eluate [data not shown and Fig. 2(C)]. These experiments demonstrate that equivalent non-covalently bound products are obtained from the retentate of the microconcentrator and the eluate of the spin column.

All the minor impurities appearing in the original ESI mass spectrum of DFMK (**1**) [Fig. 2(A)] are absent after treatment by the GPC spin column and/or microconcentrator [Fig. 2(C)], indicating that they did not specifically bind to CMVP [Fig. 2(B)]. Hence, the method described for characterizing non-covalent binding is applicable to the analysis of mixtures of compounds; non-covalently bound inhibitors will be selectively co-eluted with CMVP whereas other low M_r components will be retained by the GPC spin column resin and/or passed through the microconcentrator membrane.

Rapid screening demonstration of the specificity of non-covalently bound complexes

The previous illustration of the rapid screening methodology, using size exclusion methods coupled with ESIMS, demonstrated the ability to characterize non-covalently bound complexes. In the following, we shall further demonstrate the use of this rapid screening technique to characterize non-covalent binding of inhibitors (**2** and **3**) to specific sites in CMVP by comparing the non-covalent binding affinities of the inhibitors with

mutants A144L, S132A and E122V/A144G. CMVP A144L represents wild-type CMVP (with respect to enzymatic activity) and is the reference protease for the binding studies. Serine residue 132 is the active site residue predicted to be responsible for nucleophilic attack of the DFMK and TFMK classes of inhibitors and is likely to be essential for the non-covalent binding of these inhibitors to CMVP. Mutation of S132 to an alanine residue in CMVP S132A inactivates the protease and is likely to prevent binding of the DFMK and TFMK class of inhibitors to the protease. Mutation of E122 to a valine residue in CMVP E122V/A144L has also been shown to destroy the enzymatic activity of CMVP,[71] but the effect on inhibitor binding was not known.

The ESI mass spectrum for inhibitor TFMK (M_r 545) (**2**) [prior to passage through a spin column, Fig. 3(A)] exhibits the characteristic molecular ions $[M + H]^+$, $[M + H_2O + H]^+$, $[M + H_2O + Na]^+$ and $[M + H_2O + K]^+$ at *m/z* 546.2, 564.2, 586.2 and 602.1, respectively, in addition to one fragment ion $[M - C(CH_3)_3 + 2H]^+$ at *m/z* 490.1. In a control study, the ESI mass spectrum for the spin column eluate of pure TFMK (not illustrated) shows the absence of TFMK in the low mass region from *m/z* 415 to 620. The ESI mass spectra of the spin column eluates of TFMK incubated with CMVPs A144L, S132A and E122V/A144G (each originally prepared at a molar ratio of CMVP:TFMK of 1:40) are illustrated in Fig. 3(B), (C) and (D), respectively. All the spectra are normalized for the abundance of pure TFMK [in Fig. 3(A)] which corresponds to a 1:1 molar ratio of CMVP:TFMK. (The ion distributions for the proteases occur over the *m/z* range 750–1200 and are not depicted.) In a control study, the ESI mass spectrum of pure CMVP A144L (not illustrated) shows the presence of background peaks at *m/z* 538 and 598, which correspond to solvent complexes of acetic acid [indicated with asterisks in Fig. 3(B), (C) and (D)]. From these data, it is evident that TFMK co-elutes with CMVP A144L (with a CMVP:TFMK molar ratio of 1:1), does not co-elute with CMVP S132A and only very slightly co-eluates with CMVP E122V/A144G (with a CMVP:TFMK molar ratio of 1: < 0.05).

The specificity of these co-elution results is consistent with S132 as the active site nucleophile of CMVP and demonstrates that our screening methodology can detect specific binding of inhibitors to CMVP. From the crystal structure, the active site residues of CMVP are S132, H157 and H63.[64–67] Mutation of S132 is therefore predicted to prevent binding to CMVP of active site-directed inhibitors such as the peptidic difluoromethylene ketone (**1**) and trifluoromethyl ketone (**2**). A stabilized (reversible) hemiacetal protease–inhibitor complex is believed to have formed with CMVP A144L, as illustrated schematically in Fig. 4. However, when S132 is replaced with a lipophilic amino acid residue such as alanine, the active site is destroyed and the mutated protease is incapable of tightly binding to the inhibitor. E122 is far from the active site and is involved in a salt bridge within the protease. Hence, the conformation of protease mutant E122V may be significantly different from that of the wild-type CMVP owing to disruption of the salt bridge. CMVP E122V is enzymatically inactive[75] and we show in this study [Fig. 3(D)] that this mutant protease cannot bind the TFMK inhibitor.

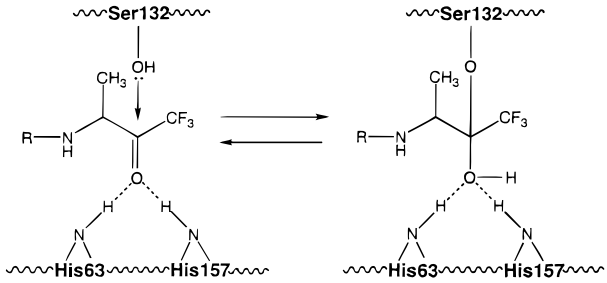


Figure 4. Scheme illustrating the stabilized (reversible) hemiacetal CMVP–inhibitor complex proposed between the triad of amino acid residues S132, H157 and H63 and peptidic trifluoromethyl ketone inhibitor TFMK (**2**). The wavy lines represent CMVP with the specific amino acid residues shown. This mechanism is also applicable to difluoromethylene ketones, e.g. DFMK (**1**).

The dibromoquinazoline (DBQ) inhibitor (M_r 489) (**3**) was identified in random screening of a chemical library. Unlike the DFMK (**1**) and TFMK (**2**) inhibitors, we could not know *a priori* if the DBQ compound is active site directed. To address this question, DBQ was incubated with each of the CMVPs A144L, S132A and E122V/A144G (with initial CMVP:DBQ molar ratios of 1:40). The ESIMS data indicated that DBQ co-eluted with CMVPs A144L and S132A (with recovered CMVP:DBQ molar ratios of 1:3.1 and 1:3.9, respectively) and did not co-elute with CMVP E122V/A144G. Two major differences are apparent in comparing the DBQ co-elution results with those of TFMK. DBQ appears to bind at several sites (three or four, from the co-elution data), in contrast to TFMK, which binds to only one site of CMVP (as suggested by the equimolar recovery of TFMK with CMVP upon passage through a spin column). Also, residue S132 is not required for binding of DBQ to CMVP, since mutant CMVP S132A bound as much (or more) DBQ than did wild-type CMVP A144L (3.9 *vs.* 3.1 DBQ molecules per molecule of CMVP).

Competition study of inhibitor mixture with CMVP

To address the question of whether DBQ (**3**) binds to the active site of CMVP, a competition experiment was performed to see whether TFMK and DBQ compete for binding to CMVP. An equimolar mixture of TFMK (M_r 545) and DBQ (M_r 489) was prepared with CMVP A144L. The molar ratio of CMVP A144L to each of the inhibitors in the mixture was 1:5:5. The CMVP–inhibitor mixture was incubated for 1 h at 25 °C and the spin column eluate was analyzed by ESIMS. The ESI mass spectrum [Fig. 5(A)] exhibited peaks for both TFMK and DBQ. For quantitation purposes, individual ESI reference spectra were obtained from mixtures prepared (without spin column analysis) with 1:1 molar ratios of CMVP A144L:TFMK [Fig. 5(B)] and CMVP A144L:DBQ [Fig. 5(C)]. From the integrated areas of reference compounds [Fig. 5(B) and (C)] and eluted compounds [Fig. 5(A)], the quantitative molar ratio of recovered CMVP A144L:TMFK:DBQ was calculated

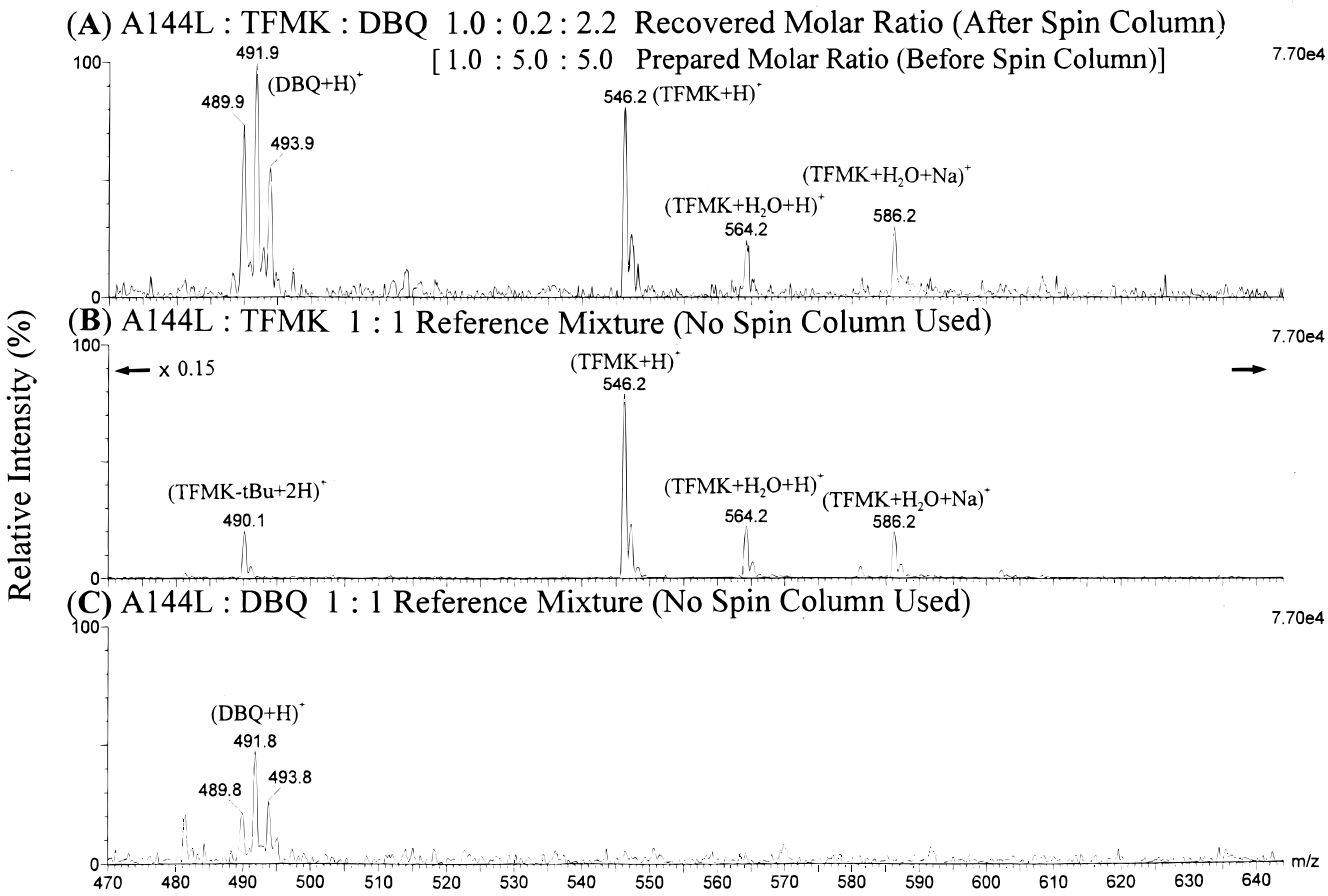


Figure 5. ESI mass spectra obtained from the rapid screening assay for a mixture of the inhibitors TFMK (**2**) and DBQ (**3**) which form non-covalent complexes with CMVP A144L. (A) ESI mass spectrum of a spin column eluate of a CMVP A144L–TFMK–DBQ mixture with a recovered molar ratio of 1.0:0.2:2.2, initially prepared with a molar ratio of 1:5:5. (B) ESI mass spectrum of a reference mixture (no spin column used) consisting of a 1:1 molar ratio of CMVP A144L:TFMK. (C) ESI mass spectrum of a reference mixture (no spin column used) consisting of a 1:1 molar ratio of CMVP A144L:DBQ. The molar ratio of CMVP A144L:TFMK:DBQ recovered after passing through the spin column was calculated from the integrated areas of the peaks in (A) with respect to the corresponding reference peaks in (B) and (C). Note that all spectra are normalized to the same scale of 7.70×10^4 ion counts (indicated in the upper right hand corner of each spectrum) and were obtained using 10 μl of the mixture where the protease concentration was 20 pmol μl^{-1} in each sample.

to be 1.0:0.2:2.2. These results suggest that DBQ prevents the binding of TFMK to CMVP. It is possible that DBQ and TFMK compete for the same site, and that DBQ was more strongly bound to that site than TFMK. Alternatively, DBQ may bind to CMVP and induce a conformational change which prevents TFMK from binding. These results suggest that additional studies are necessary to characterize more fully the unique binding properties of DBQ to CMVP.

Advantages and disadvantages of GPC spin columns and ultrafiltration microconcentrators

The methods described in Fig. 1 were evaluated for use in the rapid screening of libraries of low M_r compounds for finding molecules (potential 'drugs') which non-covalently bind to selected proteins. At present, large numbers of samples are prepared principally using GPC spin columns and to a lesser extent using ultrafiltration microconcentrators. Automated 'open access' table-top ESI mass spectrometers (Micromass Platform II) are used to detect the presence of the low M_r molecules in the GPC spin column eluate in a highly efficient and rapid manner.

A limitation in using GPC spin columns and ultrafiltration microconcentrators for preparing the protein-bound non-covalent complexes is that a dynamic equilibrium exists between the drug–protein complex, free drug and free protein. This equilibrium is disturbed as the complex passes through the spin column or is retained by the microconcentrator. For example, as the complex passes through the spin column, free drug is retained by the spin column, thereby shifting the equilibrium so as to dissociate more of the complex. Likewise, as the solvent with free drug passes through the membrane of the microconcentrator, the equilibrium must be re-established by further dissociation of the complex.

The laboratory-made GPC spin columns have been found to be very robust and are produced without channels and without resin breakthrough. On occasion, ultrafiltration membranes have been found to have pinhole leaks or burst during centrifugation, giving false negative or positive results. Another advantage of the GPC spin column is that only a fixed volume of sample is used and this volume is the volume of the eluate. The eluate is essentially the void volume, which contains only the complex and free protein while free drug is retained on the resin. As long as no open channels are

present in the resin, the amount of drug appearing in the eluate is a direct measure of the relative strength of the binding of the drug to the protein relative to the GPC material. On the other hand, with the microconcentrator, an additional step is necessary. The retained complex has to be washed, at least once, to release any free drug remaining in the retentate. However, washing of the retentate can also release, into the eluate, drug originally present as part of the complex due to the re-establishment of the equilibrium, thereby giving slightly lower quantitative yields for the drug found in the complex.

A unique feature of the GPC spin column, which we have taken advantage of in our work, is that one can design the size of the column to select compounds with desired ranges of binding constants. Empirically, with a complex of known binding constant, the size of the spin column can be adjusted just to pass the complex. Because of the equilibrium nature of the complex, drugs with weaker binding affinities will not eluate from the GPC spin column, reducing the number of possible hits to those only of a desired or greater binding affinity.

CONCLUSION

Size-exclusion methods, utilizing GPC spin columns and/or ultrafiltration microconcentrators, were coupled off-line with ESIMS to screen rapidly single compounds and mixtures for potential inhibitors of CMVP by identifying components which form non-covalent complexes with CMVP. Binding specificity was demonstrated by the use of protease mutants. Furthermore, the degree of complex specificity between the inhibitor and CMVP can be explored by competitive binding experiments of a mixture of inhibitors incubated with CMVP.

The size-exclusion separation techniques with GPC spin columns and microconcentrators coupled with mass spectrometry, described here for screening and characterizing non-covalent complexes, are each rapid, reliable and well established methods. The spin columns and microconcentrators are easy to prepare, inexpensive and disposable. The proposed methodology can rapidly screen large numbers of inhibitors since the protease complexes can be prepared individually or competitively in mixtures and fractionated in parallel via centrifugation of the spin columns and/or the microconcentrators. Since the molecular masses of the inhibitors are known and the mass spectrometer is set up to produce principally molecular ions, automated ESI infusion methods are only needed to characterize and quantitate the inhibitors either separated from, or complexed with, the protease by mass spectrometry. A potential application of this methodology is the development of human serum albumin (HSA)–drug binding assays for determining the quantitative loss of drug activity due to specific and non-specific binding with HSA.

It is expected that these methods can be extended also to screen chemical libraries and fermentation broths for components that non-covalently bind to known binding proteins. For example, using the proposed methodology, chemical libraries can be screened for compounds that form non-covalent complexes with proteins, such as the fructokinase binding protein (FKBP),[4] and fermentation broths can be screened for natural product components which form non-covalent complexes with model cell wall binding proteins containing the terminating amino acid residues –D-Ala–D-Ala.[5] To characterize isolated unknown structures, infusion alone into a low resolution ESI mass spectrometer may not be sufficient, and additional specificity and interfaces may be necessary, such as tandem chromatography, tandem MS and high resolution exact mass measurements.

Acknowledgements

The authors thank Jonathan D. Bloom, Martin DiGrandi and Russell G. Dushin for synthesizing the inhibitors used in these studies, Wei-Dong Ding and Franklin J. Moy for technical discussions and Helen Yun for literature searches.

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