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ORIGINAL RESEARCH

The effect of alkylamine additives on the sensitivity of detection for paclitaxel and docetaxel and analysis in plasma of paclitaxel by liquid chromatography–tandem mass spectrometry

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ABSTRACT: The formation of multiple molecular ions, especially due to sodium adduct ion formation, is commonly observed in electrospray mass spectrometry and may make reproducible and sensitive quantitation difficult. The objective of this work was to investigate the underlying mechanism involved in the suppression of multiple molecular ion formation and to improve the sensitivity of detection for the two anti-neoplastic agents paclitaxel and docetaxel. The results showed that alkylamine additives could significantly improve the detection of paclitaxel and docetaxel by suppression of multiple molecular ions through preferential formation of a predominant alkylamine adduct ion. Possible binding sites, binding interactions and binding competition were investigated for the sodium adduct and alkylamine adduct ions using various experimental techniques. The formation of a predominant amine adduct ion may be due to increased surface activity in the droplet. The optimal alkylamine for both analytes was octylamine, which increased peak heights of paclitaxel and docetaxel 4.8 and 3.7-fold (n = 3), respectively. The precision of the signals for the analytes was also improved 5.7-fold. A quantitative assay in plasma for paclitaxel was partially validated for the calibration range 1.0–1000 ng/mL (r = 0.9977) when using 0.05% octylamine as a reconstitution solution additive. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.5 and 0.9 ng/mL, respectively. Acceptable precision, accuracy, specificity and sample stability were demonstrated for this assay. This approach may prove useful for other analytes with similar binding sites. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: paclitaxel; docetaxel; adduct ions; alkylamine; quantitation; LC/MS/MS

INTRODUCTION

A large number of drugs have been found to be active at trace levels and there is a need for highly sensitive quantitative methods. Although high-performance liquid chromatography/electrospray ionization (ESI)/mass spectrometry has been one of the most useful techniques in biopharmaceutical quantitative analysis, poor detection sensitivity and unstable mass spectrometric signals can be problematic with a number of analytes.

Molecular ions of analytes are generally used as precursor ions when the selected reaction monitoring (SRM) detection mode is applied to quantitative LC/MS. Na^+, K^+ and NH_4^+ ions are present as background or in buffer solutions. These cations are highly acidic and readily form complexes with Lewis bases (Schug, 2002). The formation of multiple molecular ions such as $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$ rarely occurs in atmospheric pressure chemical ionization (APCI), which mostly involves proton transfer reactions (Niessen, 1999). The phenomenon, however, can be observed for many compounds in ESI, which involves the transfer of ions in the solution phase to the gas phase (Loo, 1995; Meng and Fenn, 1991; Guan *et al.*, 2003). Since an increase in the number of ion species created will disperse the signal and make sensitive and reproducible quantitation difficult (Li *et al.*, 2002), supression of the formation of multiple molecular ions in ESI needs to be addressed. Mobile phase additives can be used to improve the sensitivity of detection by formation of a single ionic species. Ammonium acetate, ammonium formate (Kamel *et al.*, 1999; Cuyckens and Claeys, 2002) or alkali metal ions (Karlsson, 1998) are commonly used mobile phase

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Abbreviations used: BA, benzoic acid; H/D, hydrogen/deuterium; MtBE, methyl-*t*-butyl ether.

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additives to promote the formation of $[M+NH_4]^+$, $[M+Na]^+$ or other alkali metal adduct ions. Stefansson *et al.* (1996) reported that alkylamines could suppress the formation of multimers under ESI conditions due to the formation of predominant molecular amine adduct ions. Teshima *et al.* (2002), along with Zhao *et al.* (2002), employed alkylamines as mobile phase additives to suppress the formation of multiple molecular ions when developing sensitive quantification methods for a new bone anabolic agent and for simvastatin, respectively.

Paclitaxel (Taxol) and docetaxel both consist of a C13 carbon side chain and a taxane ring (Fig. 1, P1 and P2, respectively), and demonstrate a broad spectrum of action in the treatment of a variety of human neoplastic diseases, including ovarian, breast, lung, brain cancer and melanoma (Wani *et al.*, 1971; Holms *et al.*, 1991; Forastiere, 1993). Although some LC/MS/MS methods have been developed for detection of paclitaxel and docetaxel with LOQs at ng/mL levels using $[M+H]^+$ as the precursor ion (Basileo *et al.*, 2003; Guo *et al.*, 2003; Parise *et al.*, 2003), these two compounds were shown in our work to produce somewhat unstable mass spectrometric detection due to the formation of multiple molecular adduct ions ($[M+Na]^+$, $[M+H]^+$, $[M+K]^+$ and $[2M+Na]^+$).

Furthermore, the calibration curve was nonlinear because of the formation of the $[2M+Na]^+$ adduct ion at high concentrations (Stefansson *et al.*, 1996). Mortier *et al*. (2004) investigated the effect of different mobile phase additives on adduct ion formation such as $[M+Na]^+$, $[M+H]^+$, $[M+K]^+$ and [M+primary amine+H]$^+$ for paclitaxel. They conducted a thorough comparison of different mobile phase additives, including acetic acid, formic acid, ammonium formate and a range of primary amines, and found that one major molecular ion could be obtained with dodecylamine/acetic acid.

Because the sodium adduct ion is the most abundant adduct ion in the absence of additives in the mobile phase, the current work focused on mechanistic aspects of the suppression of the sodium adduct ion by the alkylamine additive. A discussion of the effect of primary amines in the mobile phase from a structural perspective is presented in a separate model-based study (Lyer *et al.*, 2005). Included in this study are a series of experiments investigating the sodium adduct ion and alkylamine adduct ion in several ways: (1) the binding sites for the sodium adduct ion and alkylamine adduct ion; (2) the strength of the interaction for the sodium adduct ion and alkylamine adduct ions; (3) the effect of the concentration of sodium; and (4) the surface activity of the droplet. The strength of an interaction was evaluated indirectly by investigation of the binding stability of the parent complex in the gas phase. Collision-induced dissociation (CID) has been generally used to determine the binding stability of the complex (Akashi *et al.*, 2005; Arai *et al.*, 2004; David and Brodbelt, 2003; Cole, 1987. p. 130). The cone voltages ($V_{50\%}$), at which the abundance of the precursor ion was half of its maximum value, were compared for the amine adduct ion and sodium adduct ions to determine relative stability. Multiple stage mass spectra MS^3 can be generated using a triple quadrupole mass spectrometer through in-source CID (Makowiecki *et al.*, 2001). Although the sodium ions may migrate during decomposition of the complexes and make ascertaining the specific binding site difficult, multiple stage mass spectra were utilized to obtain useful information about sodium binding

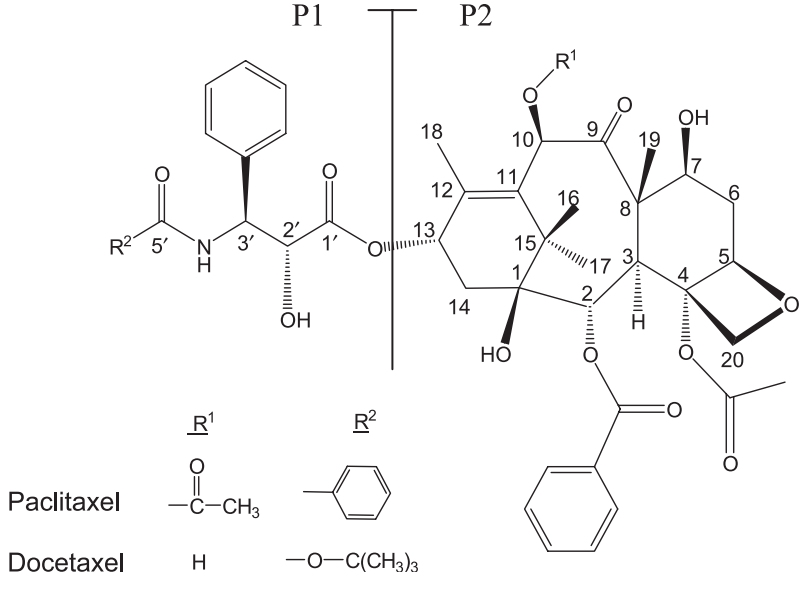


Figure 1. Chemical structures of paclitaxel and docetaxel. The P1 and P2 moieties form two major fragment ions.

sites. The binding sites for the alkylamine ion were investigated by evaluation of amine adduct ion formation for analogs of paclitaxel. Hydrogen/deuterium (H/D) exchange combined with mass spectrometry is a valuable tool to study protein–ligand interaction and protein–protein interactions (Nabuchi *et al.*, 2004; Zhu *et al.*, 2004). Recently, Lorenz and co-workers applied this approach to demonstrate the non-covalent dimerization of paclitaxel in solution and to investigate a drug–drug complex as being formed in solution or in the gas phase (Lorenz *et al.*, 2001, 2002). This study applied a H/D exchange experiment to investigate the involvement of hydrogen bonds in the non-covalent complex formed with octylamine.

A sensitive quantitative assay using octylamine in the injection solution was established to demonstrate the applicability of this approach. The assay was partially validated with regard to the precision, accuracy, specificity, extraction recovery and sample stability in the calibration range 1.0–1000.0 ng/mL.

EXPERIMENTAL

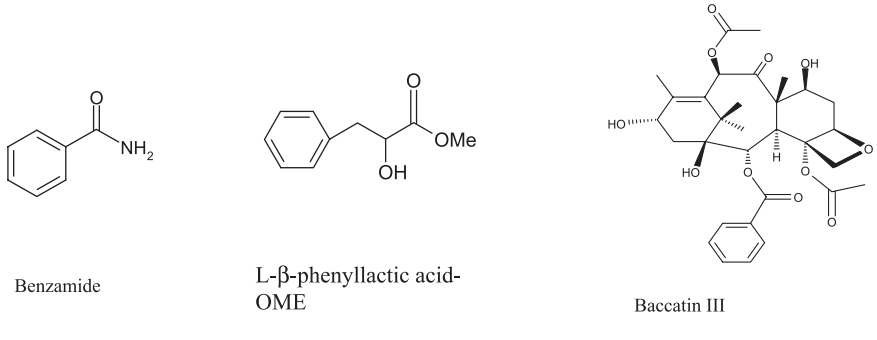
Materials. Paclitaxel and docetaxel were obtained from Sigma (St Louis, MO, USA) and Bdent Global (Lower Hutt, New Zealand), respectively. The series of alkylamines [ethylamine (70% solution in water), butylamine, hexylamine, octylamine, nonylamine, decylamine, dodecylamine, tetradecylamine, hexadecylamine, octadecylamine, diethylamine, dibutylamine, dimethyloctylamine and triethylamine] analogs of paclitaxel (benzamide, L-β-phenyllactic acid-OME and baccatin III) and deuterium water were purchased from Sigma Aldrich Chemical Co. (Milwaukee, WI, USA). Methanol, acetonitrile, methyl-*t*-butyl ether (MtBE) and dichloromethane were HPLC-grade and were purchased from Burdick & Jackson Chemicals Limited (Muskegon, MI, USA). Sodium acetate (Anhydrous) was purchased from J. T. Baker (Phillipsburg, NJ, USA). Acetic acid was obtained from Mallinckrodt (Paris, KY, USA). Water was purified using a Milli-Q system (18.2 MΩ cm). Tripotassium EDTA human plasma was obtained from Biochemed (Charleston, SC, USA).

Adduct ion formation when using alkylamine as mobile phase or reconstitution solution additives. A Micromass Quattro Triple Quadrupole mass spectrometer (Manchester, UK) was coupled to two Shimadzu LC10ADvp high-pressure pumps (Columbia, MD, USA), a Shimadzu DGU-14A solvent degasser (Columbia, MD, USA), a Shimadzu SCL10Avp system controller (Columbia, MD, USA) and a Shimadzu SIL-10ADVP robotic autosampler (Columbia, MD, USA). Separations for paclitaxel and docetaxel were performed using a 2.1 × 100 mm Waters Xterra® C18 (3.5 µm) column (Milford, MA, USA) at a flow rate of 0.250 mL/min. The mobile phase consisted of acetonitrile:water 70:30 v/v. The parameters for the MS/MS system were set as follows: spray capillary, 3.50 kV; cone, 12 V; extractor, 2 V; source temperature, 120°C; ESI probe temperature, 350°C. The desolvation gas flow was 150 L/h. Ions were scanned in the positive ESI mode.

Mixtures of 5 µg/mL each of paclitaxel and docetaxel in acetonitrile:water (70:30, v/v) were separated using a Waters Xterra™MS C18 column. Five primary amines (ethylamine, butylamine, hexylamine, octylamine and decylamine), two secondary amines (diethylamine and dibutylamine) and two tertiary amines (dimethyloctylamine and triethylamine) at concentrations of 1.56×10^{-6} g/mL (w/v) to 1.56×10^{-4} g/mL (w/v) in the mobile phase were investigated.

Alkylamine adduct ions could be formed when alkylamine additives were added to the reconstitution solution. Aliquots of 100 ng/mL each of paclitaxel and docetaxel were dissolved in acetonitrile:water (60:40) with 3 mM alkylamine and 0.1% acetic acid. The relationship between the carbon chain of the amine and the intensity of $[M+amine+H]^+$ was studied using six primary amines (ethylamine to decylamine). This investigation was then extended to the longer alkyl chain amines dodecylamine, tetradecylamine, hexadecylamine and octadecylamine.

Comparison of the amine adduct ion and the sodium adduct ion using LC/MS/MS. The same LC/MS/MS system as described above was used for comparison of the alkylamine adduct ion and sodium adduct ion. A relatively high cone voltage of 50 V was used, and the in-source CID was employed to elucidate the possible binding sites for the sodium adduct ion. Three structural analogs of paclitaxel, benzamide (possessing R2, Fig. 3), L-β-phenyllactic acid-OME (the same moiety contained in the structures of paclitaxel and docetaxel) and baccatin III (possessing R1, Fig. 2), were investigated to suggest the binding site for the



Benzamide

L-β-phenyllactic acid-OME

Baccatin III

Figure 2. Chemical structures of benzamide, L-β-phenyllactic acid-OME and baccatin III.

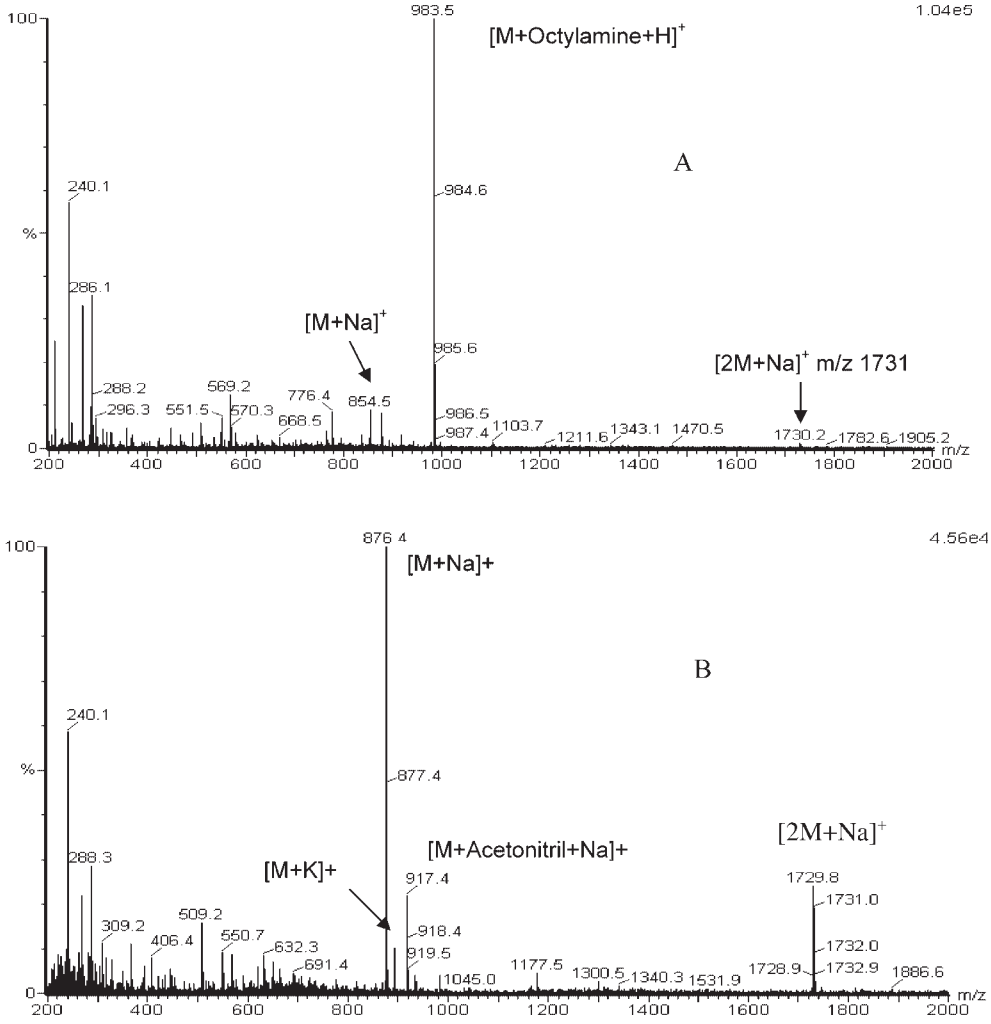


Figure 3. Mass spectra of paclitaxel with (A) and without octylamine (B). Mobile phase, water:acetonitrile, 30:70 v/v. Paclitaxel was detected at 500 ng/mL.

alkylamine adduct ion. All three analogs were prepared in 70:30 acetonitrile:water (v:v) with 0.005% octylamine and were analyzed using full ion scans.

Relative binding stabilities of the sodium adduct ions and alkylamine adduct ions were compared using in-source CID. Full ion scans from *m/z* 100 to 900 were detected in triplicate at each cone voltage increased from 15 to 60 V. Plots of the abundance of the parent complex $[M+Na]^+$ and $[M+octylamine+H]^+$, and their major fragment ions $[M+H]^+$, $[P1+Na]^+$ or $[P1+H]^+$ and $[P2+Na]^+$ or $[P2+H]^+$ (P1 and P2 are shown in Fig. 1), vs applied cone voltage were generated and $V_{50\%}$ values were calculated.

Competition for formation of the sodium adduct ion and alkylamine adduct ions was also investigated using a Waters XTerra™MS C18 column. A mixture of paclitaxel and docetaxel in acetonitrile:water 70:30 was used as a control solution, and the signals of $[M+Na]^+$ and $[M+octylamine+H]^+$ were compared with that of a solution containing the additives (3 mM octylamine alone and or 3 mM octylamine with varying concentrations of sodium acetate from 110 mM).

Q-TOF for solution phase H/D exchange experiments. A Micromass Q-Tof micro™ (Manchester, UK) mass spectrometer was employed for deuterium exchange experiments. Ions were monitored using the positive detection mode. The ESI conditions on the Q-Tof mass spectrometer were: capillary, 4.0 kV; cone, 30.0 V; extractor, 1.0 V; source temperature, 110.0°C; desolvation temperature, 110.0°C. Two syringe pumps (Harvard, Quebec, Canada) with 1000 µL syringes (Hamilton, Bonaduz, Switzerland) were connected to the spray tip of the mass spectrometer using a tee. One syringe pump infused deuterium water, and another pump was used to infuse the analyte, octylamine or a mixture of the two. The flow rate for each pump was 20 µL/min.

Validation of the paclitaxel assay. The quantitative assay was validated using a liquid chromatography–mass spectrometry (LC/MS) system, consisting of an HP 1100 Pump (Waldbronn, Germany), a LEAP Technologies CTC Analytics LC PAL autosampler (Carrboro, NC, USA), and an Applied Biosystems API 3000 triple quadrupole mass spectrometer (Foster City, CA, USA). All separations for paclitaxel and docetaxel were performed using a 2.1 × 50 mm Waters Xterra® RP-18, 3.5 µm column (Watford, Hertfordshire, UK). The flow rate was 0.3 mL/min. The mobile phase consisted of methanol (mobile phase B):water (mobile phase A). Following

an isocratic period of 0.1 min at 40% B, a steep linear gradient was employed up to 95% B at a rate of 0.8 min and held at 95% B for 1.7 min (0.8–2.5 min). After switching back to the initial condition at 40% B (2.5–2.8 min), the program finished in 1.2 min (2.8–4.0 min). The injection volume was 25 µL. The ion spray voltage (IS) was 4500 V; other instrument parameters were optimized. The ESI probe temperature was set at 450°C and the turbo gas flow rate was 8.0 L/min. The transitions of 983.46→130.21 and 937.53→130.21 were used to detect paclitaxel and docetaxel, respectively.

Human plasma samples were prepared according to the following procedure: a 200 µL volume of plasma was transferred to borosilicate glass culture tubes and mixed with 25 µL of 500 ng/mL internal standard. Two milliliters of the extraction solution (90:10 MtBE:dichloromethane, v/v) were added into each sample tube. After the samples were vortex mixed for 10 min and centrifuged at 3000 rpm for 5 min, the organic layer was transferred to a new set of tubes and was dried under dry nitrogen at 45°C. The residue was reconstituted with 200 µL of reconstitution solution 0.05:0.1:50:50 octylamine:acetic acid:methanol:water, v/v/v/v.

Calibration standard samples were prepared at concentrations of 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100, 250, 500 and 1000 ng/mL, in tripotassium EDTA-treated human plasma. Controls of 1.0, 2.5, 50.0 and 800.0 ng/mL were prepared and stored at –20°C. The method was evaluated with regard to precision, accuracy, selectivity, extraction recovery and sample stability.

RESULTS AND DISCUSSION

Adduct ion formation when the alkylamine is used as a mobile phase additive or a reconstitution solution additive

The formation of multiple molecular ions of the analytes was suppressed by formation of the alkylamine-adduct ion (Fig. 3). Ions at *m*/*z* 569.2 and 527.2 are characteristic fragment ions $[P2+H]^+$ (Fig. 1) for paclitaxel and docetaxel, respectively. The same characteristic fragment ions at *m*/*z* 569.5 and 527.2 are produced from their respective primary amine molecular adduct ions. Corresponding fragments of the sodium adduct ion could be observed at *m*/*z* 591.4 and 549.4. When the effects of different primary amines on the formation of analyte amine adduct ions were compared using the SRM detection mode, transition based on this characteristic fragment pathway was employed. These two characteristic fragment ions, however, were too weak to be detected for secondary and tertiary amine adduct ions. A different stronger product ion $[amine+H]^+$ was therefore employed when evaluating the effect of secondary and tertiary amines.

The sodium adduct ion was suppressed in all cases; however, not all the sensitivities of detection for the alkylamine adduct ions were improved. A summary of the formation of alkylamine adduct ions is listed in Table 1. Sensitivity was increased with an increase in the alkyl chain length of the primary amines (except in the case of decylamine for docetaxel). The same phenomenon was observed for secondary amines. The response of the dibutylamine adduct ion was stronger than that of the diethylamine adduct ion for paclitaxel. However, the responses of both adduct ions were decreased for docetaxel compared with that of the $[M+Na]^+$ without the amine additives. All adduct ions demonstrated ion suppression with the use of tertiary amine additives. Competition between these strong basic electrolytes themselves and other analytes might result in ion suppression. This result is consistent with the observation of Stefansson *et al.* (1996) when they applied alkylamines to regulate the formation of multimers in ESI: primary > secondary > tertiary amine. In a separate model-based study (Lyer *et al.*, 2005), it was demonstrated that a model describing the possible hydrogen bonding interaction and/or hydrophobic effects between alkylamines and the analytes (paclitaxel and docetaxel) agreed well with the observed results.

Table 1. Effect of alkylamines on the formation of adduct ions

	Paclitaxel		Docetaxel	
	A	B	A	B
Primary amine				
Ethylamine	+	+	+	+
Butylamine	+	+	+	+
Hexylamine	+	+	+	+
Octylamine	+	+	+	+
Decylamine	+	+	+	–
Secondary amine				
Diethylamine	+	–	–	–
Dibutylamine	+	–	–	–
Tertiary amine				
Dimethyloctylamine	0	–	–	–
Triethylamine	0	–	–	–

Amines were added into the mobile phase (acetonitrile–water, 70:30 v/v) within the concentration range 1.56×10^{-6} to 1.56×10^{-4} g/mL (w/v). Responses of the amine adduct ions were compared at their optimal concentrations. A and B are two qualitative parameters used to assess the effect of the alkylamine on the formation of the amine adduct ions. A, formation of the alkylamine adduct ion: + = formed; – = not formed; 0 = only slightly formed. B, comparison of the response of the amine adduct ion to that of the sodium adduct ion without the amine additive: + = increase; – = decrease.

The optimal alkylamine as a mobile phase additives for both analytes was determined to be octylamine, which increased the peak height of paclitaxel and docetaxel 4.83- and 3.72-fold (n = 3) respectively. The precision of detection was also improved with octylamine as a mobile phase additive. The RSD of six replicate injections were compared for samples that yielded signal–noise ratios of approximately 10. When 4.5×10^{-5} M octylamine was used as the mobile phase

additive, the RSD for paclitaxel and docetaxel was 5.7 and 3.0%, respectively. Without addition of octylamine to the mobile phase, the RSD of detection for paclitaxel and docetaxel was 32.8 and 17.1%, respectively. Given that precision was compared at essentially the same signal–noise level, the improvement in precision is probably due to suppression of the formation of multiple molecular ions.

Although the sensitivity of detection was improved significantly when using octylamine as a mobile phase additive, a decrease in the response of the analytes was observed after multiple injections, especially for matrix-based plasma samples. The intensity of docetaxel was decreased 49.4% after a further 15 injections. Accumulation of octylamine in the LC/MS system may have caused suppression of ionization for the analytes. Therefore, this approach was modified, using an alkylamine solution as the reconstitution solution instead of in the mobile phase. Acetic acid at a concentration of 0.1% (v/v), was also added to the reconstitution solution to avoid split peaks that were observed when octylamine was assed with the reconstitution solution alone.

When the hydrocarbon chain of the alkylamine was increased, the log of the intensity of $[M+alkylamine+H]^+$ was shown to be linear with carbon number of the alkyl chain of the alkylamine through a 10 carbon chain length (Fig. 4). The signal of the alkylamine adduct ion began to decrease, however, for tetradecylamine in the reconstitution solution when this experiment was extended to longer hydrocarbon chain alkylamines. This decrease in signal could be due to either a less favorable interaction between the amine or an effect on the ionization efficiency through a change in the surface activity of the solution. Octylamine was selected as the reconstitution solution additive for the validation, because it could improve the sensitivity of detection and is more volatile than alkylamines with longer alkyl chains.

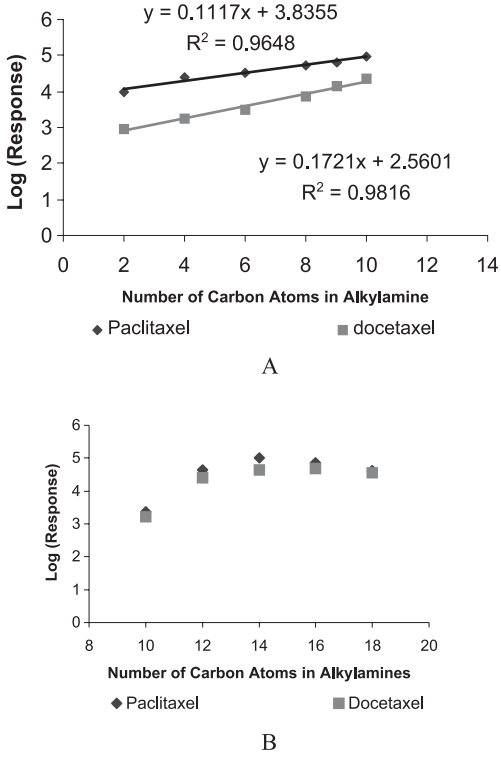


Figure 4. Relationship between sensitivity and the length of the alkyl-chain when using the alkylamine as a reconstitution solution additive. (A) Alkyl-chain from carbon 2 to carbon 10. (B) Alkyl-chain from carbon 10 to carbon 18.

Binding sites for the sodium and alkylamine adducts

The formation of sodium adduct ions can be suppressed through the formation of alkylamine adduct ions. This observation suggests that competition may exist between formation of the two adducts. An important aspect of this study was therefore to find out whether the two adducts bind to the same site on the analytes. Also, establishment of the binding sites can serve as guide in application of the approach for other analytes.

Alkali metal adduct ions of organic compounds are observed in fast atom bombardment (FAB) ionization, matrix-assisted laser desorption ionization (MALDI) and ESI mass spectrometry of various compounds. Previous work has investigated the binding site of sodium with various analytes (Morisaki *et al.*, 2002; Ngoka *et al.*, 1999) and proposed that the sodium ion is bound to the oxygen atoms of the analyte. The mass spectra of paclitaxel and docetaxel demonstrated that sodium ions were carried to product ions when the precursor ion of paclitaxel and docetaxel sodium adducts were fragmented. This means that the product ions still offer the binding sites for sodium ions. For example, according to the observed product ions $[benzamide+Na]^+$ at m/z 144, we can predict that the benzamide group offers a sodium binding site. However, the benzamide group cannot be determined as the only and original binding site for sodium ions due to the fact that sodium ions may migrate during dissociation of the adduct.

Structure P2 (see Fig. 1) contains more oxygen functional groups, and is prone to loss of ester groups. The loss of the ester group did not affect the sodium ion binding to the rest of the taxane ring, which still contained oxygen functional groups. Therefore the elucidation of the potential sodium ion attachment site for P2 was difficult. It is very possible, however, that all the electron-rich centers are involved in the sodium binding. This possibility is supported by the observation that more fragment ions resulting from elimination of water were observed in the MS/MS spectrum of the $[M+H]^+$ ion than in the MS/MS spectrum of $[M+Na]^+$ (Fig. 5).

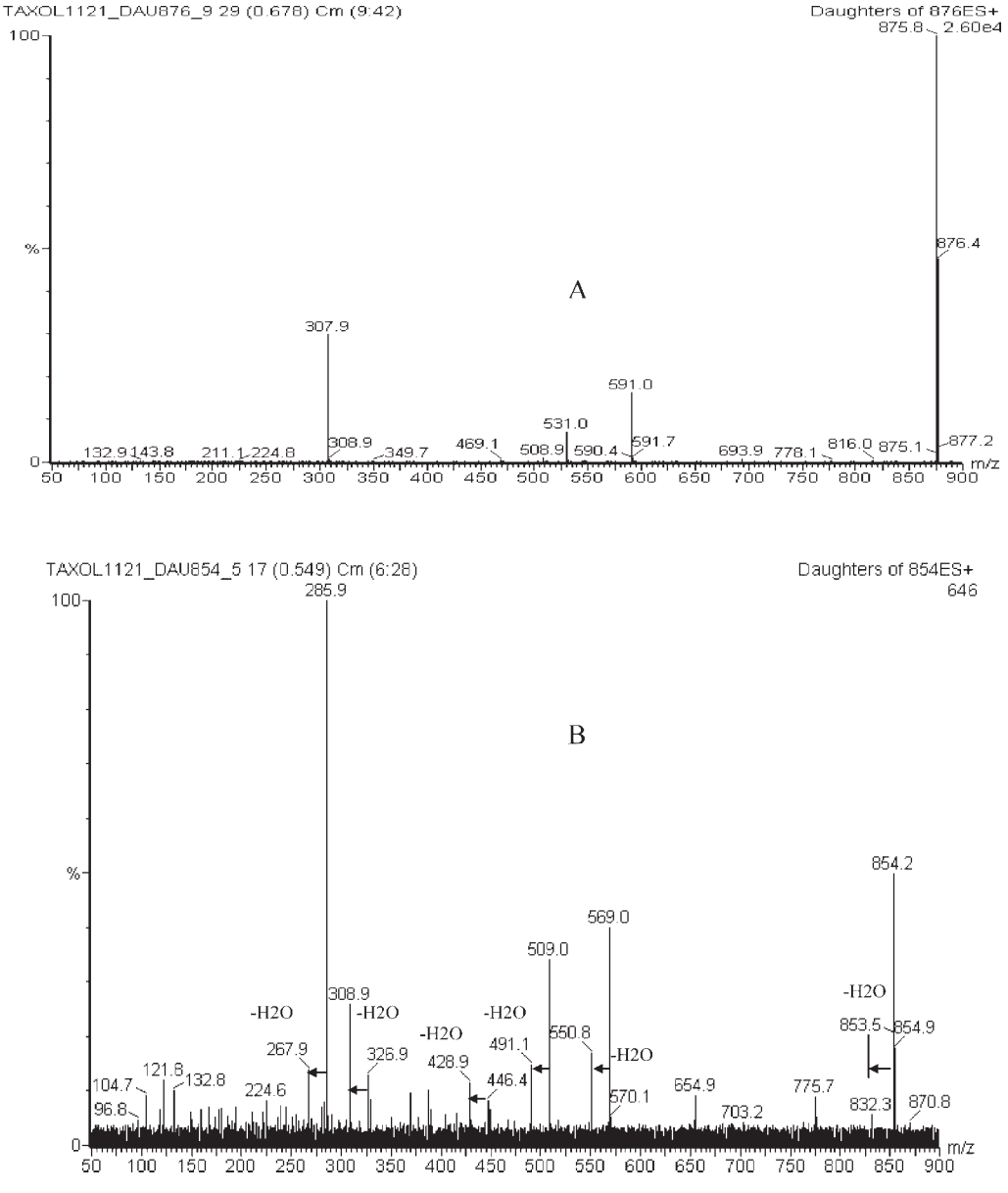


Figure 5. Product ion scan of paclitaxel. (A) Product ions of $[M+Na]^+$. (B) Product ions of $[M+H]^+$. The arrows in the figures represent the loss of water upon fragmentation.

At least six pairs of ions observed in the spectrum of $[M+H]^+$ demonstrated a difference in m/z of 18, such as m/z at 854.2→832.3, 569.0→550.8, 509.0→491.1, 446.4→428.9, 326.9→308.9 and 285.9→267.9. This may be because fewer oxygen atoms are free to bind if they are involved in binding with sodium. This is consistent with the results observed in a previous study of taxanes using FAB MS (Madhusudanan *et al.*, 1997). The analytes may form a complex with sodium ions similar to what occurs with crown ethers. A structure similar to sodium–crown ether complex has been demonstrated for the alkali–metal complex of salinomycin by X-ray diffraction (Miao *et al.*, 2003).

Although paclitaxel and docetaxel have similar chemical structures, different behaviors were observed when forming alkylamine adduct ions (Table 1). The slight differences in structure are at the substituent on the C13 side chain and the 10 position substitution of the taxane ring (R2 and R1 shown in Fig. 1). These differences may result in different binding strengths for paclitaxel and docetaxel. Among the three analogs of paclitaxel, only baccatin III (which possesses a taxane ring) formed an octylamine adduct ion. The data demonstrates that the taxane ring offers a binding site for the alkylamines. The only difference between the two taxane rings of paclitaxel and docetaxel is the substitutions at the 10 position. Therefore, the 10-ester and 10-hydroxyl groups for paclitaxel and docetaxel might play a role in binding. They are not the only critical functional groups involved in binding with the

alkylamine, however, since no alkylamine adduct ions were formed for acetylphenyl salicylate and salicylic acid, both of which possesses an aromatic ring and either an ester or hydroxyl group. Primary amines are known to form strong complexes with crown ethers, which also form a similar complex with sodium ions (Steed and Atwood, 2000). It is possible that the hydrophobic taxane ring and functional groups of oxygen offer multiple binding sites for the alkylamine. In his study of protonated amine–polyether complexes, David and Brodbelt (2003) pointed out that greater stability of the non-covalent complexes was observed with an increase in the number of optimal hydrogen bonds. Our results were consistent with this observation since the effect of alkylamines on the formation of the amine adduct ion was shown to be in the order of: primary amine > secondary amine > tertiary amine. Alkylamines can also form adduct ions with multiple oxygen groups of steroids such as fluticosone, beclomethasone and budesonide. This may also indicate that the hydrophobic ring and multiple oxygen groups are important in formation of the alkylamine adduct ions.

Competition between the sodium adduct ion and the alkylamine adduct ion

An equilibrium partitioning model was proposed by Enke to describe the relationship between the responses of a simple analyte and solution composition (Enke, 1997). Sherman and Brodbelt (2003) extended this model to host–guest complexation in ESI. The model assumes that ions between the surface layer and the interior layer of the ESI droplet can partition and exist in equilibrium. Different ionic species in the solution compete for the limited number of excess charge sites on the surface of the droplet. The ions, which form the surface excess charge at the time of droplet formation, are more likely to be detected in the mass spectra. The competition that exists for the sodium adduct ions and alkylamine adduction ions is not only due to the same binding sites (multiple oxygen group adjunct to hydrophobic ring), but also due to the limited number of excess charge sites on the surface of the droplet. This section discusses the competition between the sodium adduct ion and alkylamine adduct ion relating to concentration and droplet surface effects along with the binding stability of the adduct ions in the gas phase.

Zhao *et al.* (2002) proposed that: the relative abundance of a particular adduct ion depended on how stable this adduct ion was relative to other competing adduct ions. They postulated, therefore, that the reason for suppression of the sodium adduct ion by the amine adduct ion was the higher binding energy with the alkylamine than with sodium. It is known that hydrogen bonds, probably involved in alkylamine adduct ion formation, are very weak with a binding energy of only about 5 kcal/mol (Pine, 1987). Hoyau *et al.* (1999) summarized the binding energies of Na^+ with some small biological molecules in the gas phase which were generally larger than 20 kcal/mol. These results are inconsistent with a displacement model and therefore it was necessary to compare the binding stability for the octylamine adduct ion with that of sodium adduct ion.

In-source CID was used to investigate the binding stability of the adduct ion. Fragment ions were produced in the electrospray interface region by increasing the cone voltage. A decrease in the precursor ions could be due to two possible processes: the breakdown of the interaction between octylamine or sodium ion with the analytes (paclitaxel or docetaxel), or the breakdown of the covalent bonds in the analytes. Therefore, a full ion scan was collected to find out the source of the decrease of precursor ions. The changes in intensity of $[M+H]^+$, $[fragment+octylamine+H]^+$, $[fragment+Na]^+$ and $[fragment+H]^+$ ions were monitored. Sodium adduct ions were found to be more difficult to dissociate than the alkylamine adduct ions (Fig. 6). The abundance of the sodium adduct ion increased gradually until the cone voltage approached 60 V. The abundance of the alkylamine adduct ions was maximal at 35 V and $V_{50\%}$ was at 45 V. Therefore, this experiment is not consistent with the hypothesis that suppression of the sodium adduct ion is due to competitive exchange with the alkylamine based on binding stability as proposed by Zhao *et al.* (2002).

An alternative explanation for suppression of the sodium adduct ion was further investigated. If the sodium adduct ion and octylamine adduct ion of paclitaxel are formed in solution, there may exist a kinetic equilibrium as follows: paclitaxel + $Na^+ \leftrightarrow$ $[paclitaxel+Na]^+$ and paclitaxel + octylamine + $H^+ \leftrightarrow$ $[paclitaxel+octylamine+H]^+$. The concentration of the analyte can play an important role in competition. The ubiquitous presence of sodium from glass and reagents was found to be about 2–10 μM (Ma and Kim, 1997), around 1000-fold lower than the concentration of added octylamine. When octylamine is added into the mobile phase, octylamine adduct ions displace other ions from the droplet surface. The results shown in Fig. 7 support this hypothesis. When the sodium ions in solution were increased, the intensity of the $[M+Na]^+$ ion was increased, whereas the intensity of the $[M+octylamine+H]^+$ ion was decreased. The intensity of the $[M+Na]^+$ ion was stronger than $[M+octylamine+H]^+$, when the concentration of sodium ions was higher than that of octylamine (3 mM in solution).

Although a higher concentration of sodium can increase the intensity of the $[M+Na]^+$ ions, the intensity observed when adding sodium concentrations at 10 mM alone were still not as strong as the $[M+octylamine+H]^+$

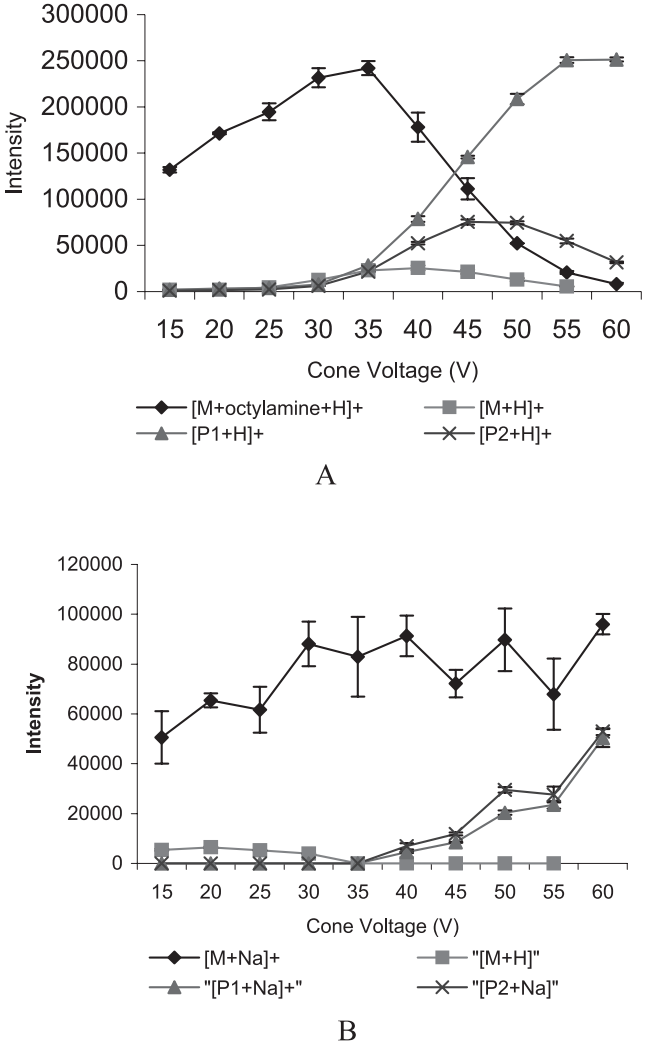


Figure 6. Comparison of the thermal stability for sodium and alkylamine adduct ions. All parameters of the mass spectrometer were set to optimized values and the cone voltage was increased from 15 to 60 V in steps of 5 V. (A) The effect of cone voltage on the abundance of the paclitaxel octylamine adduct ion and its fragment ions; (B) the effect of cone voltage on the abundance of each of the sodium adduct ions. P1 and P2 structures (see Fig. 1, $[P1+Na]^+$ and $[P2+Na]^+$) are two major fragment ions.

ion at an octylamine concentration of 3 mM. This indicates that other factors may be involved in the competition between the sodium adduct ion and alkylamine adduct ion.

In the electrospray process, the surface activities of ions, an important factor in the ion entering into the gas phase, is related to the experimental sensitivity coefficient directly (Cole, 1987, p. 33). When Schug investigated adduct ion formation in ESI MS for benzoic acid derivatives, 10-fold enhancement in the response was observed for tert-butyl benzoic acid (BA) compared with unsubstituted BA. The authors proposed that high hydrophobicity of tert-butyl BA resulted in higher droplet surface activity and higher ionization efficiency (Schug and McNair, 2003). The surface activities of tetraalkylammonium ions are known to increase with increasing size of the alkyl group (Cole, 1997). After the alkylamine forms a complex with the analyte, the surface activity of the complex may be increased and this increase demonstrates a positive correlation with the length of alkyl chain. As observed above, the response of the alkylamine adduct ions was shown to be more intense with a larger alkyl chain. In the same way, the $[M+octylamine+H]^+$ ion may have higher surface activity than the $[M+Na]^+$ ion after the complex is formed; it would therefore be depleted more easily from the droplets into the gas phase and achieve higher ionization efficiency (Cole, 1987, p. 49).

Interaction between the alkylamine and the analytes

Blaghen *et al.* (1999) reported that sodium ions bind with paclitaxel in solution. The formation of a solution complex of paclitaxel with alkylamine was also confirmed in our work by changing the mobile phase composition and solution-phase H/D exchange experiments.

Adjustment of pH demonstrated a significant effect on the response of the octylamine adduct ions. The response was decreased in the mobile phase at pH = 7 and pH = 3.5. The paclitaxel–octylamine adduct ions yielded the maximum response in acetonitrile–water while methanol–water was optimum for the docetaxel–octylamine adduct ion. Since the mobile phase affected the intensity of the octylamine adduct ions for paclitaxel and docetaxel, it is likely that the non-covalent complex with the octylamine and the analyte is formed in solution rather than being produced in the ion-molecule reaction in gas phase.

The complex formed between the alkylamine and paclitaxel is a non-covalent complex, which may involve hydrogen bonding and/or hydrophobic interactions (Lyer *et al.*, 2005). The hydrophobic effect, however, is not important without an aqueous matrix in gas phase, where the hydrogen bonding may become significant due to the lack of dispersion by the solvent (David and Brodbelt, 2003). In this section, hydrogen bonding between the alkylamine and paclitaxel was demonstrated using the H/D exchange experiment as well.

H/D exchange experiments are based on the hypothesis that, if a hydrogen bond interaction is formed in solution, the deuterium exchange of the active hydrogen atom involved in a hydrogen bond will be blocked from exchange. Consequently, the H/D exchange number in the presence of hydrogen bonding will be less than in its absence.

The average masses of the ions were calculated using equation (1) (Lorenz *et al.*, 2001):

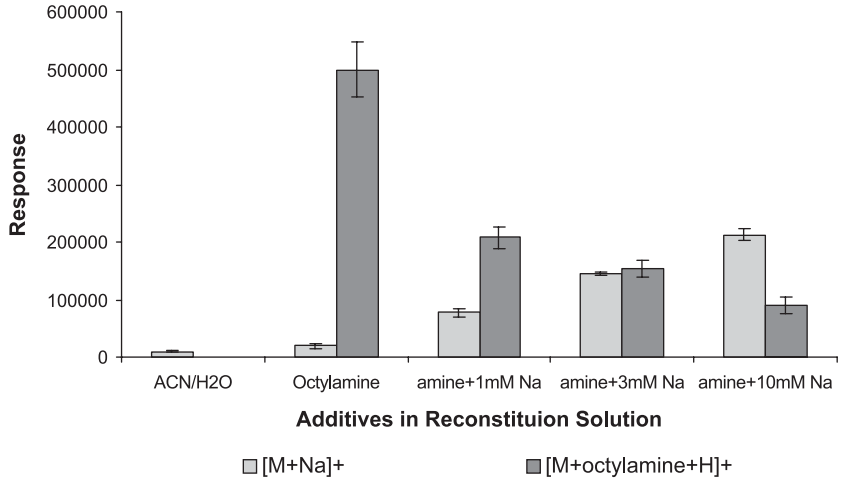


Figure 7. The effect of Na^+ concentration on $[M+Na]^+$ and $[M+octylamine+H]^+$ formation for paclitaxel. Samples in acetonitrile–water were used as a control solution, and the signals of $[M+Na]^+$ and $[M+octylamine+H]^+$ were compared with that of a solution containing 3 mM octylamine alone and or 3 mM octylamine with varying concentrations of sodium acetate.

$$M_{avg} = [RA_{(M1)}/TA]M_1 + [RA_{(M2)}/TA]M_2 + \ldots [RA_{(Mn)}/TA]M_n \quad (1)$$

where M_{avg} is the average mass of the ion, $RA_{(Mn)}$ is the relative abundance of the specified isotopic peak, TA is the total abundance of all isotopic peaks in the distribution, and M_1, M_2, M_n are the masses of the first, second and nth peaks, respectively, in the isotopic distribution. An average number of H/D exchanges for the analyte and non-covalent complex was obtained by subtracting the average masses for the non-deuterated analyte and non-covalent complex, respectively, from the average masses for the deuterated analyte and non-covalent complex.

Paclitaxel has four exchangeable hydrogen atoms and the primary protonated amine, octylamine, possesses three exchangeable hydrogen atoms. The H/D exchange reaction occurs very quickly and, although the mixture time of analyte with deuterium water was controlled by the size and length of the tubing, the H/D exchange time could not be less than 5 s due to the limitation of the instrument interface design. At a high concentration of deuterium water (10%), more than 90% of the active hydrogens in paclitaxel (98.1%) and the protonated amine (91.0%) can be changed. To control for a case in which the hydrogen and deuterium exchanges so fast that the active hydrogens involved in the hydrogen binding react as well, the concentration of deuterium water was diluted. The average H/D exchange number for paclitaxel was lowered to 26.0% when using 0.2% deuterium water. Figure 8 shows the percentage difference in H/D exchange number vs deuterium concentration. The relative difference in the average H/D exchange number was calculated as $(C_{theoretical} - C_{experimental})/C_{theoretical}$. $C_{theoretical}$ represents the exchange number of the paclitaxle–octylamine complex calculated from the sum of that for paclitaxel and docetaxel. $C_{experimental}$ represents the exchange number detected by the experiment. At higher deuterium concentration, the relative difference in these two H/D exchange numbers is only 4.4%. Although the H/D exchange occurred more slowly at lower deuterium concentrations, the difference in these two H/D exchange numbers, which reflect hydrogen bond involvement, became obvious. The relative differences were around 15% for paclitaxel and docetaxel when 0.2% deuterium water was used. A paired t-test was employed to evaluate the data, which demonstrated that the average number of H/D exchanges for the sum of octylamine and analyte was significantly larger than that of the mixture of octylamine and analyte. In

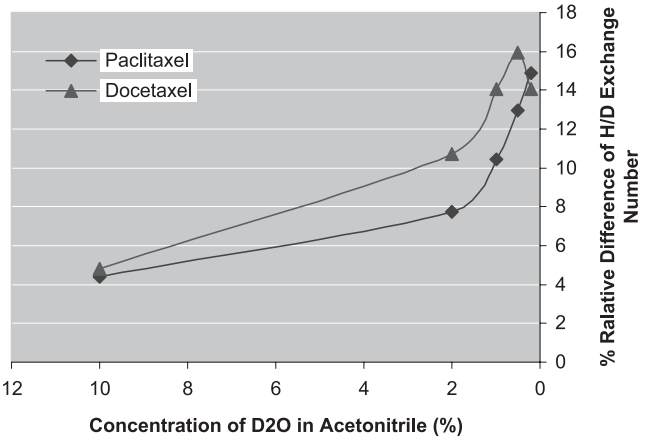


Figure 8. Solution phase H/D exchange experiment showing the relative difference in the average H/D exchange numbers versus the concentration of deuterium water.

addition to the mobile phase composition experiment, this experiment demonstrated that the alkylamine complex is formed in solution and demonstrated that hydrogen bonding exists between octylamine and analyte.

When two compounds form hydrogen bonds, the greatest stabilization of the hydrogen bonds is achieved when the two compounds have similar basicities (Davidson *et al.*, 1979). Gas-phase basicities of the amines vs paclitaxel/docetaxel, therefore, are critical for the stability of the adduct ions. Gas-phase basicities for primary amines from ethylamine to decylamine are from 210.0 to 214.3 kcal/mol (Hunter and Lias, 1998); gas-phase basicities are not known for paclitaxel and docetaxel. If the amines have significantly higher gas-phase basicities, the adduct ions cannot be stable in the gas phase because it will form predominant $[amine+H]^+$ ions and a neutral analyte. The mass spectra of the alkylamine adduct ion demonstrates that $[M+amine+H]^+$ was observed as the predominant precursor molecular ion and $[M+H]^+$ could be observed as a moderately abundant product ion. Therefore, the gas-phase basicities of alkylamines are not significantly higher than those of paclitaxel and docetaxel. Three small analogs of paclitaxel were used to ascertain the binding site for alkylamine above. Their inability to form stable hydrogen bonds with the amines could be due to lower gas phase basicity related to the size of the compound. However, the gas-phase basicity of benzamide (205.8 kcal/mol; Hunter and Lias, 1998) is not significantly lower than that of alkylamines. The absence of adduct ion formation with the amines is much more likely to be due to the lack of active functional groups in small analogs.

Results for method validation

A quantitative method for paclitaxel was established based on the results of the studies described above. This assay can potentially be used to determine docetaxel while employing paclitaxel as the internal standard. The retention times for paclitaxel and docetaxel were both 1.40 min. The SRM mode was used to establish a quantitative method for paclitaxel in plasma. The selected product ion at m/z 130.21 is the protonated octylamine ion. This method improved the sensitivity of detection through yielding one predominate precursor ion and producing abundant product ions. Since the interaction between the alkylamine and the analyte is a non-covalent interaction, which is readily broken to form fragment ions, containing an electronegative nitrogen atom, very abundant $[alkylamine+H]^+$ ions could be formed. Conversely, some LC/MS methods use acid to suppress the formation of $[M+Na]^+$ and form the $[M+H]^+$ ion. Fragmentation of $[M+H]^+$ did not produce very strong product ions, however, and did not improve the sensitivity of detection further. The limit of detection (LOD) and the limit of quantification (LOQ) for this method were 0.5 and 0.9 ng/mL, based on three and five times the standard deviation of the blank, respectively. Considering that the matrix ion-suppression for paclitaxel is not significantly different, it is possible that the limit of quantitation could be lowed further using a 0.5 mL volume of sample.

Table 2. Precision and accuracy of quality control samples

	Nominal concentration			
	1.00 ng/mL	2.50 ng/mL	50.0 ng/mL	800.0 ng/mL
Intra-assay precision (n = 6)				
MEAN	1.00	2.54	51.5	814.3
%RSD	14.0	6.7	3.9	1.6
%DFN	0.0	1.6	2.9	1.8
Inter-assay precision (n = 24, 4 × 6)				
MEAN	0.99	2.45	50.8	821.0
%RSD	16.4	11.8	4.2	4.1
%DFN	–1.1	–2.1	1.6	2.6

%DFN = percentage difference from the nominal value.

The calibration curve was constructed using linear least-squares regression (weighted $1/concentration^2$) of the ratios of the paclitaxel peak area to that of the internal standard plotted vs paclitaxel concentration. The assay was linear over the concentration range of 1.0–1000 ng/mL with a correlation coefficient of 0.9977. The linear equation produced a peak ratio = $-0.0104 + 0.0047 \times$ concentration, and all concentration residuals were within 5.65%. The precision and accuracy of the method were calculated as the relative standard deviation (%RSD) and the percentage difference from nominal (%DFN), respectively. Table 2 summarizes the precision and accuracy of the quality control samples.

Human plasma samples containing tripotassium EDTA from six individuals were extracted and analyzed for paclitaxel and the internal standard to evaluate the selectivity of the method. There were no significant chromatographic peaks detected at the mass transitions and retention times of the analytes or their internal standards which would interfere with quantitation. A small amount of crosstalk from docetaxel was observed in the channel for paclitaxel. The contribution to the paclitaxel detection channel from docetaxel at its IS working concentration was 13.3% of the lower limit of quantitation for paclitaxel. This cross talk was within the requirements of the FDA guidance (<20% of LLOQ) for bioanalytical validation. This crosstalk could be decreased by lowering the concentration of the IS. No crosstalk was observed for detection of docetaxel from paclitaxel.

Matrix effects were evaluated for six individual human plasma lots (n = 3 per lot) by comparing the chromatographic peak areas of the analyte and the internal standard in solution with those of the analyte

Table 3. Recovery calculated from a spiked blank plasma (%)

Concentration of paclitaxel	Paclitaxel ($n = 6$)	Internal standard (docetaxel, $n = 6$)
2.5 ng/mL	86.3 ± 15.5	80.1 ± 12.1
50.0 ng/mL	91.4 ± 13.4	86.8 ± 8.6
800.0 ng/mL	88.0 ± 7.4	86.2 ± 12.9

or internal standard spiked into extracts of plasma blanks (Matuszewski *et al.*, 2003). The mean matrix effect was 43.5% with a 10.3% RSD. The effectiveness of the IS for correction of this matrix effect was demonstrated in the six individual sources of matrix. These results demonstrated a mean area ratio of paclitaxel to docetaxel of 0.031 with 6.7% RSD. The RSD of the analyte to IS area ratios was considered acceptable.

Additional selectivity samples, fortified with paclitaxel at the low-concentration quality control level (nominally 2.5 ng/mL), were prepared from six individual human plasma lots and analyzed to evaluate potential matrix effects on quantitative results. A seventh lot (SPF 7), prepared from the matrix used to prepare the calibration standards and quality controls, was also analyzed as a control. Among the seven individual pools, six of seven of the back-calculated values at the low QC level were less than 15% (–1.2–12.1%) and the back-calculated value for the other was 18.3%. These results indicate that matrix suppression did not significantly compromise the accuracy of the assay.

Extraction recovery was determined by comparison of the peak areas for paclitaxel (and docetaxel) extracted from plasma vs the areas from direct injection of standard spiked into extracts of the plasma blank. Extraction recovery is listed in Table 3. Average extraction recovery was 88.6% for paclitaxel and 84.4% for docetaxel.

Samples were considered to be stable if the mean difference between the initial and final results were less than 15%. All samples demonstrated acceptable freeze–thaw stability through three cycles, 24 h bench stability and 72 h prepared sample stability.

CONCLUSION

Primary amine additives can significantly improve the sensitivity of detection for paclitaxel and docetaxel by suppression of the formation of multiple molecular ions. With only one predominate molecular ion formed, the sensitivity of detection was improved up to 4.8-fold and the precision of the signals for the analytes was also improved 5.7-fold. Secondary amines and tertiary amines were much less suitable as a means of improving the sensitivity of detection. When alkylamines were used in the reconstitution solution, there was a positive relationship between the length of the hydrocarbon chain of the alkylamines ($C \leq 10$) and the intensity of the alkylamine adduct ions. The binding sites for the sodium ions were identified as the electron-rich centers on the analytes, while the binding sites for the alkylamine involved multiple elements, such as the hydrophobic taxane ring and the oxygen functional groups. Formation of the alkylamine adduct ions in the solution phase and hydrogen bonding interactions were demonstrated using an H/D exchange experiment. Suppression of the sodium adduct ion is likely to be due to competitive exchange with the alkylamine, and suppression by the alkylamines could have been due to the relatively high concentration of alkylamine additive used. The higher response of the alkylamine adduct ion could be due to the high surface activity of the complex and its effect on ionization efficiency in ESI. A sensitive analytical method was established for quantitation of paclitaxel based on the suppression of multiple molecular ion species with a lower limit of detection of 0.5 ng/mL. This approach may prove useful for improving the sensitivity and the reproducibility of detection for other analytes with similar binding sites.

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