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Determination of active ingredient within pharmaceutical preparations using flow injection mass spectrometry

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Abstract

Two separate pharmaceutical blends, one containing 2% caffeine by weight, the other containing 2% creatine by weight, and 200 mg caffeine tablets were examined in this study. The purpose of the analyses was to determine the feasibility of using flow injection mass spectrometry for the quantitative analysis of active ingredient within a drug product or other form of pharmaceutical preparation. For more precise and accurate measurements, it was necessary to incorporate an internal reference within the samples. Further, flow injection analysis showed to provide quicker, more facile method development than the application of chromatographic separation. Samples were analyzed over an analyte concentration range of 5.0–15.0 μg/mL. Analyte selectivity was obtained through the observance of the $(M+H)^+$ ions generated by positive electrospray ionization of each of the analytes (*m*/*z* 195 for caffeine and *m*/*z* 132 for creatine), and accurate quantitation was achieved by determining the ratio of the analyte response versus the response of the incorporated reference compound. Sample-to-sample precision in these measurements was less than 3%, recovery values were shown to be accurate to within ±3% of the actual values, and both analytical methods proved to be linear over the assay range ($R^2 \geq 0.999$). Due to the excellent selectivity and low detection limits available to mass spectrometric detection, flow injection mass spectrometric analysis could be particularly applicable for analysis of formulations that contain either low doses of active ingredient, active ingredient with low solubility, or active ingredient that does not possess a strong chromophore. Additionally, this type of methodology shows to be conducive for rapid method development.

Keywords: Active pharmaceutical ingredient; Drug product; Electrospray ionization; Content uniformity; Dissolution; Flow injection analysis

1. Introduction

There are a number of analytical tests and measurements, which are necessary to perform during the development, manufacture, and release of a drug product. Among these many tests, there are several which are concerned solely with the identification and quantitation of the active pharmaceutical ingredient (or the drug substance) within its present form or environment. For instance, these analytical tests and measurements include content uniformity analysis of the drug substance within a pharmaceutical blend [1], assay of the drug substance within the formulated drug product [2], dissolution profile testing [3], and cleaning validation of manufacturing vessels [4]. Currently, the workhorse for this type of analytical testing is high-performance liquid chromatography (HPLC). In order to effectively determine the content of the active pharmaceutical ingredient, it is often necessary to separate that component from the pharmaceutical matrix in which it exists. There are numerous examples, however, in which non-chromatographic techniques have been used to perform analysis on formulated products. Such methods include the use of voltammetry [5], fluorimetry [6], UV spectrophotometry [7], near-infrared absorption spectroscopy [8], laser-induced breakdown spectroscopy [9], or colorimetric detection [10]. At present, however, the use of these detection methods without chromatographic separation are not applicable to a wide range of compounds or do not demonstrate

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similar performance qualities to that now demonstrated by HPLC or similar separation techniques (e.g. gas chromatography).

HPLC has been coupled with a number of different detection schemes. Perhaps, the most common detection scheme within a pharmaceutical analysis laboratory is ultraviolet/visible absorption spectroscopy (UV–vis) [11]. This technique is applicable for many pharmaceutical compounds, and LC–UV–vis methods typically demonstrate high analytical performance (i.e. robustness, accuracy, precision). The major limitation with UV–vis detection is the lack of sensitivity for molecules containing no chromophore. Other spectroscopic techniques used in conjunction with HPLC, such as fluorescence [12], chemiluminescence [13], or refractive index [14] detection, are effective, but for a limited number of compounds only. Mass spectrometric detection, which is applicable for many pharmaceutical compounds and is often capable of achieving low detection limits [15], is another analytical technique that has been coupled with HPLC. However, many HPLC separations are incompatible with mass spectrometry because of the buffers or additives present in the mobile phase. Further, mass spectrometric analysis often lacks the precision typically required for the analysis of pharmaceutical products. Yet, a number of analytical problems arise in which LC–UV–vis analysis does not yield an adequate response. This typically occurs with formulations that contain either a low dose of the active ingredient, an active ingredient that is not very soluble in aqueous or organic solvents, or active ingredient that does not contain a chromophore. The purpose of this study is to address these frequent challenges by attempting to develop a mass spectrometric method capable of determining the content of active pharmaceutical ingredient within a pharmaceutical preparation and to do so with relatively high analytical performance.

One way in which precision and accuracy have been improved in LC–MS analysis has been to include an internal reference. It is common within bioanalytical assays to use an isotopically labeled form of the analyte as an internal reference for LC–MS analysis [16]. Isotopically labeled forms of the analyte are most effective for use as internal references because differences between the analyte and reference, such as chromatographic retention, ionization, or ion fragmentation, will result in imprecision and inaccuracies in the measurements. In this study, we aim to use a similar methodology, but isotopically labeled forms of the analyte are not used as internal references, since we judge that the expense and effort to synthesize these compounds would be too much for the type of analysis we wish to do here. Instead, we demonstrate how non-labeled reference standards can be chosen quickly and developed into the analytical method. Further, chromatographic separation was not deemed necessary. As mentioned previously, one of the concerns when attempting to find a suitable reference compound is obtaining similar chromatographic retention to that of the analyte. By using flow injection analysis, we have essentially eliminated this variable. The analyte and the reference compound will elute simultaneously and thus subjected to the exact same chemical environment upon analysis.

Three separate analyses were carried out in this study: the analysis of caffeine within a constructed pharmaceutical preparation and within commercially available tablets, and the analysis of creatine within a constructed pharmaceutical preparation. Caffeine (structure in Fig. 1) belongs to a small subset of compounds referred to as methylxanthines, which are found naturally in cola nuts, coffee, tea, cacao beans, mate, and other plants. Caffeine is useful as a cardiac stimulant and also as a mild diuretic and has been formulated in numerous drug products [17]. Components of the pharmaceutical matrix used in this study are similar to those used in many of these commercial products. Creatine (structure in Fig. 1), which can be found in muscle tissue, is a central energy system metabolite, which is known to augment muscle cells by increasing intracellular energy pools [18]. Creatine is considered a nutritional supplement and is currently surrounded by much controversy due to possible hazardous side effects. This compound was chosen for this study specifically because it does not contain a strong chromophore and would be difficult to analyze with UV–vis detection.

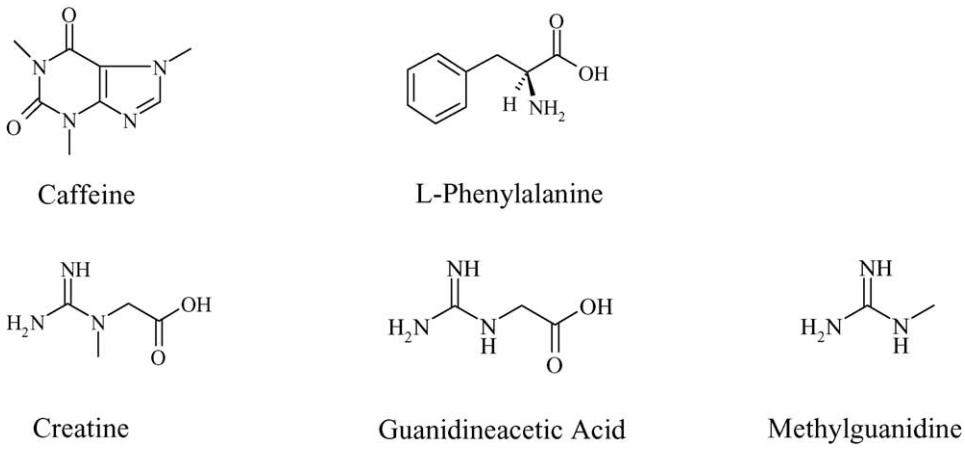

Fig. 1. Chemical structures of analytes and reference compounds used in this study.

2. Experimental

2.1. Preparation of excipient blend

An excipient blend was constructed for use as the pharmaceutical matrix for both of the active ingredients analyzed in this study, caffeine and creatine. The excipient blend was also used to approximate the formulation of the caffeine tablets for purposes of method evaluation. Therefore, many of the ingredients listed in the formulation of the Vivarin caffeine tablets are included in this excipient blend. The excipient mixture was prepared by weighing out 1200 mg microcrystalline cellulose (FMC Corporation, Newark, DE), 400 mg lactose monohydrate (Quest International, Chicago, IL), 100 mg sodium starch glycolate (Penwest, Danbury, CT), 100 mg polyethylene glycol (Sigma-Aldrich, St. Louis, MO), 100 mg polysorbate 80 (J.T. Baker, Phillipsburg, NJ), 50 mg magnesium stearate (Mallinckrodt, St. Louis, MO), and 50 mg sodium lauryl sulfate (Sea-Land Chemical, Westlake, OH).

2.2. Preparation of stock solutions

All reagent and reference solutions were prepared in a 50/50 water (Mallinckrodt, St. Louis, MO)/acetonitrile (Mallinckrodt) diluent. A standard stock solution of caffeine (1 mg/mL) was prepared by dissolving 100 mg of caffeine (Sigma-Aldrich, Milwaukee, WI) in 100 mL of diluent, and a standard stock solution of creatine (0.1 mg/mL) was prepared by dissolving 20 mg of creatine monohydrate (Sigma-Aldrich) in 200 mL of diluent. All stock solutions of reference compounds (0.01 mg/mL) were prepared by weighing out 1 mg of the reference material to 100 mL of the diluent. These reference materials included L-alanine, L-leucine, L-phenylalanine, L-glutamine, L-histidine, L-lysine, L-arginine, methylguanidine, and guanidineacetic acid (Sigma-Aldrich).

2.3. Preparation of caffeine standards and matrix samples

For the purpose of method evaluation, caffeine samples were prepared at concentrations of 5.0, 7.5, 10.0, 12.5, and 15.0 μg/mL. Six matrix samples and six standard samples were prepared at each concentration. Matrix samples of caffeine were prepared by weighing out 12.5 mg of the excipient blend into a 25-mL volumetric flask. The appropriate amount of caffeine stock solution was then pipetted to the flask. Given a target concentration of 10 μg/mL of caffeine, the amount of excipient corresponds to 2% caffeine by weight for the constructed pharmaceutical formulation. The flask was filled to volume with the diluent, and the solution was sonicated for 10 min. Next, a 5 mL aliquot was taken from the solution and filtered with the use of Acrodisc® polytetrafluoroethylene (PTFE) syringe filters with 0.45 μm pore size (Pall Corporation, Ann Arbor, MI). Then, 1 mL of the filtered solution was added to an HPLC vial. To that vial was also added 250 μL of the given reference solution (0.01 mg/mL). L-Phenylalanine was the reference used for analysis. Standard solutions of caffeine were prepared in the same manner as the matrix samples, except that the excipient mixture was not added.

2.4. Preparation of creatine standards and matrix samples

Creatine standards and matrix samples were prepared in a nearly identical fashion to that described for caffeine standards and matrix samples. Creatine samples were prepared at concentrations of 5.0, 7.5, 10.0, 12.5, and 15.0 μg/mL, and six matrix samples and six standard samples were prepared at each concentration. With a target concentration of 10 μg/mL creatine, the constructed pharmaceutical formulation also corresponded to 2% creatine by weight. The same filtration step was carried out with the creatine matrix samples, and 1 mL of the filtered solution was transferred to an HPLC vial. For analysis, guanidineacetic acid was chosen as the reference. Standard solutions of creatine were prepared in the same manner as the matrix samples, except that the excipient mixture was not added.

2.5. Preparation of caffeine tablet samples

Samples of Vivarin (GlaxoSmithKline, Pittsburgh, PA) were acquired for examination of caffeine content. These coated tablets claim 200 mg caffeine content. Individual tablets were crushed and contents were moved to 200-mL flasks. The flasks were filled to volume with the 50/50 water/acetonitrile diluent, and the solutions were sonicated for 10 min. These solutions were then diluted 100 times by pipetting 1-mL aliquots to new 100-mL volumetric flasks and filling these to volume with 50/50 water/acetonitrile diluent. Next, samples from the diluted solutions were filtered with the use of Acrodisc® polytetrafluoroethylene (PTFE) syringe filters with 0.45 μm pore size (Pall Corporation, Ann Arbor, MI). For HPLC–UV analysis, 1 mL was taken for HPLC vials. For flow injection mass spectrometric analysis, 1 mL was taken for HPLC vials, and 250 μL of 0.01 mg/mL L-phenylalanine was added to the HPLC vials.

2.6. Flow injection mass spectrometric analysis

First steps in method development for this study were to determine which reference compounds would be most effective for the given analyte. Once a reference compound was chosen, analyte samples were tested in order to evaluate the analytical performance of the method, i.e. selectivity for the analyte, system and method precision, accuracy, and linearity over the given assay range. For examination of reference compounds, three matrix and three standard samples were prepared and analyzed at the target concentration. The target concentration for each of the analytes in these studies was chosen to be 10 μg/mL. For purposes of method evaluation, analyte samples were prepared at concentrations of 5.0, 7.5,

10.0, 12.5, and 15.0 μg/mL. Six matrix and six standard samples were prepared at each concentration.

A Waters Alliance 2690 separations module was used for auto sampling and flow injection. The solvent flow, which was prepared by mixing 50 parts water, 50 parts acetonitrile, and 0.1 parts formic acid (Sigma-Aldrich, Milwaukee, WI) by volume, was set at a flow rate of 0.6 mL/min. Sample injections were 20 μL, and method run times were 2 min. The flow was interfaced to a Waters Micromass ZQ, and positive electrospray ionization was used. Three single ion monitoring channels were used for the caffeine analysis, and two channels were used for the creatine analysis. For the caffeine analysis, *m*/*z* 195 was monitored for caffeine, and *m*/*z* 166 and 120 were monitored for L-phenylalanine. For the creatine analysis, *m*/*z* 132 was monitored for creatine and *m*/*z* 118 was monitored for guanidineacetic acid. The instrumental parameters of the mass spectrometer were the same throughout this study and were as follows: capillary, 3.5 keV; cone, 25 V; extractor, 4 V; RF lens, 0.2 V; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas, 500 L/h; multiplier, −650 V; scan time, 1 s. Control of the instruments and data collection was performed with Masslynx 3.5 software.

2.7. HPLC analysis

HPLC analysis was performed for analysis of caffeine in Vivarin tablets to serve as a reference method for the flow injection mass spectrometric analysis. A Waters Alliance 2690 separations module was used. Separation was performed on a YMC Pack Pro C_{18} column (150 mm × 4.6 mm, 3 μm) with a mobile phase consisting of 4:1 water:acetonitrile with 0.1% formic acid. The flow rate was set at 1.0 mL/min and 20 μL injections were made. Retention time for caffeine was 3.5 min, and UV detection was performed at 258 nm.

3. Results and discussion

3.1. Determination of caffeine within a pharmaceutical preparation

Caffeine was initially separated from insolubles by dissolving the caffeine formulation in a 50/50 water/acetonitrile diluent, then filtering the solutions with PTFE syringe filters. Next, the caffeine solutions were spiked with an internal reference, L-phenylalanine, and injected into the solvent flow for mass spectrometric analysis. The total ion count, which was derived from the electrospray ionization of the solvent flow, was monitored as a function of time. An example is displayed in Fig. 2a. In this case, 20 μL of a 7.5 μg/mL matrix sample of caffeine was injected into the flow at time = 0. One of the main advantages of directly injecting a sample for mass spectrometric analysis is the short analysis time. In this instance, the caffeine elutes and is measured in less than 20 s from injection. A flow rate of 0.6 mL/min was used. At lower flow rates, the analyte and reference components retained much longer in the ionization source, effectively making quantitation less precise. Further, a higher flow rate was used to minimize any carryover from sample to sample. Single ion monitoring

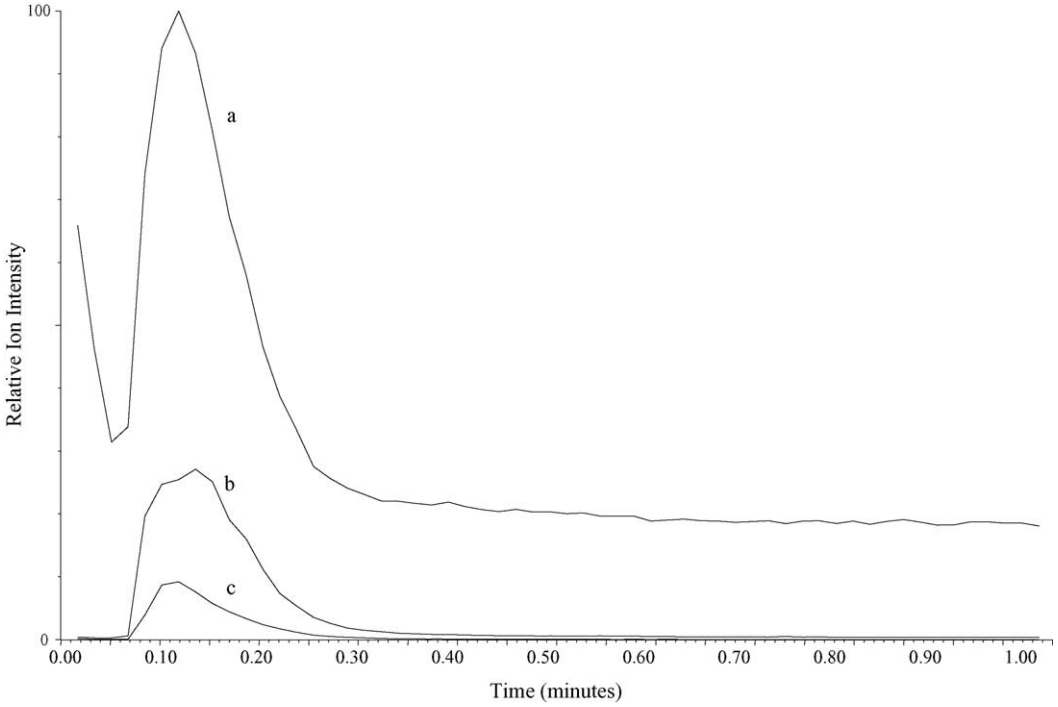

Fig. 2. Positive electrospray ionization of a 20 μL flow injection of a 7.5 μg/mL caffeine matrix sample. The ion count traces correspond to (a) total ion monitoring (scan, 50–250 *m*/*z*), (b) extracted single ion monitoring of *m*/*z* 195 (caffeine), and (c) extracted single ion monitoring of *m*/*z* 166 (L-phenylalanine).

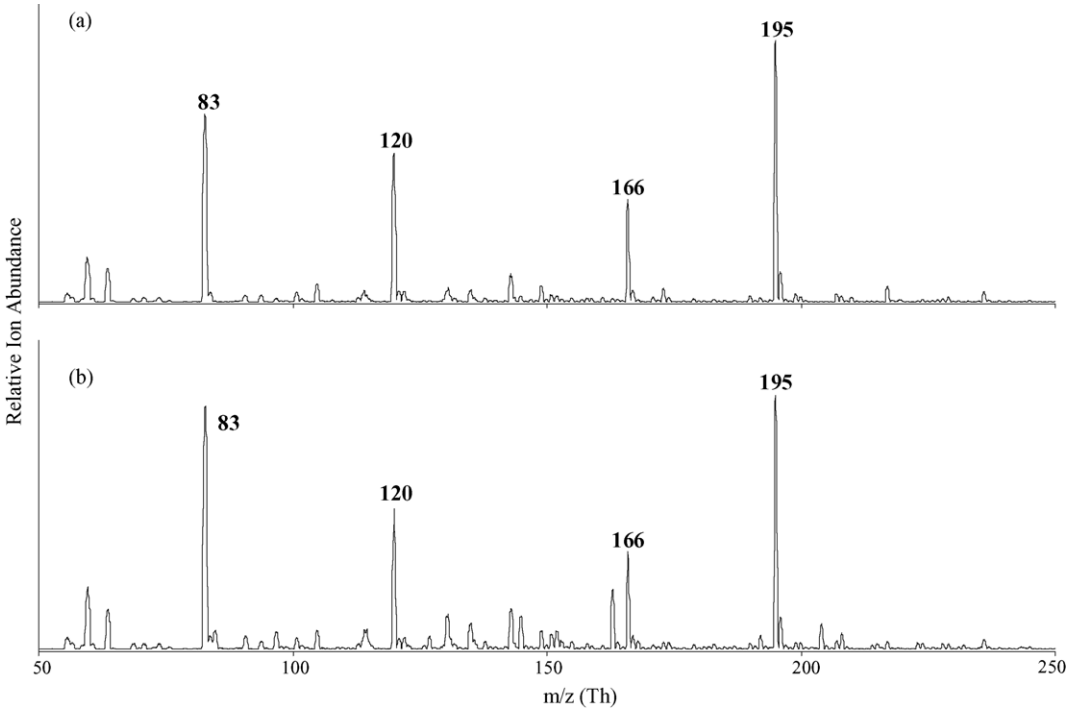

Fig. 3. Positive electrospray ionization mass spectra acquired from 7.5 μg/mL (a) caffeine standard and (b) caffeine matrix samples.

was extracted from the total ion data. The ion count trace of *m*/*z* 195 represents detection of caffeine (Fig. 2b), and the ion count trace of *m*/*z* 166 represents detection of L-phenylalanine (Fig. 2c). From the monitoring of these single ions it is evident that L-phenylalanine and caffeine elute over approximately the same time interval. Mass spectra were summed over the time interval of 0.05–0.3 min. This integration produced the mass spectrum which is shown in Fig. 3b. Similarly, the results from a standard sample of caffeine are illustrated in Fig. 3a. As mentioned previously, the peak at *m*/*z* 195 represents the $(M + H)^+$ ion of caffeine, and the peak at *m*/*z* 166 represents the $(M + H)^+$ ion of L-phenylalanine. The peak at *m*/*z* 120 represents the $(M - 46 + H)^+$ fragment ion from L-phenylalanine. The peak at *m*/*z* 83 is the dimer formed from acetonitrile, $(H_3CCN)_2H^+$. Notice that the spectrum in Fig. 3a is quite similar to that in Fig. 3b, with the exception of a few additional peaks that are present in the spectra of the matrix sample. This result shows that ionization of excipient components in this particular pharmaceutical matrix under positive electrospray ionization is not significant. This lack of interference from the pharmaceutical matrix demonstrates the compatibility of using flow injection mass spectrometry for the analysis of active ingredient in pharmaceutical formulations.

Spectra were shown above which resulted from scanning the quadrupole analyzer of the mass spectrometer from 50 to 250 *m*/*z*. However, single ion monitoring was used for the analysis and three channels were used, one for the MH^+ ion of caffeine (*m*/*z* 195) and the others for the MH^+ ion of L-phenylalanine (*m*/*z* 166) and the fragment ion of L-phenylalanine (*m*/*z* 120). Table 1 displays the results from the caffeine analysis method. The samples were examined over the concentration range of 5.0–15.0 μg/mL. Part (a) of this table shows the results obtained if only the intensity of the caffeine peak is used for the analytical response. It is obvious in looking at this data table that low recovery values (55–75%) were obtained. Recovery values were determined by dividing the response of the matrix samples by the response of the standard samples. This means that the caffeine signal for the matrix samples is much less than the caffeine signal for the standard samples, which was expected. Though the ionization of excipients in the matrix was not significant, suppression of the ionization of caffeine did occur. Another result to point out from part (a) is the method precision. The precision values range from 4 to 6% over the assay range.

In part (b) of Table 1, the analytical response for caffeine was determined by calculating the ratio response, where the ratio response = (area response of *m*/*z* 195)/(area response of *m*/*z* 166 + area response of *m*/*z* 120). The recovery values for part (b) range from 100 to 102%. The suppression of the caffeine ion due to the presence of excipients in the matrix samples is offset in this analysis because the ions generated from the reference, L-phenylalanine, are similarly suppressed. Therefore, the ratio response determined from a matrix sample is approximately equivalent to that determined for a standard sample of the same concentration. In this example, L-phenylalanine serves as a suitable reference, and the data validates that caffeine standards can be used for calibration of caffeine matrix samples. Fig. 4 illustrates the linearity

Table 1
Comparison of the precision and recovery results obtained from the analysis of caffeine either by (a) single ion monitoring of the caffeine peak (*m*/*z* 195) or (b) determining the ratio response, which equals the ion intensity for caffeine (*m*/*z* 195) divided by the summed ion intensities for the reference, L-phenylalanine (*m*/*z* 120 and166)

Caffeine (μg/mL)	(a) SIM response for caffeine (in-matrix samples)			(b) Ratio response for caffeine (in-matrix samples)		
	Area response (*m*/*z* 195)	Precision (%R.S.D., *N* = 6)	%Recovery	Ratio response	Precision (%R.S.D., *N* = 6)	%Recovery
5.0	6341381	3.94	75.64	0.697	3.20	101.3
7.5	8291343	4.21	66.46	1.059	2.36	101.7
10.0	9236570	6.27	59.61	1.344	2.07	100.4
12.5	10123726	5.59	55.76	1.635	1.77	100.3
15.0	12521431	4.55	59.13	1.950	1.73	100.3

curve for both the caffeine standards and matrix samples. The method in part (b) proves to be linear over the assay range, and the two curves are essentially the same. The R^2 value for the matrix samples is 0.9993, while the R^2 value for the standard samples is 0.9985. Another benefit provided by the use of an internal reference in this analysis is an improvement in precision. The method precision values in part (b) range from 1 to 3% over the assay range. These values are definitively lower in this case than in part (a). Imprecision in the ionization of caffeine from sample to sample is captured by similar effects to the reference component.

One variable was determined to be very important for the development and effectiveness of this method. This variable is the relative amount of reference compound versus the amount of analyte present in the samples. It was determined that if the analyte was present in much greater amount than the reference, or if the reference was present in much greater amount than the analyte, then the effectiveness of this method was diminished. For this reason, the method is limited to a linear dynamic range of only 2, perhaps 3 orders of magnitude. However, the limits of detection for caffeine or the reference, L-phenylalanine, were on the order of about 0.05 μg/mL, so this method could have been developed at much lower sample concentrations if needed, provided that the reference concentration was similarly decreased. The selectivity provided at low detection limits is an important advantage for using this mass spectrometric technique, the ability to assay low dose formulations, especially for those compounds that do not contain a chromophore. In this method, we have shown that 10 μg/mL caffeine samples can be analyzed effectively for a formulation containing 2% of the active ingredient.

3.2. Comparative analysis of caffeine in tablets

For further examination of the flow injection mass spectrometric analysis method, commercially available caffeine tablets were assayed by the flow injection mass spectrometric method and a comparative HPLC–UV method. The Vivarin samples claimed 200 mg caffeine content. It is important to mention that many of the ingredients present in the formulation of the Vivarin caffeine tablets were deliberately placed in the excipient blend constructed for the method

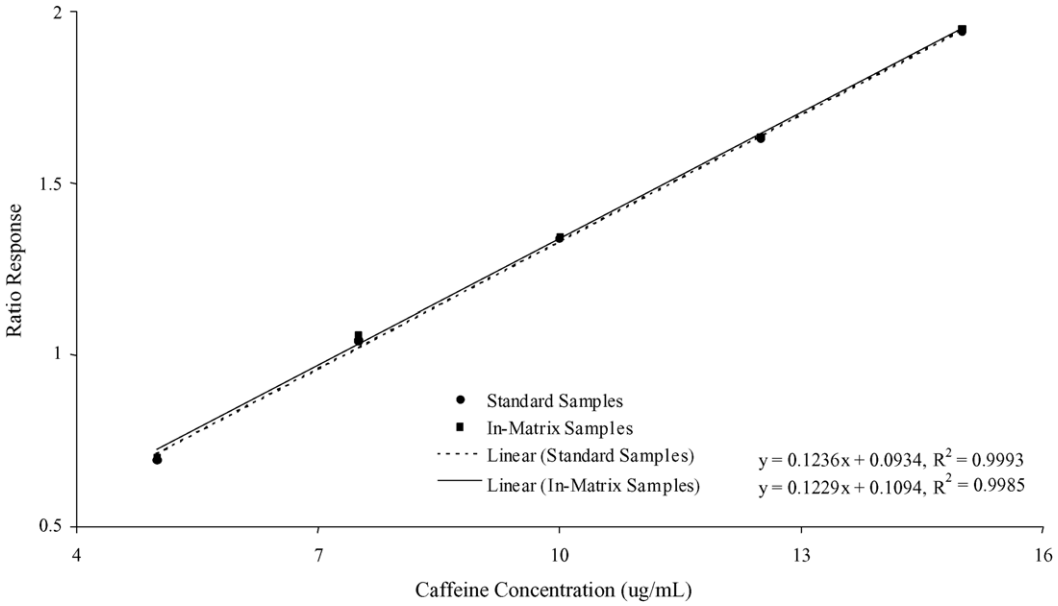

Fig. 4. Linear plot for the assay of caffeine matrix samples, where the *y*-axis represents the ratio response.

Table 2
Comparative results of caffeine analysis of Vivarin tablets

Samples	HPLC–UV (mg)	Flow injection MS (mg)
1	223.4	223.1
2	219.7	216.5
3	217.9	217.3
4	233.1	218.9
5	219.5	217.8
6	217.4	210.8
Average	221.8	217.4

evaluation. However, the relative amounts of these components were not known. For best results, one would develop a method with understanding of the exact formulation. Six different tablets were crushed, then individual samples were prepared from each at a target concentration of 10 μg/mL. These samples were then analyzed by both methods. Results are shown in Table 2. The average caffeine content in the tablets as determined by HPLC–UV was 222 mg, while that determined by the flow injection mass spectrometric technique was 217 mg.

3.3. Determination of a reference compound

To this point we have not discussed how and why L-phenylalanine was chosen as the reference compound. Would other compounds perform as well? What properties made L-phenylalanine suitable as a reference for the caffeine analysis? First, there are two obvious properties which the reference compound must have. One, as a matter of analyte selectivity, the reference compound should yield ions at different *m*/*z* values than those formed by the analyte. Second, as discussed previously, the reference and analyte compounds should elute over the same time interval. Since chromatography is not used in this method, it would be expected that different components should elute together. However, even at the high flow rate used, 0.6 mL/min, there are some compounds that could retain in the ionization source. This peak broadening could introduce error to the measurement, so any such compound should be avoided as a potential reference compound.

For the caffeine analysis, L-phenylalanine worked well as the reference compound. To define this, ionization of L-phenylalanine was suppressed similarly to the ionization of caffeine when matrix samples were examined. Therefore, the ratio response for caffeine is approximately the same for the matrix and standard samples. This enables standards of caffeine to be used for calibration. Method development showed that this was not the case for all reference compounds tested. A series of amino acids were tested individually as possible reference compounds to be used for the caffeine analysis. Table 3 lists these compounds along with their recovery results. To test each compound as a reference, three standard and three matrix caffeine samples were prepared, then analyzed by flow injection mass spectrometry. The ratio of caffeine ion intensities versus ion intensities of a given trial compound was determined from the resulting mass spectra. Results for the matrix and standard samples were compared by calculating a percent recovery value. The recovery value for L-phenylalanine was 101% (Table 3). This result correlates well with the accuracy that was obtained in the caffeine analysis method (Table 1). However, when L-arginine was tested as a reference compound, the recovery was 79%. Why is the recovery so low for L-arginine? The low percent recovery is indicative of the fact that the ion intensity for L-arginine is suppressed to a lesser extent than the ion intensity for caffeine when these two components are analyzed within the matrix samples. The next question, then, is what is different about L-arginine and L-phenylalanine such that the ionization of these two compounds would behave differently, and can we predict these differences?

Table 3
A series of L-amino acids were screened against caffeine by determining the percent recovery of caffeine from the matrix samples

Reference	PA (kJ/mol)	%Recovery
L-Alanine	901.6	107
L-Leucine	914.6	106
L-Phenylalanine	922.9	101
L-Glutamine	937.8	93
L-Histidine	988	97
L-Lysine	996	89
L-Arginine	1051	79

These results are correlated with the gas-phase proton affinities of the amino acids [19].

It is typical for most small pharmaceutical compounds that the protonated form of the analyte, MH^+, or its dimer, M_2H^+, will be formed upon ionization in positive electrospray ionization. It stands to reason, then, that a compound with greater proton affinity, or greater basicity, is more readily protonated, or ionized. L-Arginine is a very basic compound. In terms of gas-phase ion energetics, the proton affinity of L-arginine is 1051 kJ/mol [19], much more basic than L-phenylalanine (922.9 kJ/mol). Therefore, if these two compounds are competing for protons with other components in a solution or in an electrospray event, ionization of the more basic compound, L-arginine, will be less affected. In fact, Table 3 shows that the gas-phase proton affinities can be used as a reasonable predictor for which compounds will match up best with caffeine in terms of ionizability. For instance, from the compounds that were tested, L-alanine has the lowest gas-phase proton affinity (901.6 kJ/mol). As one would predict from the table, L-alanine was suppressed to a greater extent than was caffeine and yielded the highest percent recovery. In regards to developing a flow injection mass spectrometric method for analysis of a given analyte, it is evident that an appropriate reference compound should be tested into that method. Those compounds which have similar ionizability as the analyte would be most successful, and known properties of the test compounds, such as the gas-phase proton affinity or the solution-phase basicity, would be useful in predicting that ionizability.

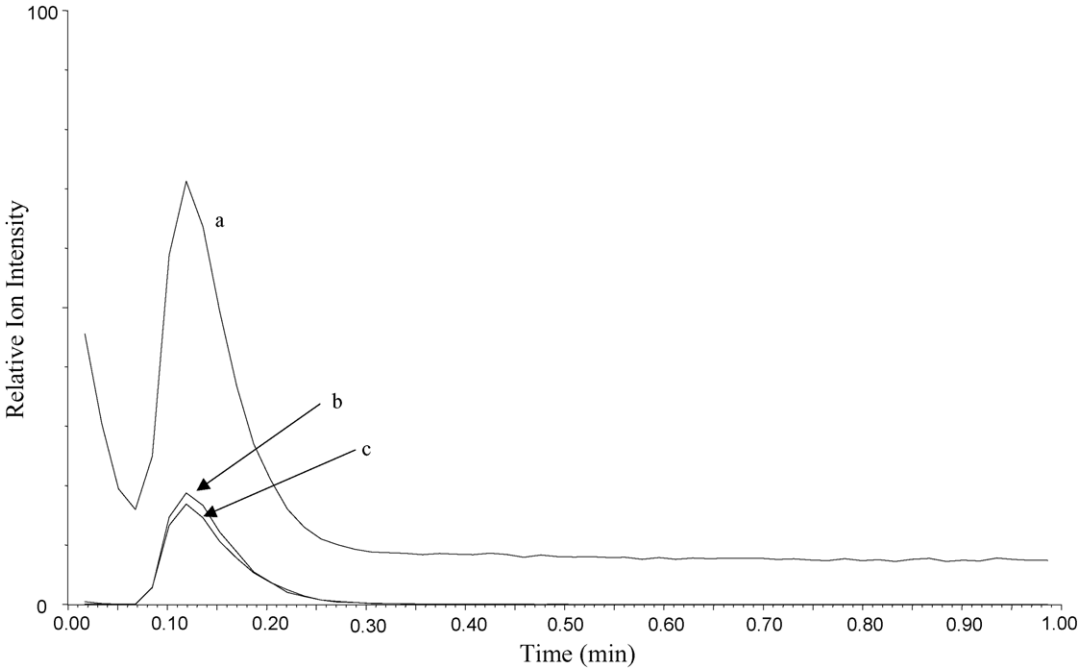

Fig. 5. Positive electrospray ionization of a 20 μL flow injection of a 10 μg/mL creatine matrix sample. The ion count traces correspond to (a) total ion monitoring (scan, 50–400 *m*/*z*), (b) extracted single ion monitoring of *m*/*z* 118 (guanidineacetic acid), and (c) extracted single ion monitoring of *m*/*z* 132 (creatine).

3.4. Determination of creatine within a pharmaceutical preparation

Flow injection mass spectrometric analysis was also attempted for a second analyte, creatine. Creatine (structure in Fig. 1), because of its strong basicity, proved to be a more difficult analyte in terms of finding a suitable reference compound. The list of compounds in Table 3 were attempted, but most were not basic enough, while L-lysine and L-arginine were too basic. So, two other compounds, methylguanidine and guanidineacetic acid (structures in Fig. 1), were tested against creatine, since their structures are similar to that of creatine and would likely have similar ionizability. Methylguanidine yielded a percent recovery of 104% and guanidineacetic acid yielded a percent recovery of 103%. Guanidineacetic acid was chosen as the reference compound for validation of this method.

The same excipient blend used for the caffeine formulation was also used for the creatine formulation. The same solvent system, the same filtration step, and the same mass spectrometric method were also used. Monitoring of the total ion count as a result of electrospray ionization of the solvent flow is displayed in Fig. 5a for the injection of a 10 μg/mL matrix sample of creatine at time = 0. Again, elution of the sample components occurs within 20 s of injection. Monitoring of single ions was extracted from the total ion counts. Specifically, the ions at (b) *m*/*z* 118, which represent guanidineacetic acid and at (c) *m*/*z* 132, which represent creatine, are shown in Fig. 5. Monitoring of the single ions shows that the analyte and reference elute over the same duration of time. Fig. 6b illustrates the mass spectrum which results from summing the mass spectra recorded over the time frame of 0.05–0.3 min for the 10 μg/mL creatine matrix sample. Similarly, the results from a creatine standard

Table 4
Comparison of the precision and recovery results obtained from the analysis of creatine by (a) single ion monitoring of the creatine peak (*m*/*z* 132) or (b) determining the ratio response, which equals the ion intensity for creatine (*m*/*z* 132) divided by the ion intensities for the reference, guanidineacetic acid (*m*/*z* 118)

Creatine (μg/mL)	(a) SIM response for creatine (in-matrix samples)			(b) Ratio response for creatine (in-matrix samples)		
	Area response (*m*/*z* 132)	Precision (%R.S.D., *N* = 6)	%Recovery	Ratio response	Precision (%R.S.D., *N* = 6)	%Recovery (adjusted)
5.0	1891634	5.91	87.1	0.508	2.02	106.3 (103.2)
7.5	2773733	9.66	91.1	0.727	2.11	103.3 (100.3)
10.0	3553028	7.59	85.6	0.981	1.95	104.3 (101.3)
12.5	3871343	6.05	89.4	1.207	2.55	101.0 (98.1)
15.0	4810740	5.04	86.7	1.468	1.59	103.2 (100.2)

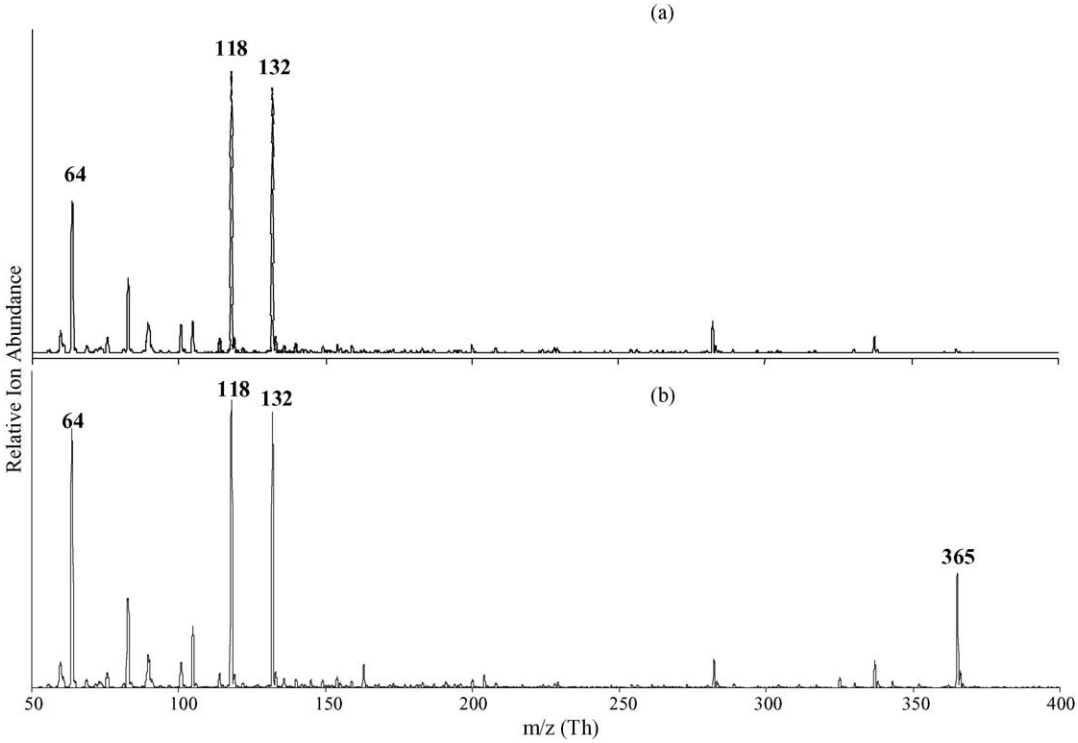

Fig. 6. Positive electrospray ionization mass spectra acquired from 10 μg/mL (a) creatine standard and (b) creatine matrix samples.

sample are illustrated in Fig. 6a. The quadrupole mass analyzers were scanned from 50 to 400 *m*/*z*. The peak at *m*/*z* 132 represents the $(M + H)^+$ ion of creatine, while the peak at *m*/*z* 118 represents the $(M + H)^+$ ion of guanidineacetic acid. The peak at *m*/*z* 64 stems from the mobile phase, and the peak observed at *m*/*z* 365 originates from some component in the excipient mixture. The ratio response in this analysis is defined as ratio response = (area response of *m*/*z* 132)/(area response of *m*/*z* 118). As in the case of the caffeine analysis, the ratio of the ions representing creatine and the ions

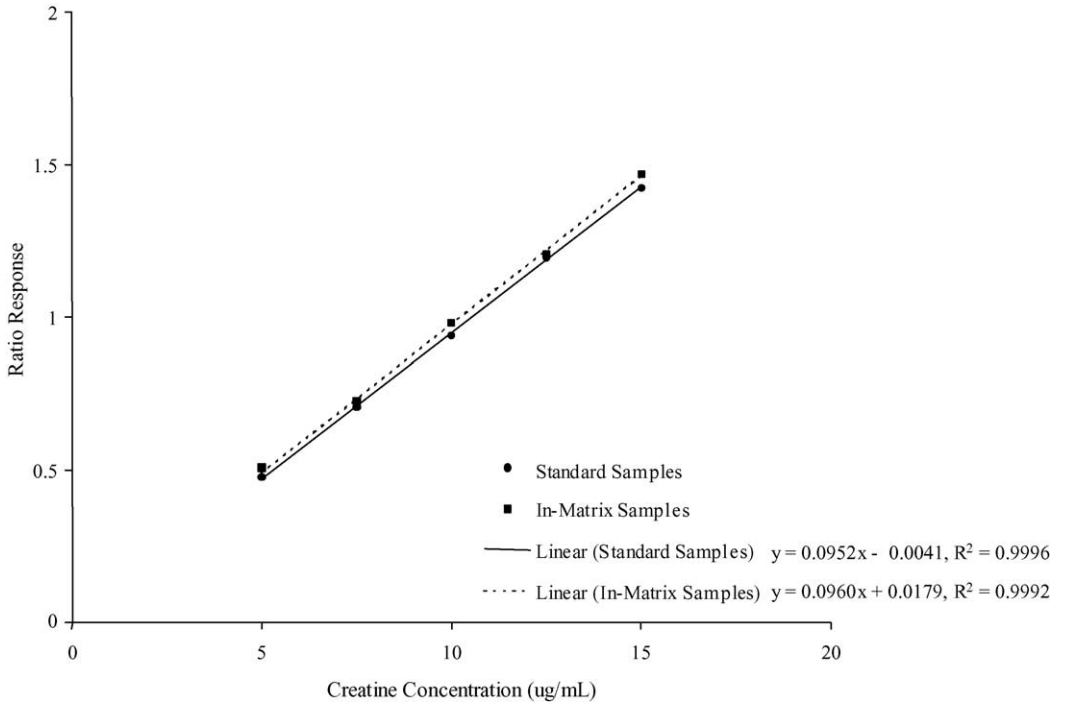

Fig. 7. Linear plot for the assay of creatine matrix samples, where the *y*-axis represents the ratio response.

representing the reference standard is similar for both the standard and matrix samples. For assay of creatine, samples were examined over the concentration range of 5.0–15.0 μg/mL. Table 4 showss the recovery and precision results from (a) monitoring only the creatine ion response and (b) recording the ratio response for creatine. In respect to monitoring only the creatine response, recovery values were approximately 90%. The presence of excipients in the sample suppressed the ionization of creatine, though not to a large extent. The method precision of these measurements ranged from 5 to 10%. By determining the ratio response, part (b) shows that the precision values were improved to 1–3% R.S.D., about the same precision values achieved when determining the ratio response in the caffeine analysis. The recovery values from part (b) range from 101 to 106%. Fig. 7 is a plot of ratio response as a function of creatine concentration, and it shows that the data is linear over the assay range for both the creatine standards ($R^2 = 0.9996$) and creatine matrix samples ($R^2 = 0.9992$). We infer from the high recovery values that the ionization of guanidineacetic acid is slightly more suppressed than the ionization of the creatine within the matrix samples. We notice that the slopes of the two lines are nearly the same. Though the recovery values for creatine are high, they are predictably high. When guanidineacetic acid was chosen as the reference compound from initial screening analysis, a 103% recovery was obtained. This recovery value is an empirically determined characteristic of the relative ionizabilities of guanidineacetic acid and creatine when these components are contained within the pharmaceutical matrix being tested. Therefore, we can apply an adjustment factor in order to accurately determine the creatine content. The target recovery, 100%, divided by the measured recovery for guanidineacetic acid as reference, 103%, equals 0.97. This adjustment factor is multiplied against the measured recoveries in the method validation. The adjusted recovery values (shown in parentheses, Table 4) now range from 98 to 103%, a more accurate determination.

4. Conclusion

This study has demonstrated that flow injection mass spectrometric analysis is useful for the quantitative analysis of active ingredient within a pharmaceutical preparation. The premise of the experiment is that by choosing a suitable reference compound and by using the ratio response for analysis, then interferences in the ionization of the sample caused by the presence of matrix components, can be offset, thus providing accurate quantitation. Though these interferences from the matrix required a reference compound to be used, they were not as significant as one would expect from more complex samples, such as a biological matrix. This is probably because the typical major components present in a solid dose pharmaceutical preparation (i.e. sugars and cellulosic materials) are rather transparent to positive electrospray ionization. For this reason, chromatographic separation did not show any added advantages, so flow injection analysis was used.

For the two analytical methods evaluated, recoveries were accurate to within ±3%, method precisions were less than 3% R.S.D., and linearity ($R^2 \geq 0.999$) was achieved over the assay range. One advantage of this technique is the application towards low dose formulations. In this study, we successfully analyzed two formulations where the active was 2% of the total, and the methodology is applicable at even lower dosage strengths. Another advantage of this technique is the universality of the mass spectrometric technique. The majority of small pharmaceutical compounds are ionizable by positive electrospray ionization. This is especially applicable to compounds that do not have chromophores and might otherwise be difficult to analyze. Finally, this type of analytical methodology is conducive to rapid method development.

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