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Assay of ezlopitant, a substance P receptor antagonist, and metabolites in biological matrices by gas chromatography with mass spectrometric detection: simultaneous analysis of a benzyl alcohol and alkene

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Abstract

A method for the analysis of the substance P antagonist ezlopitant and two active metabolites in serum using solid-phase extraction followed by GC–MS analysis is described. The linear dynamic range was 1.0 to 100 ng/ml and precision and accuracy over this range were within 15%. Upon injection of reconstituted sample extracts into the hot injector port of the gas chromatograph, the benzyl alcohol metabolite undergoes a small amount of spontaneous dehydration to the alkene metabolite. We have incorporated an additional hexadeuterated internal standard of the benzyl alcohol into the assay to permit measurement of the extent of dehydration in each sample. This novel approach should be generally applicable to the simultaneous determination of benzyl alcohols and corresponding alkenes by GC–MS when the possibility exists that the alcohol can undergo spontaneous dehydration to the alkene in the injector port of GC instrumentation.

Keywords: Ezlopitant; Substance P

1. Introduction

Ezlopitant, (2*S*,3*S*-*cis*)-2-(diphenylmethyl)-*N*-{(2-methoxy-5-isopropylphenyl)methyl}-1-azabicyclo-[2.2.2]octan-3-amine (CJ-11,974, Fig. 1), represents a novel, potent, non-peptidic antagonist of the substance P receptor similar in structure to the well-characterized analogue CP-96,345 [1]. It is currently being investigated as a potential therapy for disorders in which substance P is believed to play a role, such as inflammatory diseases, pain, and emesis [2]. In addition to the parent compound, two metabolites of ezlopitant arising from dehydrogenation (CJ-12,458, Fig. 1) and benzylic hydroxylation (CJ-12,764, Fig. 1) are believed to also contribute to the antagonism of the substance P receptor in vivo. In order to understand the pharmacokinetics of ezlopitant in humans and preclinical species, a sensitive assay for ezlopitant was required. Additionally, in order to better understand pharmacokinetic/pharmacodynamic relationships, determination of circulating concentrations of the active metabolites was required. Thus, it was necessary to develop an assay that would quantitate ezlopitant and both active metabo-

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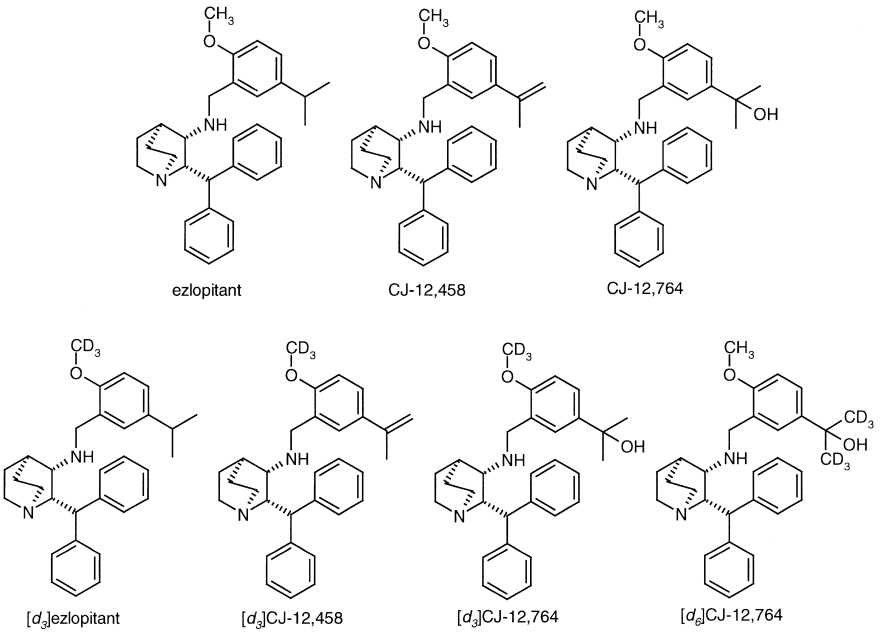

Fig. 1. Structures of ezlopitant, metabolites CJ-12,458 and CJ-12,764, trideuterated ISTDs and hexedeuterated CJ-12,764.

lites. For ease of use, it was desirable to devise a method that would measure all three compounds simultaneously.

In early work, an assay for ezlopitant was developed using gas chromatography with nitrogen-specific detection that was sensitive to 1.0 ng/ml in dog serum (Reed-Hagen et al., unpublished data). Thus, despite its relatively high molecular weight, ezlopitant possesses suitable volatility to permit gas chromatographic analysis. When it became apparent that the active metabolites of this compound also needed to be quantitated, the GC technique was extended to include the metabolites in the analysis. However, in order to provide better selectivity, electron-impact mass-selective detection was chosen as the detection method. Described in this report is an analytical procedure that simultaneously quantitates ezlopitant and the two active metabolites, CJ-12,458 and CJ-12,764. In this assay, serum samples are subjected to solid-phase extraction (SPE) on a cyano SPE cartridge followed by GC with electron-impact MS detection, monitoring fragment ions specific to each analyte and corresponding trideuterated internal standards (ISTD).

One problem encountered in the analysis of alcohols by gas chromatographic methods lies in the potential for this class of compounds to undergo thermally catalyzed spontaneous dehydration to alkenes. Mechanistically, the proclivity of alcohols at carbon centers adjacent to sp^2 hybridized carbons, such as benzylic alcohols, to undergo this process is even greater than that of typical aliphatic alcohols. Thus, it can be anticipated that the simultaneous quantitation of benzylic alcohols and their corresponding alkenes by GC will be confounded by the unknown extent of spontaneous dehydration that can occur in the heated injector port during injection. In order to account for this in the assay for ezlopitant and metabolites, a novel approach was taken in that an additional internal standard of the benzylic alcohol was added to monitor the extent of dehydration. Thus, in addition to its general utility in the specific quantitation of ezlopitant and metabolites, the assay procedure described herein also outlines a general approach to simultaneously monitor benzylic alcohols and alkenes when the potential exists for conversion of one analyte to the other during the assay procedure.

2. Experimental

2.1. Reagents

All reagents (methanol, acetonitrile, and water) used were HPLC grade or equivalent. Triethylamine (+99%) and toluene (+99%) were purchased from Aldrich (Milwaukee, WI, USA). Helium was of ultra-high purity. Control human serum was obtained from in-house donors or commercial sources. Ezlopitant, CJ-12,458, and CJ-12,764, were from Pfizer. Trideuterated internal standards were kindly provided by Dr. Kunio Satake, Pfizer, Nagoya, Japan. Hexadeuterated CJ-12,764 was prepared starting with hexadeuterated acetone, and the procedure will be described at a later date (Obach, manuscript in preparation). Stock solutions were made in methanol using volumetric pipettes and volumetric flasks. Standards were refrigerated when not in use. All glassware was subjected to gas-phase silylation using hexamethyldisilazane prior to use [3].

2.2. Construction of standard curves

Concentrated (1.0 mg/ml) stock solutions of ezlopitant, CJ-12,458, and CJ-12,764 were made in methanol. These stock solutions were combined and diluted to generate a solution containing equal concentrations of all three analytes at 10.0 μg/ml. From this stock, standard curve samples in human serum were prepared at concentrations of 100, 50.0, 25.0, 10.0, 5.00, 2.50, and 1.00 ng/ml in duplicate. Quality control samples were prepared from separate stock solutions in an analogous manner.

2.3. Sample preparation

The serum sample preparation procedure utilizes 3 ml/500 mg end-capped cyano solid-phase extraction (SPE) cartridges (Isolute, Jones Chromatography, Lakewood, CO, USA). Samples were processed with a Vac-elut SPS 24 sample processing manifold. Prior to extraction, samples, standards, and quality control samples were spiked with 50 μl of ISTD solution (mixture of $[d_3]$ezlopitant, $[d_3]$CJ-12,458, $[d_3]$CJ-12,764 and $[d_6]$CJ-12,764 at 1 μg/ml). SPE cartridges were conditioned by washing with one column volume methanol followed by 4 ml water (It is critical that the cartridges not be allowed to dry during conditioning). Aliquots of serum (1.0 ml) were added to the top of the SPE bed and aspirated through. Following sample addition, the bed was washed with 4 ml water and one column volume methanol. Analytes were eluted from the cartridge with two column volumes of 1% triethylamine in acetonitrile (prepared fresh daily). The eluent was evaporated to dryness under a gentle stream of nitrogen at 30°C with a TurboVap concentration workstation (Zymark, Hopkinton, MA, USA). Residues were reconstituted in 20 μl of toluene and transferred to limited volume inserts in crimp-topped vials.

2.4. Gas chromatography

The analysis was performed on a Hewlett-Packard 5890 gas chromatograph equipped with electronic pressure control (EPC) and a mass-selective detector utilizing electron-impact ionization and selective-ion monitoring (Hewlett-Packard, Palo Alto, CA, USA). The autosampler tray was cooled to 12°C using a circulating water bath. Splitless injection (2 μl) was accomplished utilizing an inlet pressure program to enhance sample delivery onto the column. Instrument settings are listed in Table 1.

The initial column head pressure was held at 40

Table 1
Instrument settings

Injection parameters	
Pulsed injection	40 p.s.i.; 0.8 min
Injection volume	2 μl
Septum purge valve	2–4 ml/min
Injection liner	Hewlett-Packard single taper
Septum	Supelco Thermogreen
Zone temperatures	
Injection port	260°C
MS transfer line	280°C
GC oven (initial)	170°C
Capillary column	
Manufacturer	Hewlett-Packard
Phase	Ultra-1 (dimethylsiloxane)
Internal diameter	0.2 mm
Length	12 m
Phase film thickness	0.32 μm
Carrier gas	Helium (99.999% purity)

p.s.i. for 0.8 min and then dropped to 18.2 p.s.i. at a rate of 99 p.s.i./min. Constant flow was then maintained at 0.88 ml/min. The oven temperature was programmed to hold at an initial temperature of 170°C for 2 min, followed by a linear temperature increase to 280°C at a rate of 50°C/min, and then held at 280°C for 5.8 min. The temperature was then raised to 325°C and held for 2 min for a total analysis time of 12.9 min. Under these conditions, the retention times for ezlopitant, CJ-12,458, and CJ-12,764 were 6.9, 7.5, and 8.3 min, respectively.

2.5. Mass spectrometry

A Hewlett-Packard 5972 mass-selective detector operated in the electron-impact mode was used to detect the analytes. Ions characteristic of each analyte and internal standard were monitored as outlined in Table 2.

A manual tuning procedure was followed which maximized ion current transmission in the m/z region of interest. Perfluorotributyl amine (PFTBA) was used as the calibration compound and introduced into the source. Abundance of several ions characteristic of PFTBA (m/z 264, 314, 326) were maximized by ramping the potential of source elements (repeller ion focus, entrance lens offset, entrance lens, and X-ray) and selecting the optimum values. Selected ions of PFTBA were changed back to the standard values of m/z 69, 219, and 502 to calibrate the peak widths and mass axis. During chromatographic runs, the ionization source was on only between 5 and 9 min in order to capture peaks of interest while extending source lifetime.

Table 2
List of analytes and internal standards and their respective fragment ions monitored for their quantitation

Compound		m/z
Ezlopitant	Analyte	287
[d_3]Ezlopitant	ISTD	290
CJ-12,458	Analyte	285
[d_3]CJ-12,458	ISTD	288
[d_5]CJ-12,458	DHSTD	290
CJ-12,764	Analyte	303
[d_3]CJ-12,764	ISTD	306
[d_6]CJ-12,764	DHSTD	309

2.6. Calculations

Data collection and integration was accomplished with Hewlett-Packard MS ChemStation software. Standard curves for ezlopitant and CJ-12,764 were constructed by plotting analyte/ISTD peak area ratios vs. intended concentrations and fitting the data using a weighted ($1/x$) linear regression. For CJ-12,458, a more complicated method to determine the peak area ratio was required to account for the extent of dehydration of CJ-12,764 and [d_3]CJ-12,764 ISTD to CJ-12,458 and [d_3]CJ-12,458 ISTD, respectively. The following formula to correct for the extent of dehydration is derived as follows. Ordinarily, a peak area ratio simply consists of the area of the unknown analyte divided by the area of a fixed quantity of an isotopically labelled internal standard:

$$\text{CJ-12,458 ratio} = \frac{A_{\text{CJ-12,458}}}{A_{\text{[d3]CJ-12,458}}}$$

where A represents the peak area for the given analyte listed in the subscript. The isotopically labelled internal standard must possess enough difference in mass from the analyte such that natural abundance frequencies of carbon-13 in the analyte do not interfere with the quantitation of the internal standard. (Usually, three deuterium atoms are adequate for most organic compounds of molecular mass <1000.)

However, since a portion of the peak areas of both CJ-12,458 (alkene) and [d_3]CJ-12,458 ISTD can also be due to dehydration of the benzylic alcohol (CJ-12,764) and its trideuterated internal standard ([d_3]CJ-12,764), respectively, this should be subtracted from the observed peak areas for CJ-12,458 and [d_3]CJ-12,458. To determine the amount of peak area of alkene that arises per peak area observed for the alcohol, the amount of peak area for pentadeuterated alkene ([d_5]CJ-12,458) that arises per peak area of hexadeuterated alcohol ([d_6]CJ-12,764) is determined:

$$\text{correction factor for dehydration} = \frac{A_{\text{[d5]CJ-12,458}}}{A_{\text{[d6]CJ-12,764}}}$$

The area observed for pentadeuterated alkene [d_5]CJ-12,458 must be corrected for the natural abundance of two carbon-13 atoms in the fragment ion being

monitored for the trideuterated internal standard of alkene CJ-12,458. Since there are 18 carbon atoms in the fragment ion being monitored, and the natural abundance of carbon-13 is 1.08%, 1.9% of alkene [d_3]CJ-12,458 ISTD will possess two carbon-13 atoms and thus yield the same fragment ion as pentadeuterated alkene [d_5]CJ-12,458. Therefore, the correction factor for dehydration described above must be further modified to reflect this:

$$\text{correction factor for dehydration} = \frac{A_{[d5]CJ\text{-}12,458} - 0.019A_{[d3]CJ\text{-}12,458}}{A_{[d6]CJ\text{-}12,764}}$$

The amount of observed peak area for alkene CJ-12,458 that arises via dehydration of alcohol CJ-12,764 in the injector port is thus equal to the amount of peak area observed for CJ-12,764 multiplied by the correction factor:

$$\text{amount of CJ-12,458 peak area due to CJ-12,764 dehydration} = \left(\frac{A_{[d5]CJ\text{-}12,458} - 0.019A_{[d3]CJ\text{-}12,458}}{A_{[d6]CJ\text{-}12,764}}\right)A_{CJ\text{-}12,764}$$

and a corresponding term for the trideuterated CJ-12,458 ISTD is

$$\text{amount of [d}_3\text{]CJ-12,458 peak area due to [d}_3\text{]CJ-12,764 dehydration} = \left(\frac{A_{[d5]CJ\text{-}12,458} - 0.019A_{[d3]CJ\text{-}12,458}}{A_{[d6]CJ\text{-}12,764}}\right)A_{[d3]CJ\text{-}12,764}$$

Subtracting these values from the observed peak area values for CJ-12,458 and [d_3]CJ-12,458 ISTD yields the following expression for the corrected peak area ratio for CJ-12,458:

$$\text{corrected CJ-12,458 ratio} = \frac{A_{CJ\text{-}12,458} - [(A_{[d5]CJ\text{-}12,458} - 0.019A_{[d3]CJ\text{-}12,458})/A_{[d6]CJ\text{-}12,764}]A_{CJ\text{-}12,764}}{A_{[d3]CJ\text{-}12,458} - [(A_{[d5]CJ\text{-}12,458} - 0.019A_{[d3]CJ\text{-}12,458})/A_{[d6]CJ\text{-}12,764}]A_{[d3]CJ\text{-}12,764}}$$

Rewritten using the appropriate m/z values, this equation is

$$\text{corrected CJ-12,458 ratio} = \frac{A_{m/z\ 285} - [(A_{m/z\ 290} - 0.019A_{m/z\ 288})/A_{m/z\ 309}]A_{m/z\ 303}}{A_{m/z\ 288} - [(A_{m/z\ 290} - 0.019A_{m/z\ 288})/A_{m/z\ 309}]A_{m/z\ 306}}$$

After this correction was made, standard curves for CJ-12,458 were constructed as described above. Assay accuracy and precision were assessed using quality control samples prepared independently of the standard curve samples. Accuracy and precision were determined by

$$\text{Accuracy} = \frac{100 \cdot \text{individual measured concentration}}{\text{intended concentration}}$$

$$\text{Precision} = \frac{100 \cdot \text{standard deviation}}{\text{mean measured concentration}}$$

3. Results

Mass spectra for ezlopitant and the metabolites CJ-12,458 and CJ-12,764 are presented in Fig. 2. Molecular ions for these compounds were not observed. The base peaks for ezlopitant and CJ-12,458 of m/z 287 and 285, respectively, arise via fragmentation of the diphenylmethyl moiety. This fragmentation pathway appears to predominate for CJ-12,764 as well, as exemplified by m/z 303, however a further loss of H_2O yielded the base peak of m/z 285. The m/z peaks 287, 285, and 303 were selected for monitoring ezlopitant, CJ-12,458, and CJ-12,764, respectively, due to their abundance in the spectra and also due to the fact that they represent corresponding fragmentations for these three compounds.

Representative ion chromatograms for the three analytes and the respective internal standards are presented in Fig. 3 and chromatograms from a representative blank serum sample are presented in Fig. 4. No endogenous interferences were observed in the region of elution of all analytes and internal standards. The linear dynamic range for this assay was from 1.0 to 100 ng/ml using a 1.0 ml sample aliquot. Plots of the standard curves are presented in Fig. 5. While the limit of quantitation for all three analytes was 1.0 ng/ml, the limit of detection was typically 0.1 ng/ml. However, this sensitivity could not be attained on a regular basis to permit reliable quantitation at this level.

Intra-assay accuracy and precision is summarized in Table 3. Accuracy ranged from 94 to 114% for all three compounds. Precision ranged from 0.7 to 6.0%. Inter-assay accuracy and precision from five separate analyses ranged from 103 to 111% and 1.9 to 3.3%, respectively. This assay procedure was amenable to

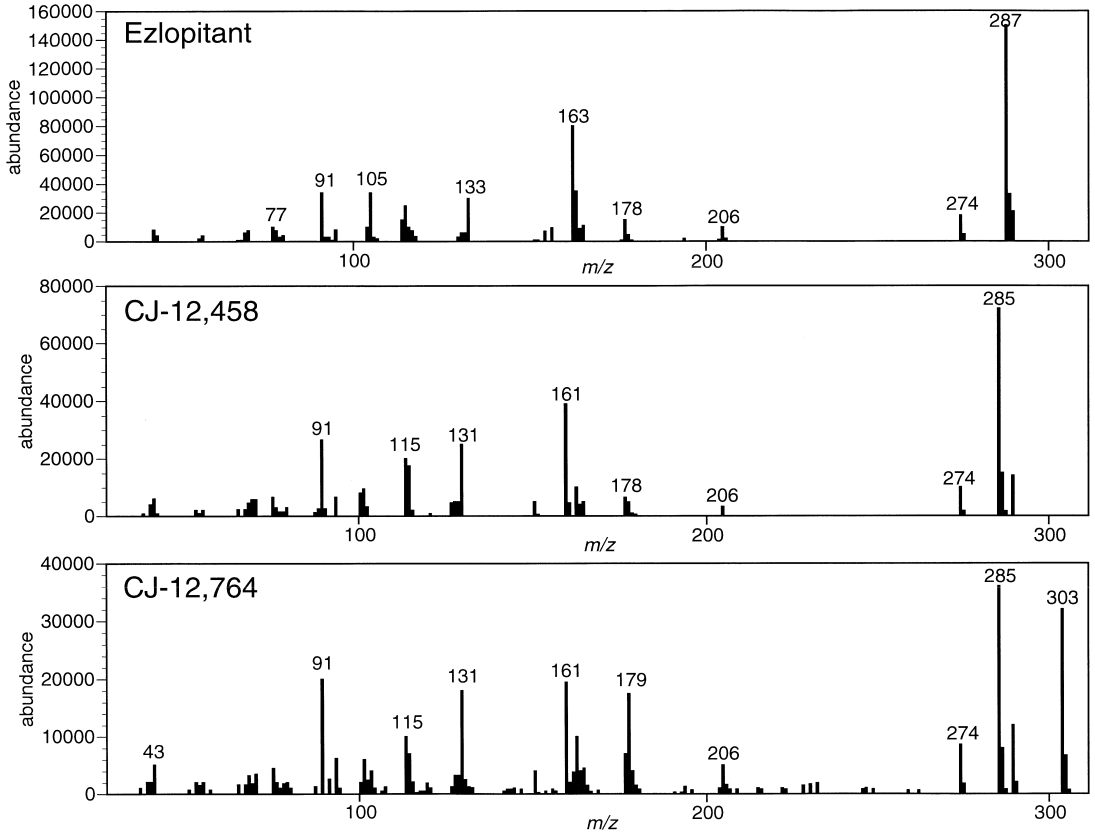

Fig. 2. Electron-impact mass spectra for ezlopitant (top), CJ-12,458 (middle), and CJ-12,764 (bottom).

analysis of samples containing analyte concentrations in excess of 100 ng/ml after 4-, 10-, or 20-fold dilution with control human serum (Table 4).

In human serum, ezlopitant, CJ-12,458, and CJ-12,764 have been demonstrated to be stable for 400 days when stored at −20°C, stable for 8 h when stored at room temperature, and stable through three cycles of freezing and thawing. Furthermore, under conditions of storage in the frozen state, intended concentrations of the analytes are projected not to deviate from initial concentrations by 15% until 600 days. After extraction and reconstitution, samples were permitted to stand on the autosampler for up to 72 h prior to injection. The analytes were stable on the autosampler throughout this period, and stability under this condition was projected to 6 days.

A comparison of CJ-12,458 concentrations calculated with and without utilizing the conversion factor for dehydration of CJ-12,764 is shown in Table 5. Typically, the extent on dehydration of CJ-12,764 was around 2 to 4%. Thus, when concentrations of CJ-12,458 were equivalent to or exceeded those of CJ-12,764, incorporation of the dehydration correction factor did not make a significant impact on the determined concentration of CJ-12,458. However, when CJ-12,764 was present in five-fold or greater excess than CJ-12,458, incorporation of the dehydration correction factor yielded lower percent residual values. For example, when the intended concentrations of CJ-12,458 and CJ-12,764 were 5.00 and 50.0 ng/ml, respectively, the residual value for CJ-12,458 was 20% if uncorrected but was only 15% if corrected for the extent of dehydration of the alcohol. The amount of dehydration of CJ-12,764

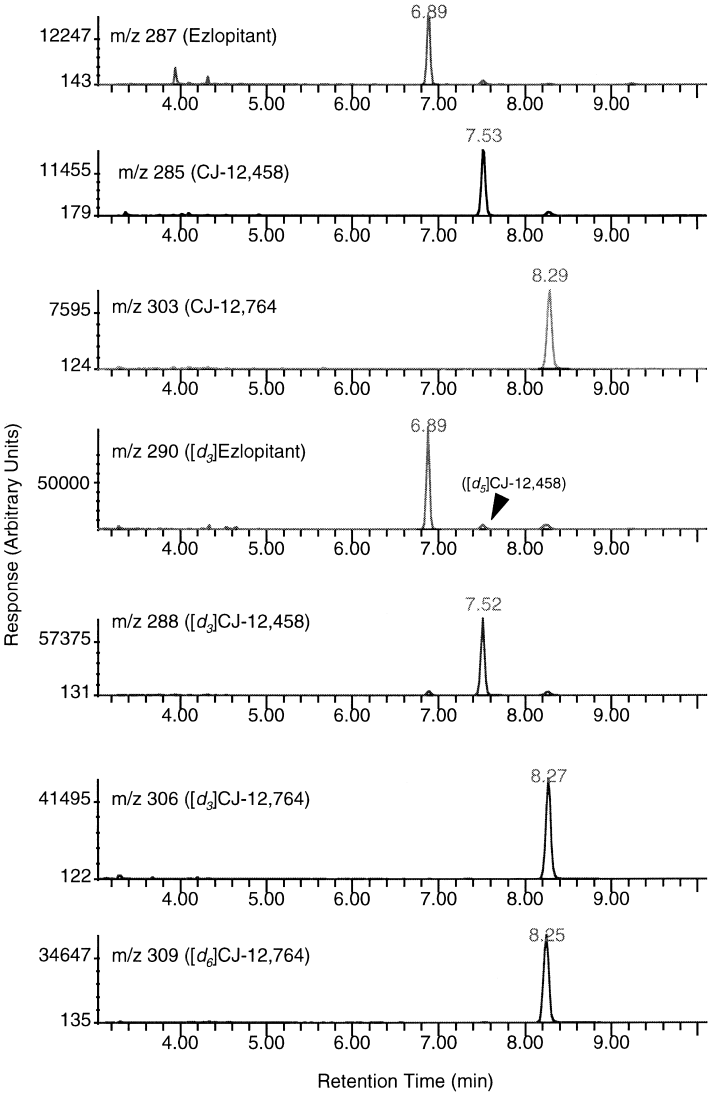

Fig. 3. Representative ion chromatograms for m/z 287 (ezlopitant), m/z 285 (CJ-12,458), m/z 303 (CJ-12,764), m/z 290 ([d_3]ezlopitant and [d_5]CJ-12,458), m/z 288 ([d_3]CJ-12,458), m/z 306 ([d_3]CJ-12,764), and m/z 309 ([d_6]CJ-12,764). Analytes are at concentrations of 10 ng/ml. The [d_5]CJ-12,458 represents approximately 2% of conversion by dehydration.

appeared to be highly dependent on the condition of the injection port liner. With a freshly changed liner, approximately 2 to 4% of CJ-12,764 underwent dehydration to CJ-12,458. When an injection port liner had become 'dirty' due to several hundred injections or due to injection of samples containing large quantities of extraneous materials, the extent of dehydration was so great (~90%) as to prohibit quantitation of CJ-12,764. There did not appear to be a circumstance where an intermediate (10–50%) extent of dehydration occurred. Thus, the dehydration conversion factor could not be tested under a condition where more than 2–4% dehydration occurred.

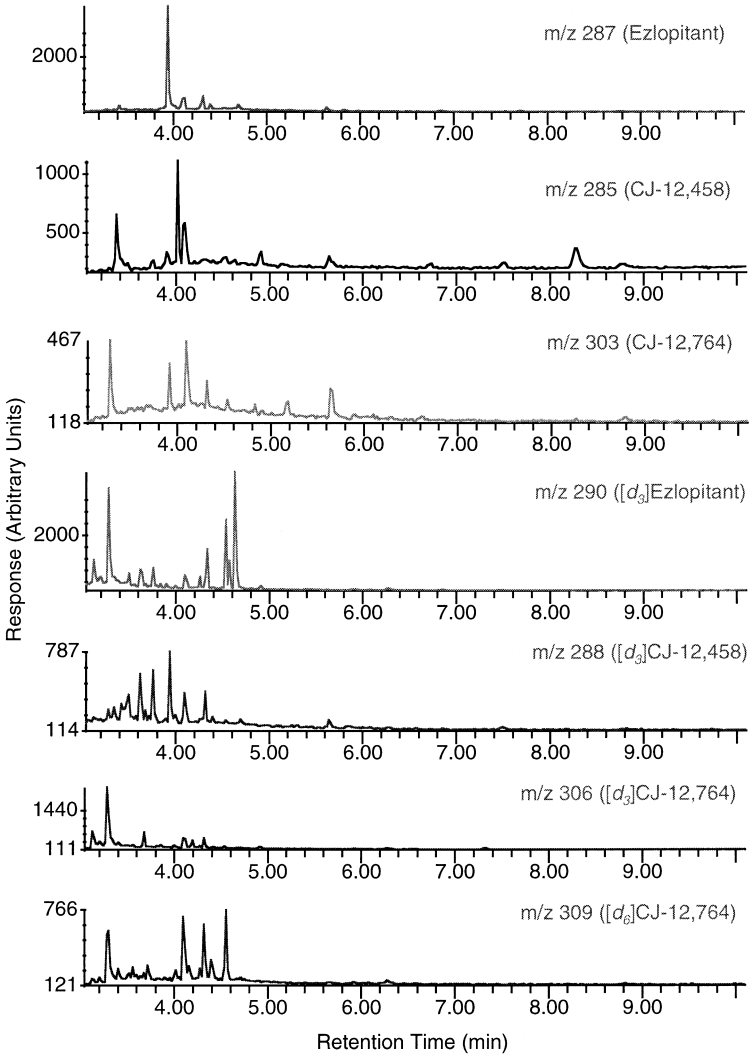

Fig. 4. Representative ion chromatograms for ions of interest for analytes and internal standards in a blank serum sample.

4. Discussion

Ezlopitant represents a useful tool to explore the physiological functions of substance P in animals and in clinical studies. It is therefore required that a method be available to measure the circulating concentrations of ezlopitant and active metabolites in order to relate circulating concentrations to observed effects. The described assay has been utilized in the determination of ezlopitant and metabolite concentrations in human serum samples after administration of ezlopitant (unpublished data). Furthermore, we have used it in the analysis of ezlopitant and metabolite concentrations in animal serum and plasma (Reed-Hagen et al., manuscript in press). Endogenous interferences in animal serum and plasma are not present, and all other characteristics of the assay appear to be the same for this assay when using similar matrices (e.g., serum or plasma) from different species.

The identity of the solid-phase extraction cartridges used for this assay is of significant importance. The cyano chemistry appears to retain the analytes, even through a methanol wash. Addition of

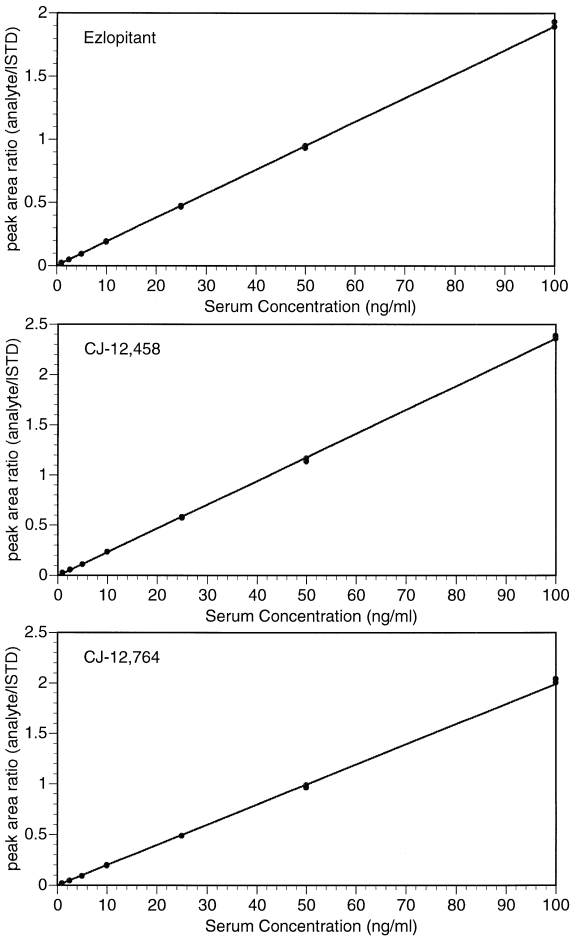

Fig. 5. Standard curves for ezlopitant, CJ-12,458, and CJ-12,764 in human serum. The r^2 and intercept values were 0.9997 and 0.0017, respectively, for ezlopitant; 0.9995 and 0.0006, respectively, for CJ-12,458; and 0.9994 and 0.0020, respectively, for CJ-12,764.

triethylamine to acetonitrile results in effective elution of the analytes. Recoveries of all three analytes through the SPE procedure ranged from 40 to 100% for all three analytes, and were dependent on the concentration, however recoveries of each analyte matched the corresponding internal standard. Such a SPE washing procedure permits obtaining a very clean sample extract which thus assists in prolonging the time before maintainance of the GC–MS is required (e.g., ionization source cleaning, injector liner and septum changing). Free silyl groups in the SPE columns used in this procedure were end-capped which reduces the binding of amine groups to the resin. This assay cannot be conducted using non-end-capped cyano SPE cartridges. The temperature under which SPE eluates were evaporated was also important in this assay. In preliminary attempts in assay development, evaporation under N_2 was conducted at 60°C to expedite removal of the elution solvent. However, recovery for all three analytes was extremely poor and it was found that the recovery was substantially improved if the temperature was lowered to 30°C. The reason for the loss at the higher temperature is unknown.

The dehydration of alcohols to alkenes is a common reaction in organic chemistry, typically catalyzed by protic or Lewis acids [4]. Due to the fact that the alcohol of CJ-12,764 is both tertiary and benzylic, this compound can readily undergo dehydration to CJ-12,458. Naturally, this could complicate any analytical procedure in which both the alcohol and alkene need to be simultaneously quantitated. For gas chromatography, the elevated temperatures utilized during injection and chromatographic separation provide a high potential for dehydration of alcohols, especially tertiary benzylic alcohols. In the assay described in this report, a novel approach was taken to correct for spontaneous dehydration of CJ-12,764 to CJ-12,458: incorporation of an additional isotopically labelled internal standard ([d_6]CJ-12,764) to determine the extent of dehydration in each separate injection (DHSTD). A formula was derived to correct the peak area ratio of CJ-12,458 analyte/internal standard. A correction for CJ-12,764 itself is not necessary since the analyte and internal standard both undergo dehydration to the same extent. However, since samples contain differing ratios of CJ-12,764 to CJ-12,458, but the same ratio of [d_3]CJ-12,764 ISTD to [d_3]CJ-12,458 ISTD, a correction factor is required for the proper calculation of CJ-12,458 concentrations in unknown samples. The formula for this correction simply attempts to determine the amount of the peak area of CJ-12,458 that arises through dehydration of CJ-12,764 in the sample so that it can be subtracted. It also does the same for the internal standard [d_3]CJ-12,458 (from [d_3]CJ-12,764). In order to determine this, [d_6]CJ-12,764 is added to each sample and the peak area of [d_5]CJ-12,458 is measured. The detector response for [d_5]CJ-12,458 (m/z 290) arises from

Table 3
Intra- and inter-assay accuracy and precision for the analysis of ezlopitant and metabolites in human serum. Intra-assay accuracy and precision were determined from five replicate analyses of quality control samples. Inter-assay accuracy and precision were determined from duplicate analyses of quality control samples conducted on five separate occasions

	Intra-assay ($N = 5$)				Inter-assay ($N = 5$)			
Ezlopitant								
Intended conc. (ng/ml)	1.00	2.70	43.0	70.0	100	2.70	43.0	70.0
Mean	0.94	2.90	42.5	76.0	102	3.00	43.4	74.7
SD	0.05	0.05	0.5	1.2	0.7	0.08	0.8	1.5
Accuracy (%)	94	107	99	109	102	111	103	107
Precision (%)	5.0	1.9	1.2	1.7	0.7	3.0	1.9	2.1
CJ-12,458								
Intended conc. (ng/ml)	1.00	2.70	30.0	70.0	100	2.70	30.0	70.0
Mean	1.13	2.97	31.5	78.0	104	2.88	31.3	74.9
SD	0.03	0.04	0.2	1.6	1.3	0.08	0.8	1.9
Accuracy (%)	113	110	105	111	104	107	104	107
Precision (%)	3.0	1.5	0.7	2.3	1.3	3.0	2.7	2.7
CJ-12,764								
Intended conc. (ng/ml)	1.00	2.70	30.0	70.0	100	2.70	30.0	70.0
Mean	1.14	2.97	31.3	78.0	103	2.92	31.2	74.3
SD	0.06	0.04	0.2	2.3	0.7	0.09	0.6	1.8
Accuracy (%)	114	110	104	111	103	107	104	107
Precision (%)	6.0	1.5	0.7	3.3	0.7	3.3	2.0	2.6

two sources: dehydration of the [d_6]CJ-12,764 internal dehydration standard and also the natural abundance of [d_3]CJ-12,458 ISTD containing two ^{13}C atoms in the fragment ion that is monitored (1.9%). Thus the amount of peak area measured for CJ-12,458 resulting from dehydration of CJ-12,764 is represented by the term

$$\left(\frac{A_{[d5]CJ\text{-}12,458} - 0.019A_{[d3]CJ\text{-}12,458}}{A_{[d6]CJ\text{-}12,764}}\right)A_{CJ\text{-}12,764}$$

A corresponding term exists for [d_3]CJ-12,458 for use in determination of the corrected analyte/ISTD peak area ratio for CJ-12,458. Derivation of this correction factor requires that there is no deuterium isotope effect for the dehydration reaction since the deuterium atoms are incorporated in the positions of dehydration. Such an isotope effect was not observed. Incorporation of this correction factor provided better agreement between intended and measured concentrations of CJ-12,458 when the ratio of CJ-12,764 to CJ-12,458 in the sample was high.

The high chromatographic resolution between CJ-12,458 and CJ-12,764 suggests that the dehydration of CJ-12,764 occurs prior to introduction onto the column, and not during chromatography. If dehydration occurred while the compound was on the column, it would be expected that either tailing of

Table 4
Accuracy and precision of the analysis of ezlopitant and metabolites in humans serum after dilution with control human serum when concentrations exceed the upper limit of quantitation

	Intended serum conc. (ng/ml)		
	300	500	1000
Dilution:	1:04	1:10	1:20
Analyte: ezlopitant			
Mean	266	486	906
SD	6	3	30
Accuracy (%)	89	97	91
Precision (%)	2.4	0.7	3.3
Analyte: CJ-12,458			
Mean	271	480	898
SD	6	5	23
Accuracy (%)	90	96	90
Precision (%)	2.3	1.0	2.6
Analyte: CJ-12,764			
Mean	273	471	896
SD	7	7	22
Accuracy (%)	91	94	90
Precision (%)	2.5	1.6	2.5

Table 5
Comparison of calculated concentration values for CJ-12,458 in the presence of CJ-12,764 when including or ignoring the extent of dehydration of CJ-12,764

Intended concentration (ng/ml)		Ratio CJ-12,764/ CJ-12,458	Mean calculated CJ-12,458 concentration (ng/ml) (% residual in parentheses)	
CJ-12,458	CJ-12,764		Uncorrected	Corrected
5.00	50.0	10	6.00 (20)	4.25 (15)
15.0	15.0	1	14.2 (5.3)	14.3 (4.7)
50.0	5.00	0.1	50.1 (1.9)	52.3 (4.6)

the CJ-12,458 peak or blending of the CJ-12,458 and CJ-12,764 peaks would be observed. Attempts were made to lower the extent of dehydration. Reconstitution of SPE eluates in toluene containing triethylamine resulted in less dehydration, either by coating catalytically active surfaces in the injector port or through basification of the reconstitution matrix. However, enough of a decrease of this reaction was not provided to merit incorporation of injection of a solvent such as TEA onto the GC column. Also, lowering the injection port temperature was attempted to lessen the extent of dehydration. While somewhat less dehydration occurred at lower injector port temperatures, delivery of analyte to the column was also decreased. The injector port temperature used in the assay was the optimal temperature for both sensitivity and extent of dehydration.

In summary, the GC–MS procedure described in this report is a robust method for quantitating the substance P antagonist ezlopitant, and its metabolites in biological matrices with high (1.0 ng/ml) sensitivity. Furthermore, this method describes a novel approach in the simultaneous quantitation of tertiary benzylic alcohols and alkenes when the former spontaneously dehydrates to the latter in gas chromatographic analysis. It is proposed that this approach would be generally applicable in similar situations.

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