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Development and validation of a selective and sensitive bioanalytical procedure for the quantitative determination of gaboxadol in human plasma employing mixed mode solid phase extraction and hydrophilic interaction liquid chromatography with tandem mass spectroscopic detection

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Abstract

A selective and sensitive hydrophilic interaction liquid chromatography tandem mass spectrometric bioanalytical method for the quantitative determination of gaboxadol in human heparinized plasma was developed and validated. Gaboxadol and the stable isotope labeled internal standard were extracted from plasma by mixed mode solid phase extraction and analyzed on an Asahipak NH_2P HPLC column with a mobile phase composed of 70% acetonitrile and 30% ammonium acetate (20 mM, pH 4). The analytes were detected by a SCIEX API 4000 triple quadropole instrument using turbo electrospray ionization and multiple reaction monitoring negative mode. The method was validated over the concentration range of 0.5–100 ng/mL. The intra-day precision of the assay, as measured by the coefficient of variation (CV%), was within 4%. The intra-day assay accuracy was found to be within 2.2% of the nominal concentration for all the standards. The average recovery of gaboxadol was about 87% and the ion suppression was approximately 8%. To eliminate late eluters including the glucuronides, a "front cut" column switching procedure was added to the chromatographic system. The effectiveness of the column switching in eliminating the absolute matrix effect caused by late eluters was demonstrated by the low variation (CV < 3.5%) in the peak areas of the internal standard during the assessment of the inter-day precision and accuracy and no significant relative matrix effect was observed as illustrated by the excellent intra-day precision (CV < 1.5%) from the assessment of standard samples prepared in five different lots of control plasma. The described bioanalytical method has been successfully utilized for the analysis of gaboxadol in post-dose samples (>8000) from various clinical studies. Inter-day precision and accuracy were assessed from the daily mean ($n = 2$) of QC values from 52 runs, i.e. more than 3000 samples. The inter-day precision of the assay, based on the coefficient of variation of QC, ranged from 2.1 to 5.1%. The inter-day assay accuracy was found to be within 4% of the nominal concentration for all QC samples.

Keywords: Gaboxadol; HILIC; LC–MS/MS; SPE; Matrix effect; Validation

1. Introduction

Gaboxadol (Fig. 1) is a heterocyclic analogue of γ-aminobutyric acid and a selective extrasynaptic $GABA_A$ agonist (SEGA). The compound, also known as THIP, was under development for treatment of insomnia in a joint partnership between Merck and H. Lundbeck A/S.

Gaboxadol is a small, polar compound and a zwitter ion. Past attempts to develop an applicable method for bioanalysis utilized a time consuming pre-column derivatization step [1,2]. In addition, these methods did not have sufficient sensitivity to determine circulating levels of the drug.

Reversed phase liquid chromatography is currently the most commonly applied separation technique for quantitative determination of drugs in biological matrices. However, in the case of

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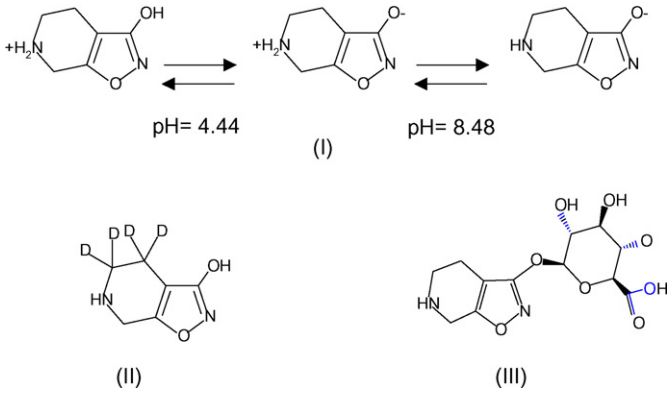

Fig. 1. Structures of (I) gaboxadol, (II) d_4-gaboxadol internal standard and (III) *O*-glucuronide-gaboxadol. Gaboxadol is a zwitterion with acidic as well as basic properties. The pK_a values are 4.44 for the alcohol and 8.48 for the amino group.

small polar compounds, it may be difficult to obtain the retention of such compounds under conditions that are compatible with mass spectrometric detection.

Recently, several papers have described the use of hydrophilic interaction chromatography (HILIC), as an alternative to reverse phase chromatography, for the bioanalysis of pharmaceuticals in plasma. Zwitterion [3,4] and bare silica columns [5–12] have been employed in these methods.

Under HILIC conditions, a polar stationary phase is utilized with an aqueous-organic mobile phase, typically with a water fraction below 50%. Once the column is equilibrated with the mobile phase, a water-enriched liquid layer is established within the stationary phase. Hydrogen bonding and dipole–dipole interactions between the analyte in the mobile phase and the water-enriched stationary phase will separate the analyte from the effluent, thus providing retention of small polar, ionic compounds [13]. Retention of small, polar and ionic compounds may be caused by the primary HILIC interaction with a weak secondary, electrostatic interaction and therefore require low buffer concentrations to elute from the column. Hence, a HILIC column operated with a mobile phase of high organic content and low buffer concentration is thus ideal for the LC–MS analysis of polar compounds.

Under HILIC conditions however, endogenous polar molecules such as phospholipids, peptides and sugars may have strong electrostatic interactions with the polar stationary phase, depending on the pH and ion strength of the mobile phase, and therefore require high buffer concentrations to disrupt these bindings. These compounds may be strongly retained on a HILIC column operated at a high organic content and a low buffer concentration and thus elute late from the column. Furthermore, these endogenous compounds are known to cause competition during ionization at the electrospray LC–MS interface, thereby potentially causing the ionization of the target analyte(s) to be suppressed [14,15].

This paper describes a bioanalytical application for the quantitative determination of gaboxadol in heparinized human plasma samples. The method utilizes mixed-mode cation exchange/reverse phase solid phase extraction for sample preparation. Analytes are separated using hydrophilic interaction chromatography and are detected using a tandem mass spectrometer equipped with a turbo ion-spray source. A "front cut" column switch technique is incorporated into the method to remove interferences from late eluting matrix components as well as late eluting phase II conjugates of gaboxadol. The method was validated, according to current regulatory requirements [16] over the analyte concentration range of 0.5–100 ng/mL. The method was successfully utilized to analyze samples collected following the oral administration of gaboxadol.

2. Experimental

2.1. Materials

Gaboxadol hydrochloride was provided by H. Lundbeck or purchased from Sigma–Aldrich (THIP). 96-well Oasis MCX (10 mg) SPE plates were from Waters (Milford, MA USA). All standard chemicals used were at the highest grade of purity. Gaboxadol-*O*-glucuronide was provided by Merck Laboratory Research.

2.2. Preparation of d_4-gaboxadol: 4,4,5,5-tetradeutero-6,7-dihydro-isoxazolo[5,4-c]pyridin-3-ol hydrochloride (internal standard)

2.2.1. 6-Benzyl-deutero-isoxazolo[5,4-c]pyridin-3-ol

To a stirred solution of 6-benzyl-3-hydroxy-isoxazolo[5,4-*c*]pyridin-6-ium bromide in ethanol/water (1:1), two times surplus of $NaBD_4$ cautiously portion wised was added, keeping the temperature under 35 °C. The mixture was stirred for 24 h, after which the mixture was filtered and the residue was washed with water. The combined filtrates were evaporated under reduced pressure until all of the ethanol had evaporated. A solid began to precipitate and the mixture was cooled in an ice-water bath with stirring for 30 min. The solution was filtered and the residue was washed with cold water. The residue was dried to give 6-benzyl-deutero-isoxazolo[5,4-*c*]pyridin-3-ol.

2.2.2. 4,4,5,5-tetradeutero-6,7-dihydro-isoxazolo[5,4-c]pyridin-3-ol hydrochloride

To a stirred solution of 6-benzyl-deutero-isoxazolo[5,4-*c*]pyridin-3-ol in ethyl acetate under a nitrogen atmosphere surplus of di-disopropylethylamin and methyl chloroformate were added and the mixture was stirred for 48 h. The mixture was cooled in an ice-water bath and an aqueous solution of ammonia (25%, w/v) was added. After 15 min the aqueous phase was separated and was adjusted to pH 1.0 by the addition of aqueous hydrochloric acid (10 M). This aqueous phase was extracted twice with ethyl acetate and these combined extracts were dried over anhydrous magnesium sulfate and evaporated under reduced pressure. The residue was dissolved in a solution of gaseous HBr in acetic acid (33%, w/v) and the mixture was stirred at 40 °C for 6 h. The mixture was cooled in an ice-water bath and ethanol was added. A precipitate formed and the mixture was stirred in the cold for a further 1 h. The mixture was filtered and the residue was washed with cold ethanol and dried. The residue was re-crystallized

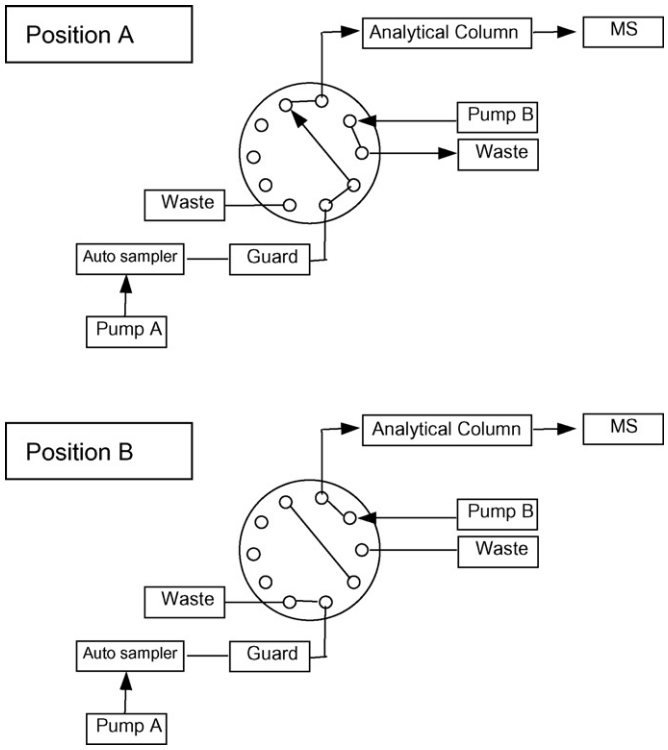

Fig. 2. Schematic diagram of the "front cut" HPLC system. A Bio Basis AX guard column (2.1 mm × 10 mm, 5 μm) and an Asahipak NH_2P-50 2D 150 mm × 2 mm HPLC column were connected via a Valco 10-port 2 position electrically actuated switching valve. The valve was programmed to conduct a "front cut" of effluent with the following settings: 0 min: valve position A, flow rate pump A: 0.25 mL/min, flow rate pump B: 0.25 mL/min. 0.5 min: valve position B, flow rate pump A: 0.25 mL/min, flow rate pump B: 0.50 mL/min. 7.5 min: valve position A, flow rate pump A: 0.25 mL/min, flow rate pump B: 0.25 mL/min. The run time was 8 min.

in aqueous hydrochloric acid to give 4,4,5,5-tetradeutero-6,7-dihydro-isoxazolo[5,4-*c*]pyridin-3-ol, hydrochloride.

2.3. Instrumentation

The method was validated and performed on a Perkin-Elmer 200 series micropump system with a Varian Prostar 430 autosampler. The standard autosampler stainless steel needle was replaced with a PEEK sample needle. The system was coupled to a Sciex API 4000 tandem mass spectrometer equipped with a Turbo Ion Spray Interface. Data acquisition software was Analyst version 1.4.

2.4. Chromatographic conditions

A Bio Basis AX (Thermo-Hypersil, Keystone) guard column (2.1 mm × 10 mm, 5 μm) and an Asahipak NH_2P-50 2D 150 mm × 2 mm (Shodex, Japan) HPLC column were connected via a Valco 10-port 2 position electrically actuated switching valve (Fig. 2). The valve was programmed to conduct a "front cut" of the effluent. The columns were operated at a flow rate of 0.25 mL/min with a mobile phase composed of 70% acetonitrile and 30% ammonium acetate (20 mM, pH 4). The run time was 8 min.

2.5. LC–MS/MS conditions

The mass spectrometer was connected to the HPLC system via a turbo ion spray interface and was operated in the negative ionization mode. The detection of the analytes was based on multiple reaction monitoring (MRM) of the de-protonated molecules $(M-H)^-$ and their major collision-induced fragments (m/z 139 → 110 for gaboxadol and m/z 143 → 110 for d_4-gaboxadol). The dwell time for both channels was 800 ms.

2.6. Preparation of standards and quality control samples

Calibration standards to cover the assay range of 0.5–100 ng/mL were prepared by adding 25 μL of 2, 1, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 μg/mL working standards to 0.5 mL aliquots of control plasma. Quality control (QC) samples were prepared by adding 1 mL of 7.5, 1.5 or 0.15 μg/mL solutions of the gaboxadol to 100 mL volumetric flasks, after which the flasks were filled to volume with control plasma. The resulting pools contained the analyte at concentrations of 75 ng/mL (High QC), 15 ng/mL (Middle QC) and 1.5 ng/mL (Low QC).

2.7. Sample preparation

A half milliliter aliquot of a human heparin plasma sample was spiked with 25 μL internal standard solution and was acidified with 500 μL 2% formic acid. The entire resulting solution was loaded on to a Waters Oasis MCX (10 mg) 96-well plate conditioned with 1 mL aliquots of methanol and water. The plate was washed with 1 mL aliquots of 2% formic acid, water, and methanol sequentially. The analytes were eluted with 250 μL of 1.2% (v/v) solution of ammonium hydroxide in methanol. The effluent was evaporated to dryness and was, to promote peak compression, reconstituted in 250 μL of methanol/acetonitrile (70/30, v/v). A 25 μL aliquot of the final extract was injected into the LC–MS/MS system.

3. Results and discussion

Gaboxadol is a small, polar, ionic compound and an unique approach was required to develop a robust, selective, and sensitive bioanalytical method. The sample preparation was based on mixed mode solid phase extraction. The effluent from the extraction column was evaporated to dryness and reconstituted in a reduced volume to increase sensitivity. The sample was then injected into a column switching HPLC system operated in the HILIC mode. Fig. 3 shows a chromatogram of a spiked human plasma standard sample containing 0.5 ng/mL gaboxadol and 5.0 ng/mL d_4-gaboxadol internal standard; the sample was extracted and chromatographed using the final assay conditions. Development, validation and application of the method are described in the following paragraphs.

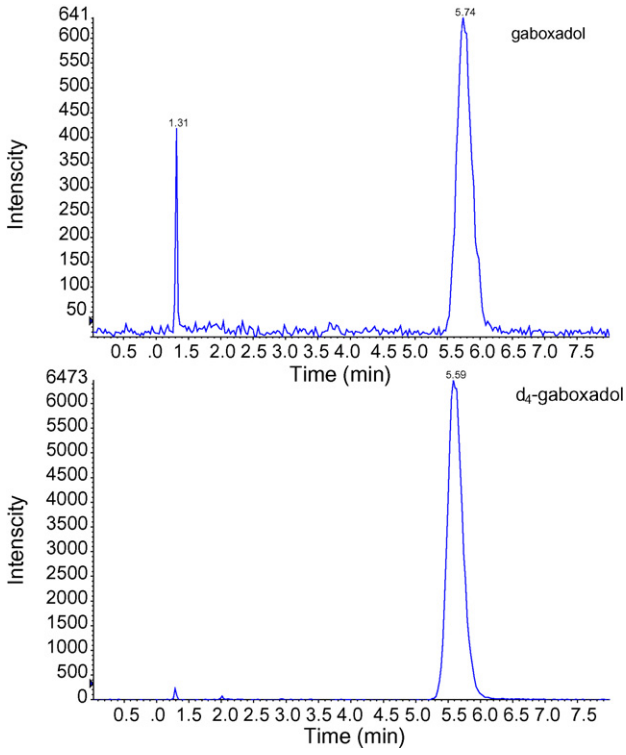

Fig. 3. Chromatogram of a LLOQ standard (0.500 ng/mL) gaboxadol containing 5.00 ng/mL d_4-gaboxadol.

3.1. Assay development

3.1.1. Chromatographic system

Use of standard reverse phase (RP) conditions resulted in minimal retention of the analytes. Hence, the use of hydrophilic interaction chromatography on bare silica columns was evaluated as an alternative to RP-HPLC. Mobile phases containing a high percentage of water (>35%) were required to elute gaboxadol from the bare silica columns. Under these conditions, column dissolution, manifested in the form of a build-up of white material on the ion-spray needle, was observed. The column dissolution resulted in steadily decreasing sensitivity over the course of a sample run. In addition, the useful lifetime of bare silica columns was limited.

Columns containing amino bonded stationary phases operated in HILIC mode were explored as an alternative to bare silica columns. The amino columns chosen for evaluation were an Asahipak NH2P-50 2D, 150 mm × 2 mm column (Shodex, Japan) based on a derivitized polyvinyl alcohol polymer, a YMC NH2, 100 mm × 2 mm column (YMC, USA) based on an amino propyl silica polymer, a Phenomenex Luna NH2, 150 mm × 2 mm column (Phenomenex USA) based on a cross linked "Prepolymer", and a Capcell Pak NH2, 150 mm × 2 mm column (Shiseido, Japan) based on a polymer encoated silica polymer. Mobile phases containing a mix of acetonitrile and ammonium acetate buffer (pH 4) were used during the column evaluation. At this pH the electrostatic interaction between the analyte and the stationary phase was low and HILIC retention was assumed to be the dominant retention mechanism.

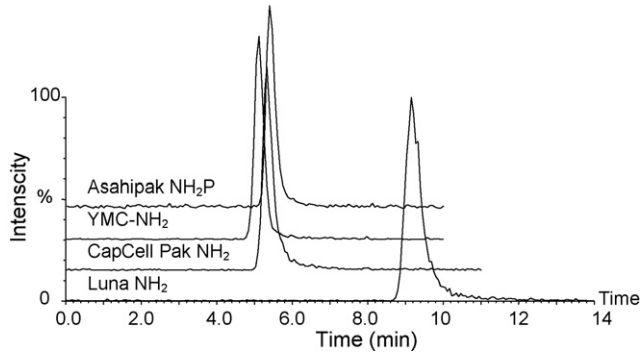

Fig. 4. Overlay of four chromatograms of gaboxadol run on four different amino HILIC columns operated at identical conditions: The columns were operated at a flow rate of 0.2 mL/min with a mobile phase consisting of 70% acetonitrile and 30% 20 mM ammonium acetate adjusted to pH 4 at ambient temperature.

The columns were evaluated and compared by retention properties towards gaboxadol, as well as the retention properties towards matrix residuals. Fig. 4 shows that the chromatographic properties towards gaboxadol at pH 4 were similar for the columns, however the Phenomenex column was more retentive than the other three columns.

Matrix interactions were evaluated by continuous post-column infusion of gaboxadol into the mobile phase [14] and injection of acetonitrile protein precipitation plasma extracts. The post-column infusion technique allowed for the comparison of the secondary HILIC interactions between the stationary phases of the amino columns and what was assumed to be different classes of endogenous components from plasma. Fig. 5 shows infusion chromatograms of injected protein precipitated plasma samples overlaid with chromatograms of gaboxadol for the four amino columns. The distribution of suppression regions of the chromatogram was very different for the four columns even though the retention of gaboxadol was similar.

Fig. 6 shows chromatograms of 160 min infusion after injection of a protein precipitated plasma sample onto the four amino columns. In Fig. 6A (Phenomenex Luna NH2) the column separated a large number of endogenous compounds, causing suppression of the electrospray ionization of gaboxadol. The silica based YMC amino propylene column (Fig. 6C) showed selectivity towards the endogenous compounds and separated the compounds into five major regions. The Capcell Pak NH2 and Asahipak NH2P columns (Fig. 6B and 6D) both showed two major regions of suppression after the injection of protein-precipitated plasma. The ionization suppressing compounds may be different classes of phospholipids with different heads and different lipid chain lengths and compositions, indicating that the retention mechanisms may be secondary, electrostatic interactions between the phospholipids and the amino columns operated under HILIC conditions [17–19].

The Asahipak column was chosen for further exploration due to its general properties of the column combined with the infusion profile in Fig. 6D. The stable baseline in the infusion chromatogram for the Asahipak column made it possible to perform approximately 10 injections before the major late eluting ion-suppression region appeared from the first injection. The

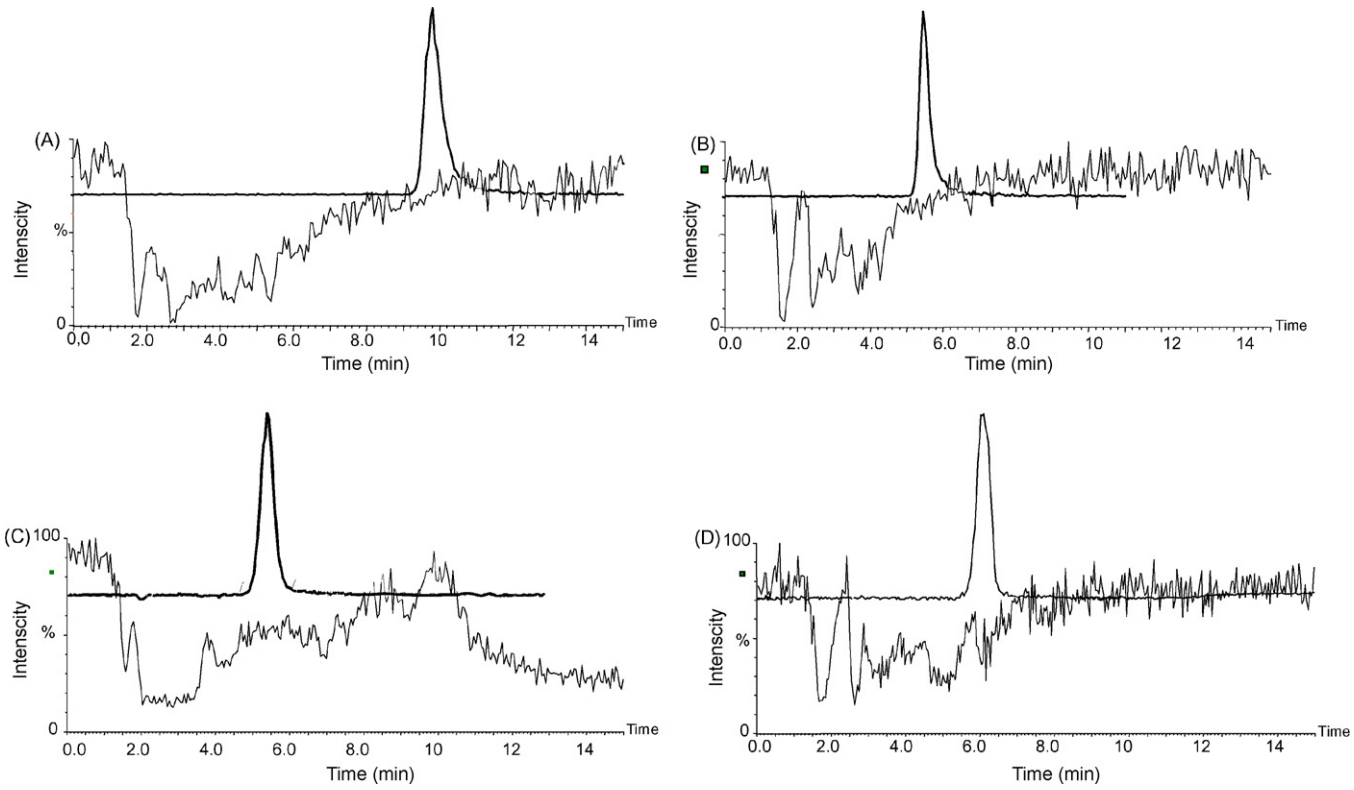

Fig. 5. Infusion profiles of a protein precipitation extract of blank plasma injected onto four different amino HPLC columns. The columns were (A) Phenomenex Luna NH2, 150 mm × 2 mm ID, (B) Capcell Pak NH2, 150 mm × 2 mm ID, (C) YMC NH2, 100 mm × 2 mm ID, (D) Asahipak NH2P-50 2D, 150 mm × 2 mm ID. The profiles are overlaid with chromatograms of gaboxadol standards injected onto the same HPLC column without infusion. The suppression of the ionization of gaboxadol in the LC–MS electrospray interface was assessed by continuous post-column infusion experiments. 1 ng/mL of gaboxadol was teed into the HPLC effluent with a flow of 1.2 mL/h. When an elevated and constant baseline was observed, protein precipitated control plasma was injected. Protein precipitation was performed by adding cold acetonitrile at a ratio of 4:1 to plasma. The comparison of columns and the infusion of precipitation samples were performed on a Micromass Quattro Ultima triple quadropole mass spectrometer, equipped with an electrospray interface. The infusion was obtained as chromatograms for 15 min and any region of ionization suppression was observed as a decrease in the baseline.

late eluting region had a duration corresponding to the run-time of one injection and resulted in an approximately 50% decrease in mass spectrometer sensitivity after the 10th injection (Fig. 7A). The mass spectrometer sensitivity for the subsequent injections were suppressed constantly and by approximately 50%.

3.1.2. Sample preparation

The protein precipitation sample preparation method was felt to be a major contributor to the presence of the late eluting components that contributed to the loss of sensitivity observed by following repetitive injections. Protein precipitation is known to be non-selective [15] and may not be applicable for sample preparation in a HILIC based method; an amino bonded HILIC column may have general weak anion properties [20–22] that may cause the strong retention of matrix residuals.

For better ruggedness and selectivity of the assay, a mixed mode (cation exchange/reversed phase) solid phase extraction procedure was thus developed. Although the SPE method provided better selectivity and high recovery for gaboxadol, absolute matrix effects resulting from the late eluting endogenous peaks (up to 3 h) were still observed under the described chromatographic conditions. This observation was confirmed by the post-column infusion of a gaboxadol after the injection of blank plasma solid phase extract (data not shown).

In addition, a small late eluting peak was observed in the gaboxadol transition following the injection of post-dose sample extracts. The late eluting peak (RT ∼24 min) was later proved to be the *O*-glucuronide of gaboxadol having undergone conversion to the aglycone within the mass spectrometer ion source (Fig. 8A).

3.1.3. Column switching system

To eliminate the late eluting peaks, a "front cut" column switching procedure was added to the chromatographic system. Samples were injected onto a guard column connected to the analytical column. After elution of the analytes from the guard onto the analytical column, the valve position was switched so that only the effluent from the first 0.5 min after the sample injection was directed to the analytical column. All effluent after 0.5 min was directed to waste through the guard column. Late eluters including the glucuronides were flushed from the guard column to waste, while the analytes were separated on the analytical column.

The effectiveness of the column switching in eliminating the absolute matrix effect caused by late eluters was demonstrated

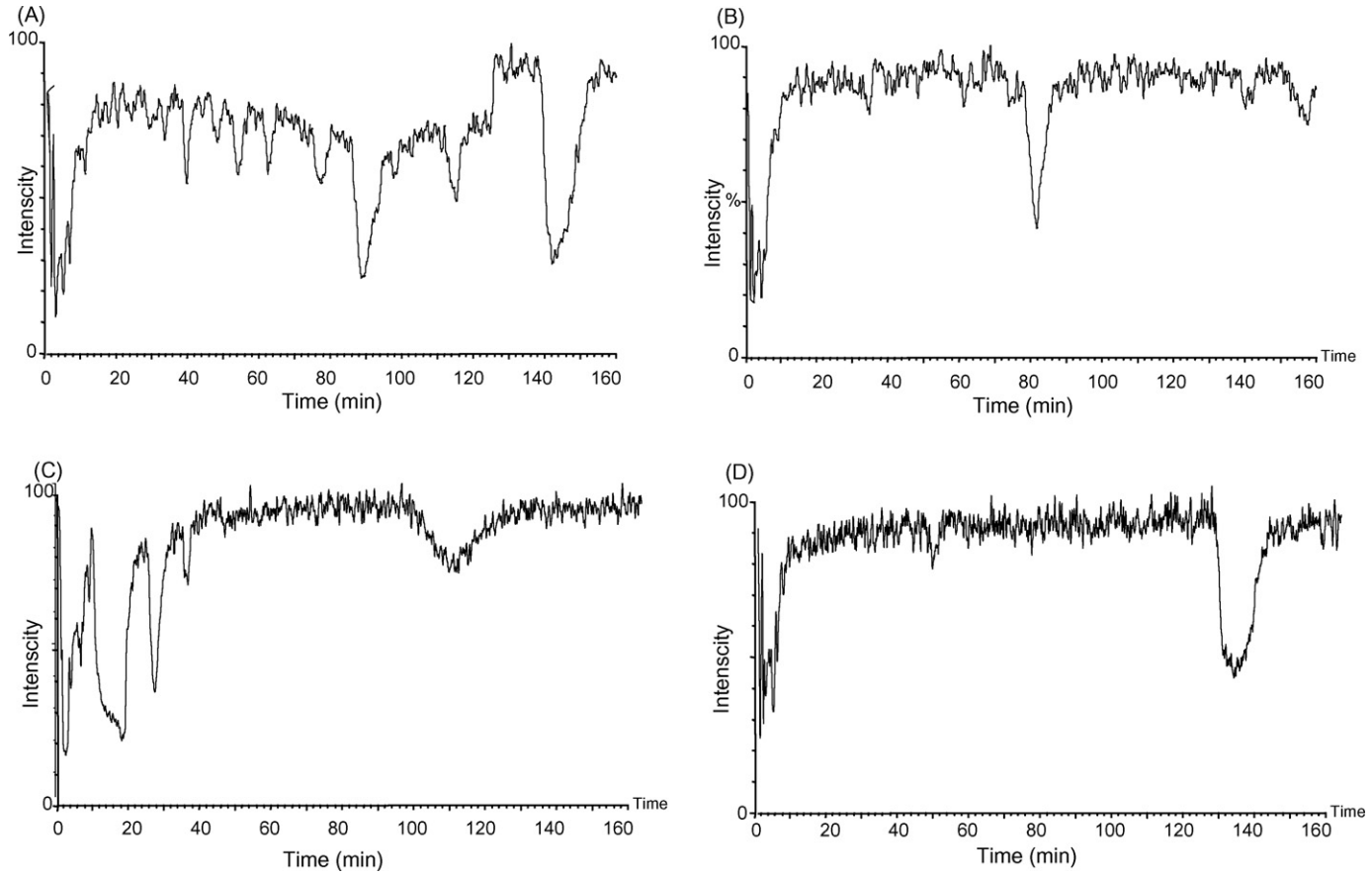

Fig. 6. A 160 min infusion profile obtained under the same conditions as described for Fig. 5.

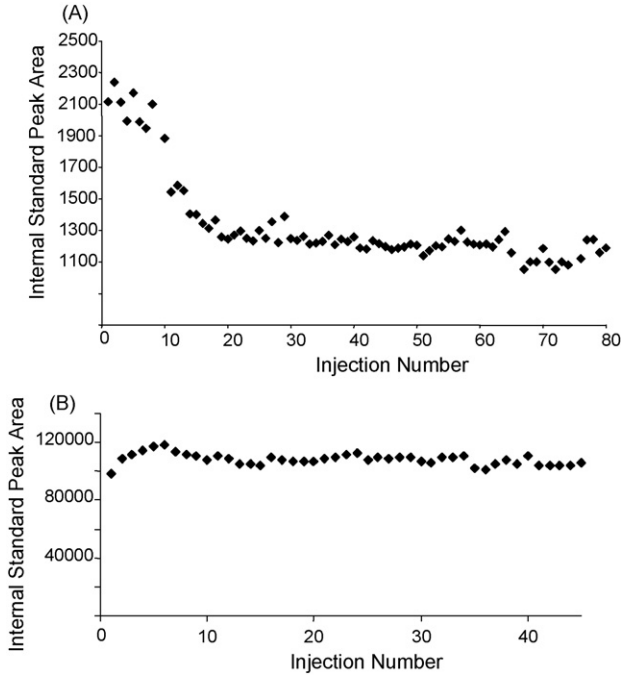

Fig. 7. (A) Internal standard peak areas observed during injection of 80 protein precipitated plasma samples. The analyses were performed on a Micromass Quattro Ultima triple quadropole mass spectrometer, equipped with an electrospray interface. (B) Internal standard peak areas observed during the determination of the inter-day precision and accuracy.

by the low variation (CV < 3.5%) in the peak areas of the internal standard during the assessment of the inter-day precision and accuracy of the column-switching method (Fig. 7B). The switching also eliminated the late eluting *O*-glucuronide, as illustrated in the chromatograms from the injection of the *O*-glucuronide standard (Fig. 8B).

3.2. Method validation

3.2.1. Method specificity

No peaks eluting at the retention times of gaboxadol or the internal standard were detected in the double blank samples prepared from six different lots of control plasma. In addition, no "cross-talk" was observed in the MRM channels used for gaboxadol and the internal standard. Representative chromatograms from the standard at LLOQ (lower limit of quantization) are shown in Fig. 3.

3.2.2. Linearity

Weighted ($1/x^2$) least squares regression calibration curves were constructed by plotting the peak area ratios of analyte to internal standard versus standard concentration. The correlation coefficient (r^2) was typically better than 0.99 over the concentration range of 0.5–100 ng/mL plasma. The deviation between the nominal standard concentrations and experimental standard concentrations was generally within 10% with the use of the weighted least squares regression.

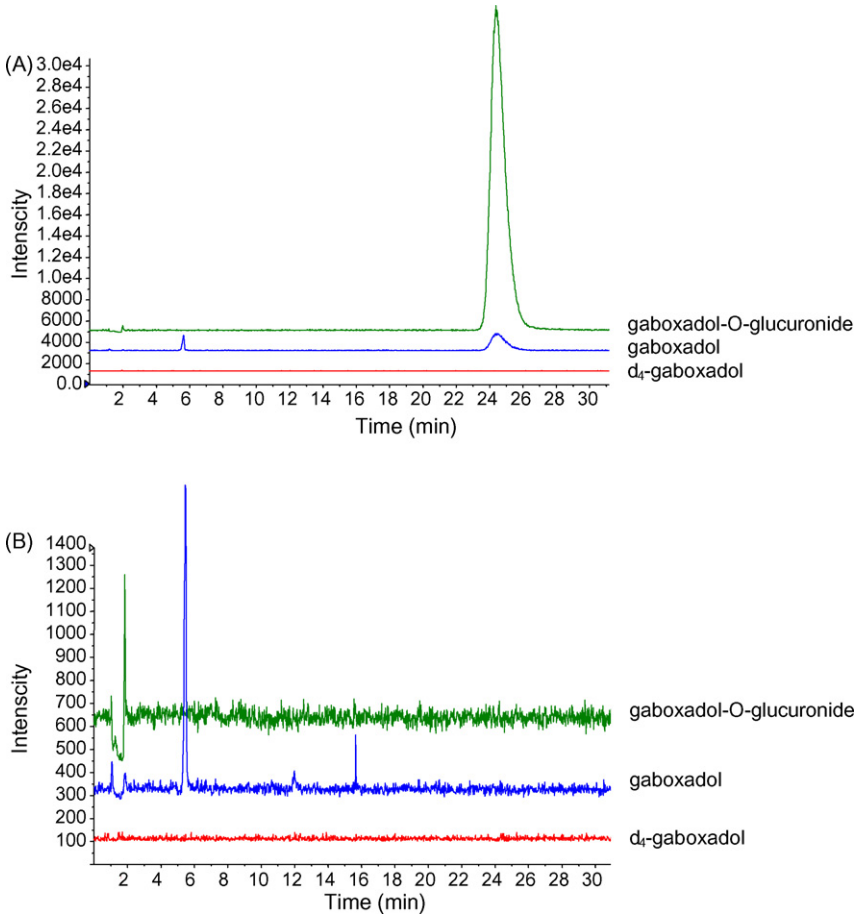

Fig. 8. (A) Chromatogram of the gaboxadol *O*-glucuronide analyzed without front cut. MS/MS transitions are shown for gaboxadol, gaboxadol-*O*-glucuronide and d_4-gaboxadol, respectively. Note that the glucuronide to some extent was converted to gaboxadol within the mass spectrometer ion source. (B) Chromatogram of the gaboxadol-*O*-glucuronide of analyzed with front cut.

3.2.3. Extraction recovery and assessment of matrix effect

Extraction recovery and the effect of the sample matrix on ionization were evaluated for gaboxadol plasma samples prepared at concentrations of 1, 5, and 50 ng/mL in five different lots of control plasma. Extraction recovery was determined by comparing the absolute peak areas of the standards extracted from spiked control plasma to control plasma extracted in the same manner and spiked post-extraction with the same standard concentration. Matrix enhancement/suppression of ionization was evaluated by comparing the absolute peak areas of post-extraction spiked samples with corresponding absolute peak areas of neat standards prepared in the reconstitution solution.

Results from the assessments are shown in Table 1. The average recovery of gaboxadol over the standard curve range of the assay was about 87%. Even though slight ion suppression (∼8%) was shown from the assessment of absolute matrix effect, no significant relative matrix effect was observed, as illustrated by the excellent intra-day precision results (Table 2) from the assessment of standard samples prepared in five different lots of control plasma. The negligible relative matrix effect was also confirmed by the low coefficient of variation (CV < 1.5%) between the slopes of the five standard curves.

3.2.4. Intra-day method precision and accuracy

Intra-day precision and accuracy were assessed by analyzing five sets of standard samples prepared in five different lots of control plasma over the concentration range of 0.5–100 ng/mL. The intra-day precision of the assay, as measured by the coefficient of variation (CV%), was within 4% for all points on the standard curve. The intra-day assay accuracy was found to be within 2.2% of the nominal concentrations for all standards (Table 2).

Table 1
Extraction recovery and assessment of matrix effect on ionization

Spiked plasma concentration[a] (ng/mL)	Extraction recovery[b] (%)	Matrix effect[c] (%)
1	86.1	88.0
5	84.8	93.2
50	91.0	95.8
5 (ISTD)	90.4	90.3

[a] $N = 5$ in different lots of plasma.
[b] Extraction recovery was calculated using the following formula: Recovery (%) = [(mean raw peak area)$_{\text{pre ext. spike}}$/(mean raw peak area)$_{\text{post ext. spike}}$] × 100.
[c] Matrix effect was calculated using the following formula: Matrix effect (%) = [(mean raw peak area)$_{\text{post ext. spike}}$/(mean raw peak area)$_{\text{neat}}$] × 100.

Table 2
Intra-day precision and accuracy data for the determination of gaboxadol in five lots of human plasma

Nominal concentration (ng/mL)	Determined conc. Mean (ng/mL, $n=5$)	Accuracy[a] (%)	Precision[b] (%)
0.5	0.503	100.5	4.0
1	0.994	99.4	3.4
2.5	2.48	99.3	1.9
5	4.91	98.3	1.8
10	10.2	102.2	1.5
25	24.8	99.1	1.6
50	50.6	101.2	2.5
100	99.9	99.9	1.3

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Coefficient of variation of peak area ratios.

Table 3
Inter-day precision and accuracy data for the determination of gaboxadol human plasma from the daily QC results

	Low QC	Middle QC	High QC
Nominal conc. (ng/mL)	1.50	15.0	75.0
Mean conc. (ng/mL) ($n=52$)	1.56	15.0	74.7
Accuracy (%)	104	99.9	99.6
Precision (% CV)	5.1	2.1	2.9

3.2.5. Inter-day method precision and accuracy

Inter-day precision and accuracy were assessed from the daily mean ($n=2$) of QC values from 52 runs, i.e. more than 3000 samples. The inter-day precision of the assay, based on the coefficient of variation of QC, ranged from 2.1 to 5.1%. The inter-day assay accuracy was found to be within 4% of the nominal concentrations for all QC samples (Table 3).

3.2.6. Analyte stability

Stability of gaboxadol under various process and storage conditions was investigated. Room temperature (RT) benchtop and freeze/thaw stability were assessed by the analysis of quality control samples that were left on the benchtop at room temperature for 4 h, or subjected to three freeze (−70 °C)/thaw (RT) cycles prior to the sample extraction. The results indicated that gaboxadol was stable in plasma under both conditions (Table 4).

Processed sample stability was evaluated by the re-injection of a group of samples consisting of a set of calibration standards and QCs that were remained on the autosampler for 34 h after the initial injections. The difference between the concentrations determined from the initial injection and the re-injection was within 8%.

Table 4
Assessment of Benchtop and freeze/thaw stability of Gaboxadol in human plasma based on the analysis of quality control samples

	Low QC	Middle QC	High QC
Nominal conc. (ng/mL)	1.50	15.0	75.0
Mean conc. (ng/mL) ($n=5$)			
Benchtop[a]	1.52 (3.8)	14.8 (1.6)	72.3 (1.8)
Freeze/thaw[b]	1.45 (1.7)	15.0 (1.9)	73.1 (3.4)

Numbers within parentheses are coefficients of variation (%CV).
[a] Benchtop at room temperature for 4 h.
[b] Three freeze (−70 °C)/thaw cycles.

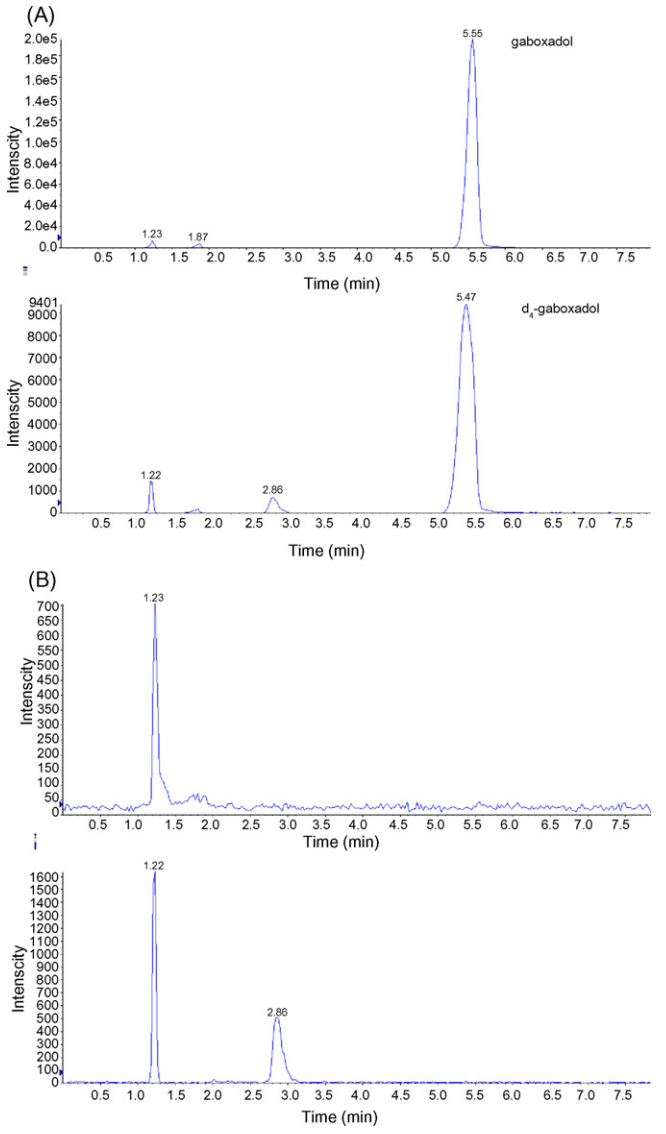

Fig. 9. Representative chromatogram of (A) gaboxadol post-dose plasma sample (conc. of gaboxadol = 69.7 ng/mL) and (B) blank pre-dose sample from the same subject.

The long-term storage of gaboxadol in human plasma was tracked by the repeated analysis of the same batch of QC samples that were stored at −70 °C for an extended period of time. The results indicated that gaboxadol was stable in human plasma for a period of at least 17 months when samples were stored at −70 °C.

3.2.7. Method application

The described SPE assay has been successfully utilized for the analysis of gaboxadol in post-dose samples (>8000) from various clinical studies. Representative chromatograms from post-dose and pre-dose samples are presented in Fig. 9.

4. Conclusion

A robust, selective, and sensitive HILIC–MS/MS method for the quantitative determination of gaboxadol in human plasma has been developed and validated.

This study has shown that HILIC may be applicable for bioanalytical assays of polar, ionic organic molecules, provided that a very selective extraction procedure is employed in combination with a stable isotope labeled internal standard.

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