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Quantitative determination of clonazepam in plasma by gas chromatography–negative ion chemical ionization mass spectrometry

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Abstract

A gas chromatographic–negative ion chemical ionization mass spectrometric (GC–NCI-MS) method for the quantitative analysis of clonazepam in human plasma is described. Clonazepam (M_r=315) was derivatized by N,O-bis-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane. A pre-conditioning procedure involving injection of a silyl-8 and ethyl acetate extraction solution from plasma reduces the interaction between clonazepam–TMS and the analytical system. The routine limit of quantification was set to be 0.25 ng/ml with an injection volume of 2 μl and a sample volume of 1 ml. The signal-to-noise ratio was greater than five. The detection limit for clonazepam can reach 0.1 ng/ml. The isotope clonazepam-d_5 was used as the internal standard.

Keywords: Clonazepam

1. Introduction

Clonazepam [7-nitro-5-(2-chlorophenyl)-3H-1,4-benzodiazepine-2(1H)-one] is an anticonvulsant and antiepileptic benzodiazepine drug. Its molecular mass is 315 and its structure is shown in Fig. 1.

Numerous methods including GC [1,2] and GC–MS [3–5] have been presented for testing clonazepam. However, when we used these methods [3–5], we encountered problems of low sensitivity and reproducibility. Under the specific conditions required by these methods, the sensitivity would drop down rapidly after several injections. It was also very hard to get a reproducible result in an assay with 50 samples or more. The present HPLC methods for testing clonazepam suffer from low sensitivities (2–5 ng/ml) [6–8].

Furthermore, the reported GC–MS methods [3–5] required that the glassware received special treatment, e.g. silylation. For a large number of samples, this treatment would be very labor-intensive. For example, two test-tubes and one GC vial are required to prepare one sample, for one thousand samples, three thousand pieces of glassware would have to be treated.

Therefore, it is necessary to develop a new method to overcome the problems stated above and to satisfy the requirements of today's pharmacokinetic studies, where the concentration of clonazepam in human plasma must be tested at as low as 0.25 ng/ml. The method described in this manuscript reaches this goal. By derivatizing clonazepam prior to analysis

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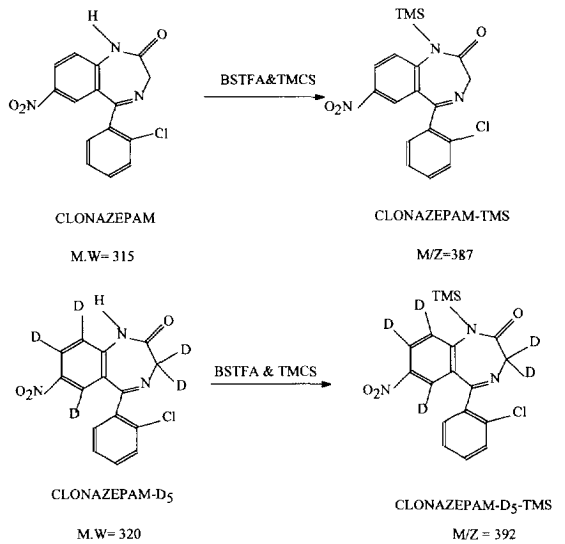

Fig. 1. Procedure for the derivatization of clonazepam and clonazepam-d_5.

and by pre-conditioning the analytical system, a detection limit of 0.1 ng/ml can be attained without any treatment of the glassware. The precision and accuracy are also greatly enhanced, both intra-assay and inter-assay, which will be discussed in detail in Section 3.

The isotopically labeled compound clonazepam-d_5 (M_r=320) (Fig. 1) was used as the internal standard for quantitative analysis.

2. Experimental

2.1. Chemicals

All pure drug compounds, clonazepam (Ro 05-4023, Lot No. 028033, purity >99.9%) and clonazepam-d_5 (Ro 05-4023-d_5, Lot No. 22072-128, atom of D>98%) were generously supplied by Hoffman-La Roche (Nutley, NJ, USA). N,O-Bis-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA with 1% TMCS) was purchased from Pierce (Rockford, IL, USA). Dichloromethane, toluene, methanol were purchased from Baxter Healthcare (McGaw Park, IL, USA). Boric acid and potassium chloride were from Aldrich (Milwaukee, WI, USA). Sodium carbonate (anhydrous) was from J.T. Baker (Phillipsburg, NJ, USA). Human plasma, potassium oxalate–NaF was from Clinical Research Associates (New York, NY, USA).

2.2. Sample preparation

To each of the 1-ml (human) plasma samples, 25 μl of the internal standard working solution (0.5 μg/ml) were added. The sample was extracted with 1.0 ml of borate buffer (1.00 *M*, pH 10.0) and 5.0 ml of toluene–dichloromethane (70:30, v/v). The test-tube was capped (snap caps) and vortex-mixed for 15 min. The sample was then centrifuged for 10 min at ambient temperature at 700 *g*. About 4.5 ml of the organic layer were pipetted into another labeled test-tube. The organic extract was evaporated to dryness under nitrogen at 60°C. Then, 30 μl of BSTFA with 1% TMCS was added to derivatize the clonazepam for 30 min at 60°C. This solution was transferred to a sample vial for analysis by GC–NCI-MS.

2.3. Method of analysis

Analysis was performed on an HP 5890 II gas chromatograph with an HP-5 column (12 m×0.25 mm I.D., 0.33 μm film thickness) and an HP 5989 MS engine equipped with a Model 7673 autosampler, a Model 59940 ChemStation and a Model 59944C (Rev. C.10.0) HP-UX ChemSystem (Hewlett Packard, Palo Alto, CA, USA).

The GC carrier gas was helium, with a column head pressure of 30 KPa at about 25°C and the flow-rate was maintained at 1 ml/min. The injector temperature was 300°C. The oven temperature programming was set from an initial temperature of 180°C to a final temperature of 300°C at a step rate of 30°C/min. With splitless injection, the injection volume was 2 μl. The running time was approximately 6 min. The spectrometer was set in the NCI and SIM acquisition mode: monitoring ions at *m/z* 387 $[\text{clonazepam+TMS}]^-$ and 392 $[\text{clonazepam-}d_5\text{+TMS}]^-$. The temperatures of the ion source and the quadrupole were 250 and 100°C, respectively. The energy of ionizing electrons and the voltage of the electrical multiplier were set at 230 eV and 2000 V, respectively. The CI gas was methane and the best operating pressure was found to be 0.173–0.25 KPa.

2.4. Calibration standard curve and quantitative analysis

A calibration standard sample was prepared, in duplicate, by adding 25 μl of the respective working solution in methanol (containing 0.25, 1.0, 5.0, 10.0, 15.0 and 25 ng of the analyte, respectively), 25 μl of the internal standard working solution in methanol (containing 12.5 ng of clonazepam-d_5) and control blank plasma to a total volume of 1 ml to a test-tube. The final concentrations of clonazepam for each point on the calibration curve were 0.25, 1.0, 5.0, 10.0, 15.0 and 25.0 ng/ml, respectively, and 12.5 ng/ml for the internal standard clonazepam-d_5. Quality control (QC) samples were run in duplicate at three different concentration levels; QC_L=2.0, QC_M=8.0 and QC_H=20.0 ng/ml. The quantitative analysis was based on the ratio of peak height of clonazepam to that of clonazepam-d_5. The accuracy and reproducibility of the method was demonstrated by the results of duplicate analyses of standard samples at six different concentrations and of the QC samples at three different concentrations in intra-assay (within one day) and inter-assay (between days).

3. Results and discussion

3.1. Summary of results

The calculation and regression of the standard curve was performed by the Drug Metabolism Laboratory Information Manager System (DM-LIMS) [9]. The calibration curve was linear for concentrations of clonazepam from 1 to 25 ng/ml and non-linear from 0.25 to 1 ng/ml. No isotopically labeled compound can be 100% pure. In our case, clonazepam-d_5 contained about 1% clonazepam, i.e. 0.125 ng/ml (clonazepam-d_5 equals 12.5 ng/ml). Considering this factor, for the lowest calibration point S1 (nominally 0.25 ng/ml), the actual concentration of clonazepam should be 0.375 ng/ml, similarly, for the second calibration point S2 (nominally 1.0 ng/ml), the actual concentration of clonazepam should be 1.125 ng/ml. Obviously, $1.125/0.375 \neq 1.0/0.25$. That is why in the low concentration part (0.25 to 1 ng/ml), the calibration curve is not linear. The background noise of m/z 387 would give the same effect at times, even if the clonazepam-d_5 were 100% pure. In the high concentration range, the factor of 0.125 ng/ml would have a negligible effect. That is why the calibration curve is linear at concentrations from 1 to 25 ng/ml. The DM-LIMS program can yield a good fit for the whole calibration range (0.25–25 ng/ml) by non-linear regression (see Table 1). This feature was also discussed in detail in our previous papers [10,11].

Three thousands samples of clonazepam were analyzed by this method. The precision, accuracy and reproducibility of intra-assay and inter-assay experiments are listed in Table 2 and Table 3, both of which were better than 10%.

3.2. Derivatization

The structure of clonazepam shows that it has a NO_2 group, plus an oxygen and a Cl, indicating that clonazepam has a strong electron affinity. Moreover,

Table 1
Calibration for clonazepam

Sample ID	Calibration concentration [A (ng/ml)]	Concentration found (average) [B (ng/ml)]	Error, (B−A)/A (%)
STD-1	0.25	0.268	7.2
STD-2	1.00	0.92	−8.0
STD-3	5.00	5.28	5.6
STD-4	10.0	10.7	7.0
STD-5	15.0	14.1	−6.0
STD-6	25.0	25.1	0.4
r^2=0.996			

Table 2
Intra-assay precision[a] and accuracy[b] from quality control samples of clonazepam in human plasma

Sample	Theor. conc. (ng/ml)	Conc. (mean±S.D.) (ng/ml)	C.V. (%)	Theor. value (%)	n
QC_L	2.0	2.09±0.114	5.47	104	5
QC_M	8.0	8.45±0.612	7.25	106	5
QC_H	20	20.3±1.17	5.78	101	5

[a] Precision is reflected by C.V. (%).
[b] Accuracy is reflected by the theoretical value (%).

it also has an active hydrogen in the N–H group which could form hydrogen bonds with the column.

In most published GC [1,2] and GC–MS [3–5] methods, underivatized clonazepam was directly injected into the gas chromatograph for analysis. However, when we repeated these methods in our experiments we found that it is very difficult to obtain symmetrically sharp peaks and reproducible results even if the silylation of the glassware is strictly performed. After several injections the sensitivity would decrease further to about 25 ng/ml. These features indicate that the clonazepam reacts with the column because of its active N–H group. Therefore, derivatization of the N–H group in clonazepam should solve this problem. From experiments with different derivatization agents, we found that BSTFA with 1% TMCS gave the best results. The derivatization procedure is shown in Fig. 1. The hydrogen in the N–H group of clonazepam was replaced by the TMS group. The m/z for $[M-H+TMS]^-$ and $[Md_5-H+TMS]^-$ were 387 and 392, respectively, which were monitored in the mass spectrometer with SIM mode. The chromatogram showed that the peak of clonazepam was symmetrically sharp. A detection limit of 0.1 ng/ml could be reached. This sensitivity was much higher than that obtained with the underivatized method. De Carvalho and Lanchote [12] reached a similar conclusion, i.e. that the sensitivity obtained using the underivatized GC method was significantly lower than that of the GC method where methylated derivatization of clonazepam was carried out.

Table 3
Inter-assay precision[a] and accuracy[b] from quality control samples of clonazepam in human plasma

Assay date	QC_L found (average) (nominally 2 ng/ml)	QC_M found (average) (nominally 8 ng/ml)	QC_H found (average) (nominally 20 ng/ml)
11/10/95	2.17	8.90	20.60
12/10/95	2.09	8.63	21.52
13/10/95	2.10	8.41	19.67
16/10/95	2.09	9.14	21.40
18/10/95	1.95	8.77	20.17
20/10/95	2.15	9.14	19.79
23/10/95	1.96	8.76	19.82
24/10/95	2.00	8.29	21.65
25/10/95	2.03	8.69	20.44
27/10/95	1.89	8.15	22.10
02/11/95	2.06	8.99	21.99
03/11/95	2.05	9.00	21.48
07/11/95	2.17	8.54	20.78
08/11/95	2.21	8.23	20.65
09/11/95	1.87	8.33	20.27
10/11/95	2.21	8.53	20.63
Mean	2.06	8.66	20.81
S.D.	0.11	0.32	0.79
C.V. (%)	5.3	3.7	3.8
Theoretical (%)	103.1	108.2	104.0
n	32	32	32

[a] Precision is reflected by C.V. (%).
[b] Accuracy and reproducibility is reflected by theoretical value (%).

The life of a capillary GC column was significantly longer in our method. One column could be used to analyze more than 500 samples without any drop in the sensitivity or significant peak broadening. Moreover, the peak shape of the chromatograph was much sharper for a derivatized sample and the signal-to-noise ratio (*S/N*) was enhanced also. The *S/N* was better than five at the limit of quantitation (0.25 ng/ml).

3.3. Pre-conditioning of the analytical system

If the analytical system was not pre-treated, the beneficial results were lost regardless of the derivatization of clonazepam with TMS. This indicates that clonazepam+TMS is still active and able to bind to the column as well as to the analytical system including the injection port, GC column, mass source and mass filter (quadrupole). Therefore, it is important to clean the injection port, mass source and mass filter thoroughly before analysis. Moreover, preconditioning of the whole analytical system by pre-conditioning the solution and silyl-8 is necessary. A pre-conditioning solution was prepared by extracting the blank plasma with ethyl acetate. This solution contained sufficient lipids to cover the active sites of the column and the contact surface of the analytical system. Injecting the pre-conditioning solution and silyl-8 four times each with an injection volume of 2 μl before performing the analysis of clonazepam would increase the sensitivity dramatically. A similar technique was employed in the analysis of flumazenil [13].

It should be emphasized that both the derivatization and the pre-conditioning procedures are necessary for this analysis. Without derivatization, pre-conditioning of the analytical system alone will not make any improvement. On the other hand, without pre-conditioning the analytical system, good results were impossible even if a derivatized sample of clonazepam–TMS was injected.

A noteworthy feature is that once the analytical system was treated, it was not necessary to treat it again for a long time, e.g. 1000 samples could be analyzed before the procedure had to be repeated. According to our experience, the pre-conditioning procedure is needed again only after cleaning the source and quadrupole.

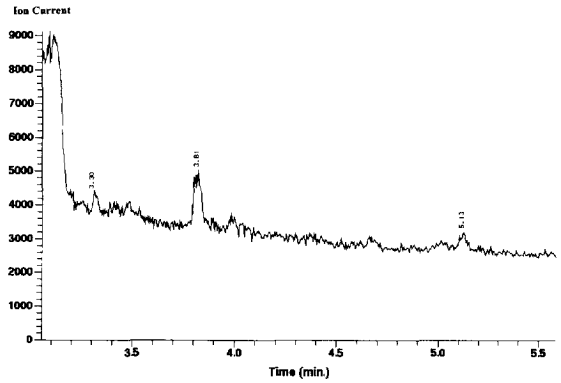

Fig. 2. GC–MS chromatogram of a blank plasma sample.

The detection limit of 0.1 ng/ml with an injection volume of 2 μl was achieved with this procedure. The routine limit of quantification was set at 0.25 ng/ml with 1 ml of plasma. This assured consistent results, even if the sensitivity is decreased because of contamination of the ion source through prolonged usage.

3.4. Chromatography

Fig. 2 shows the GC–MS chromatogram of blank plasma. The ions at t_R (retention time) 3.30 min (*m/z* 387), t_R=3.81 and 5.13 min (*m/z* 392) are from the components of plasma. Fig. 3 shows the routine limit of quantification obtained using our method. The concentration of clonazepam and its internal standard are 0.25 and 12.5 ng/ml, respectively. Fig. 4 is a typical GC–MS chromatogram obtained from a

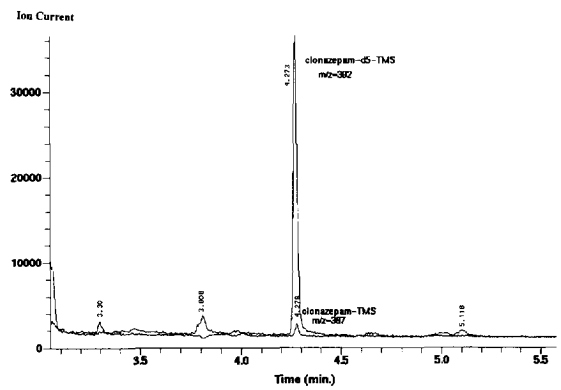

Fig. 3. GC–MS chromatogram of the standard sample that shows the routine limit of quantification for clonazepam (0.25 ng/ml).

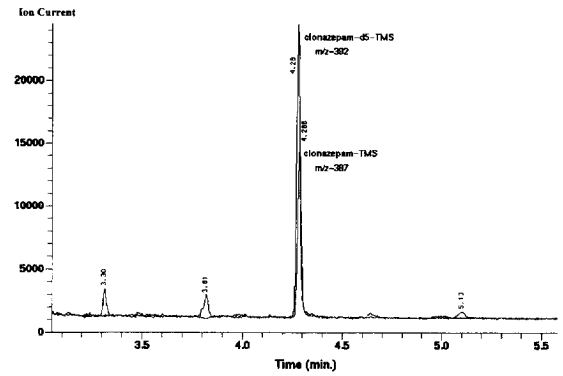

Fig. 4. GC–MS chromatogram of a test sample.

sample from a patient taken 2 h after a 2-mg dose of clonazepam was ingested; the concentration of clonazepam in plasma was found to be 6.3 ng/ml.

4. Conclusion

Clonazepam is a very difficult compound to analyze by the GC method. This manuscript introduces the techniques of derivatization and pre-conditioning in one analysis. The routine limit of quantification is set at 0.25 ng/ml with a 1-ml plasma sample. No piece of glassware needs to be treated. One column can analyze more than 500 samples. The accuracy and precision are better than 10%. This method is not only the most sensitive available for the analysis of clonazepam in plasma so far, but it is also an economical and effective method. Because of its high selectivity, this method is also suitable for the analysis of clonazepam in various biological fluids such as human urine and dog and mouse plasma.

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References

[1] G.L. Miller, H. Friedman and D.J. Greenblatt, J. Anal. Toxicol., 11 (1987) 55.
[2] W. Loescher and F.O. Al-Tahan, Ther. Drug Monit., 5 (1983) 229.
[3] W.A. Garland and B.H. Min, J. Chromatogr., 172 (1979) 279.
[4] B.H. Min, W.A. Garland, K.C. Khoo and G.S. Torres, Biomed. Mass Spectrom., 5 (1978) 692.
[5] B.H. Min and W.A. Garland, J. Chromatogr., 139 (1977) 121.
[6] B.C. Sallustio, C. Kassapidis and R.G. Morris, Ther. Drug Monit., 16 (1994) 174.
[7] D.R.A. Uges, Ziekenhuisfarmacie, 8 (1992) 34.
[8] T. Hayashi, K. Suzuki, I. Johno and S. Kitazawa, Byoin Yakugaku, 18 (1992) 79.
[9] Programmed by G.L. Rathmill, PennComp, PA, USA.
[10] D. Song, S. Zhang and K. Kohlhof, J. Chromatogr. B, 658 (1994) 142.
[11] D. Song, S. Zhang and K. Kohlhof, J. Chromatogr. B, 660 (1994) 95.
[12] D. DeCarvalho and Ver L. Lanchote, Ther. Drug Monit., 13 (1991) 55.
[13] D. Song, V. Khaykis and K. Kohlhof, J. Chromatogr. B, 663 (1995) 263.