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Determination of xanomeline and active metabolite, N-desmethylxanomeline, in human plasma by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Abstract

We have developed a method for the determination of xanomeline and its pharmacologically active N-desmethyl metabolite. The validated method uses hexane to extract xanomeline and its N-desmethyl metabolite from basified plasma. The hexane extract is dried, reconstituted, and analyzed using a liquid chromatographic–atmospheric pressure chemical ionization tandem mass spectrometry system. The method was developed to support phase II clinical trials and has proven to be extremely sensitive, fast, and rugged. The method has a limit of quantitation of 75 and 200 pg/ml plasma for xanomeline and the N-desmethyl metabolite, respectively. Sample analysis times were less than 3 min from one injection to the next.

1. Introduction

Xanomeline (Fig. 1a) tartrate is a muscarinic receptor agonist currently in phase II clinical trials [1–3]. The potency of the drug necessitated the need for analytical determination of plasma levels in the low picogram/milliliter range. Conventional techniques, such as HPLC with UV detection [4], which are used to monitor drug plasma concentrations during clinical trials, did not have sufficient sensitivity for the determination of xanomeline pharmacokinetics in the low picogram/milliliter range. As a result, a liquid chromatographic–ionspray tandem mass spectrometry method was developed and validated for the determination of xanomeline in human plasma [5]. The ionspray method was capable of quantitating xanomeline plasma concentrations as low as 75 pg/ml with an analysis time of 1.5 min for each sample. The ionspray method was used to assay approximately 600 samples as part of a clinical study on the pharmacokinetics of xanomeline.

Further clinical studies necessitated the need to monitor xanomeline and its pharmacologically active metabolite, N-desmethylxanomeline (Fig. 1b). The same analytical requirements of sensitivity, speed, and ruggedness, by which the xanomeline ionspray method was developed, were to be met for a method which could quantitate both xanomeline and its N-desmethyl metabolite.

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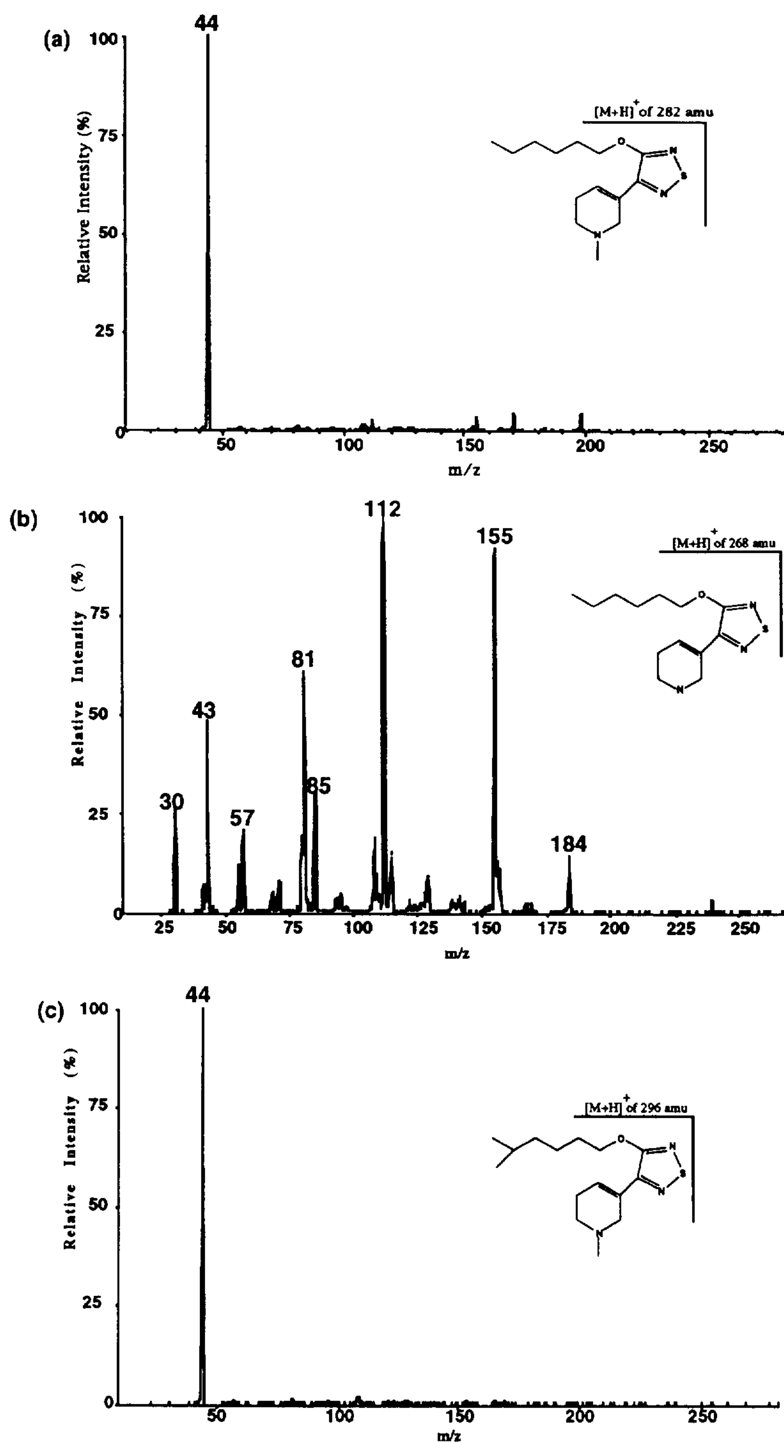

Fig. 1. Product ion mass spectra of xanomeline (a), N-desmethylxanomeline (b), and the internal standard (c).

This report describes the development, validation, and application of a liquid chromatographic–atmospheric pressure chemical ionization tandem mass spectrometry (LC–APCI-MS–MS) method capable of determining low plasma levels (pg/ml) of xanomeline and its N-desmethyl metabolite.

2. Experimental

2.1. Chemicals

Xanomeline tartrate, N-desmethylxanomeline oxalate, and the internal standard oxalate salt (compound LY282122, I.S.) (Fig. 1c) were prepared at Lilly Research Laboratories (Indianapolis, IN, USA). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI, USA). HPLC grade hexane and acetonitrile were both obtained from Baxter (Muskegon, MI, USA). Human plasma with EDTA anticoagulant was obtained from Biological Specialties Corporation (Landsdale, PA, USA). Dimethylchlorosilane (DMCS) was obtained from Pierce (Rockford, IL, USA). Ammonium acetate, sodium hydroxide, and potassium chloride were obtained from EM Science (Cherry Hill, NJ, USA).

2.2. Instrumentation and chromatographic conditions

A Shimadzu (Kyoto, Japan) SCL-10A system controller was used to program a Shimadzu SIL 10-A autoinjector and a Shimadzu LC-10AD liquid chromatographic pump. A Shimadzu solvent degasser, Model DGU-3A, was also used.

The pumps were set to deliver a mobile phase of 1% TFA in water–acetonitrile (10:90, v/v) at a flow of 1 ml/min. A 50-μl injection of each sample was chromatographed using an Applied Biosystems, (Foster City, CA, USA) RP-8 30 × 4.6 mm, column with a particle diameter of 5 μm. The eluate from the column was connected to a Sciex API III tandem mass spectrometer (Thornhill, Ontario, Canada). The mass spectrometer was operated in the heated nebulizer mode with a nebulizer temperature of 500°C. The corona discharge ionization needle was maintained at approximately 5 kV and the orifice voltage was set at 90 V. Xanomeline, N-desmethylxanomeline, and the I.S. compound were detected in the positive ion mode by multiple reaction monitoring (MRM). A collision gas mixture of argon–nitrogen (90:10) and a collisional energy of 30 eV was used to produce product ions from the protonated molecular ions. Collisional activated dissociation (CAD) of the protonated molecular ions of xanomeline ($[M+H]^+$ of m/z 282) and the I.S. ($[M+H]^+$ of m/z 296) both yielded a m/z 44 ion as the most abundant product ion for both compounds. The N-desmethylxanomeline protonated molecular ion ($[M+H^+$ of m/z 268) yielded a m/z 112 ion as the most abundant product ion. The instrument was programmed for a scan dwell time of 298 ms. The area integration for calibration curves and sample analysis were made using the Sciex Macquan software version 1.2.

2.3. Standard and quality control preparation

Prior to sample preparation all centrifuge tubes were silanized by placing 2 ml of a 10% (v/v) DMCS in hexane solution into each tube and rotating for 15 min. The solution was discarded and the tubes were allowed to dry.

Stock solutions of xanomeline, N-desmethylxanomeline, and I.S. were prepared in 0.1 *M* HCl–methanol (1:1, v/v) at concentrations of approximately 1 mg/ml. The stock solutions of xanomeline and N-desmethylxanomeline were serially diluted in 0.1 *M* HCl–methanol (1:1, v/v) to yield solutions of 5 μg/ml. The stock solution of I.S. was serially diluted in 0.1 *M* HCl–methanol (1:1, v/v) to yield a working I.S. solution of 25 ng/ml.

A stock plasma solution containing xanomeline and N-desmethylxanomeline was prepared at 25 ng/ml by transferring aliquots of the 5 μg/ml solutions into a vial containing blank plasma. Working plasma standards containing both xanomeline and N-desmethylxanomeline were prepared at 0.075, 0.200,

0.800, 1.25, 2.5 and 5.0 ng/ml by serially diluting the stock plasma solution with blank plasma.

Plasma controls containing both xanomeline and N-desmethylxanomeline were prepared at 0.075, 0.200, 2.5, and 5.0 ng/ml by making a separate weighing of each compound and diluting in 0.1 *M* HCl–methanol (1:1, v/v) and plasma as described for the plasma standard preparation.

Solutions of xanomeline, N-desmethylxanomeline, and I.S. were prepared in 1% TFA in water–acetonitrile (75:25, v/v) to test the suitability of the LC–APCI-MS–MS system.

2.4. Extraction of standards, controls, and samples

An aliquot of 1 ml of plasma was placed into a 15 ml (16 × 100 mm) silanized centrifuge tube. A 100-μl aliquot of the 25 ng/ml working internal standard solution was added and the tube was vortexed for 15 s. An aliquot of 1 ml of sodium hydroxide–potassium chloride buffer (pH 12.5) and 5 ml of hexane were added. The tubes were mixed for 20 min on a tube rotator (Scientific Industries, Bohemia, NY, USA) at 6 rpm. The tubes were then centrifuged at 2900 *g* for 10 min. The hexane layer was then removed, transferred to a clean 5-ml silanized conical centrifuge tube, and evaporated to dryness under nitrogen. The residue was reconstituted with 80 μl of 1% TFA in water–acetonitrile (75:25, v/v), briefly vortex-mixed, transferred to an autosampler vial, and vortex-mixed again to remove air bubbles prior to injection. For analysis of actual sample batches, duplicate quality controls and standards were analyzed. The overall recovery of the assay was determined to be greater than 74% for xanomeline and N-desmethylxanomeline. Recovery was estimated by comparing the peak heights (by HPLC with UV detection) of solvent standards versus plasma extracts.

2.5. Method validation

For method validation, five replicates of the plasma quality controls and duplicate plasma standards were assayed on each of three days. A weighted ($1/x$) regression was used for calibration curves. The inter- and intra-assay precision and accuracy were determined using the plasma control concentrations of 0.075, 0.200, 2.5, and 5.0 ng/ml.

3. Results and discussion

3.1. Method development

Development of the method to analyze both xanomeline and its N-desmethyl metabolite began by determining the protonated molecular ions for each analyte and the I.S. Solutions (1 μg/ml) of each analyte, and the I.S., in methanol were directly infused at 10 μl/min. Protonated ($[M+H]^+$) molecular ions of m/z 282, 268, and 296 were obtained for xanomeline, N-desmethylxanomeline, and the I.S., respectively. Product ion spectra of the protonated molecular ions were then obtained to determine the most abundant product ion.

The product ion spectra (Fig. 1) for xanomeline, N-desmethylxanomeline, and I.S. indicated that the m/z 44 ion was the most abundant product ion for xanomeline and I.S., while a m/z 112 ion was the most abundant product ion for N-desmethylxanomeline. The instrument was programmed to operate in the multiple reaction monitoring (MRM) mode in which Q1 is set to admit only the protonated molecular ions of each analyte for collisionally activated dissociation (CAD) in Q2. The most abundant product ion, from each CAD reaction of the protonated molecular ions, was allowed to pass through Q3 for detection.

The focus of development shifted to the type of inlet system to use for the analysis. The xanomeline ionspray method [5] suffered from sensitivity losses after 8 h of continuous operation. The sensitivity loss was perhaps a result of the ionspray needle being placed too close to the orifice of the instrument and/or failure of the 1 mm I.D. column to withstand more than 150 injections per day. To avoid the sensitivity loss

observed in the ionspray method [5] we considered the use of the heated nebulizer probe with corona discharge chemical ionization (APCI). The use of LC–APCI-MS–MS for the simultaneous determination of drugs and their metabolites has been demonstrated [6–9]. The heated nebulizer mode on the Sciex API III can accommodate flow-rates of 1 ml/min to the mass spectrometer without splitting the effluent. At a flow-rate of 1 ml/min we could utilize chromatographic columns of conventional diameters (i.e. 4.6 mm I.D.) and loading capacities.

To minimize analysis time and avoid carryover, a mobile phase system of 1% TFA in water–acetonitrile (10:90, v/v) was used with a RP-8 30 × 4.6 mm guard column and a 50-μl injection volume. The addition of 1% TFA was determined to be essential to eliminate analyte carryover at high analyte levels (5 ng/ml). The chromatographic column also eliminated a response observed for all three compounds when we tried to assay the plasma blanks by flow injection using this mobile phase. The mobile phase and column allowed elution of all three compounds in approximately 30 s. The ratio of water–acetonitrile (10:90, v/v) in the mobile phase was found to yield the highest signal-to-noise ratio for the analytes.

Samples were reconstituted in 80 μl of mobile phase. Solvating the residue in 80 μl of mobile phase allowed approximately 95% recovery of xanomeline from the tube without diluting the sample to the point of achieving a significant reduction in signal-to-noise.

A plasma blank reconstituted in the mobile phase showed a peak for all three traces, as was similarly observed when attempts were made to assay plasma blanks by flow injection. To reduce this solvent effect, a series of plasma blanks were reconstituted in various combinations of 1% TFA in water–acetonitrile (Fig. 2.). It was found that a plasma blank reconstituted in 1% TFA in

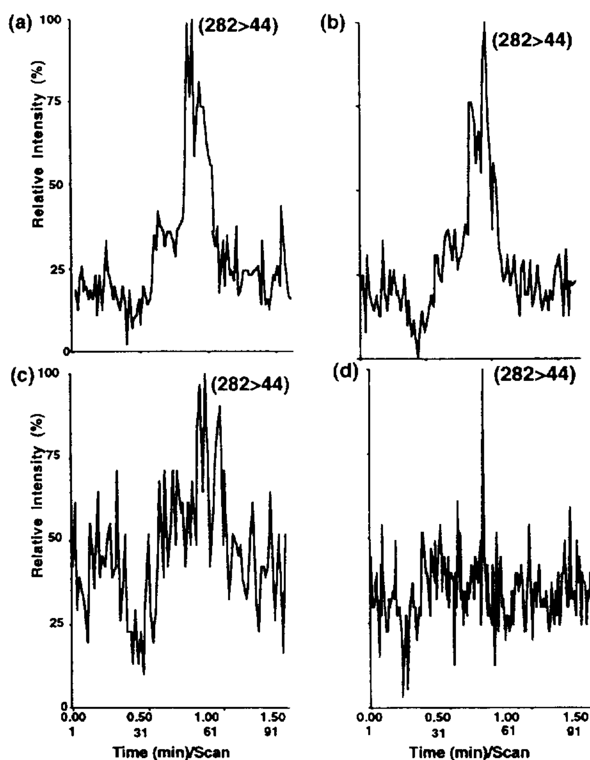

Fig. 2. Ion traces for xanomeline (282 > 44) in plasma blanks reconstituted in 1% TFA in 20:80 (a), 50:50 (b), 75:25 (c), and 70:30 (d) water–acetonitrile.

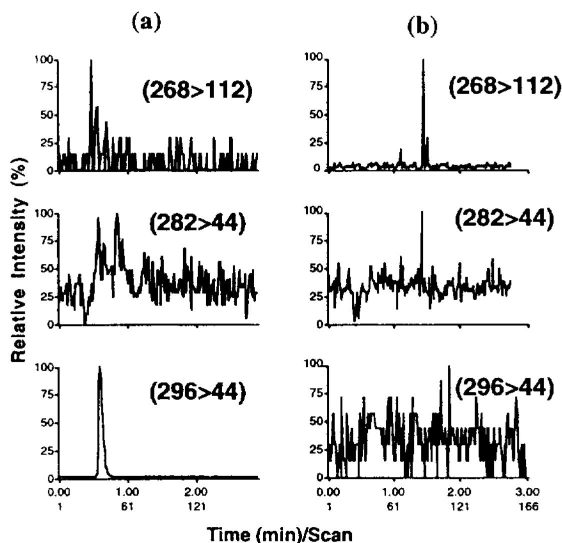

Fig. 3. Plasma blanks with 2.5 ng I.S./ml (a) and without I.S. (b) showing ion traces for N-desmethylxanomeline (268 > 112), xanomeline (282 > 44), and the internal standard (296 > 44).

water–acetonitrile (75:25, v/v) did not yield a response (Fig 2). Having met the criteria for sensitivity and speed the system was then considered ready for validation.

3.2. Method validation

On each of three days duplicate standard curves were found to have regression coefficients (weighted $1/x$) of greater than 0.99 for both xanomeline (0.075 to 5.0 ng/ml) and N-desmethylxanomeline (0.200 to 5.0 ng/ml). Xanomeline controls (Table 1) showed intra-assay accuracies ranging from 97.1 to 101% and precisions of 5.8 to 14.2% over a concentration range of 0.075 to 5.0 ng/ml plasma.

N-desmethylxanomeline (Table 2) showed intra-assay accuracies ranging from 91.4 to 102% over the 0.200 to 5.0 ng/ml plasma concentrations. The N-desmethylxanomeline controls showed intra-assay precisions of 10.4 to 17.7%.

Ion traces, for both analytes and the internal standard, are shown for plasma blanks 0.075 and 0.200 ng/ml plasma standard (Figs. 3–5).

3.3. Clinical application

The method was used for the determination of xanomeline and N-desmethylxanomeline as analytical support for a clinical study. Patients were given a single 75-mg dose of xanomeline and subsequently sampled at specific time points for the determination of the analytes. Approximately 650 analyses, including standards, controls and samples were assayed in six days. A plot of xanomeline and N-desmethylxanomeline concentrations in the plasma of a normal volunteer is shown in Fig. 6.

Table 1
Precision and accuracy of xanomeline plasma controls in method validation

Concentration (ng/ml)	n	Concentration found (mean) (ng/ml)	Accuracy (%)	S.D.	C.V. (%)
Day 1					
0.075	3	0.09	117.33	0.01	10.13
0.200	5	0.20	99.48	0.01	6.47
2.50	5	2.65	105.98	0.14	5.26
5.00	5	5.25	105.69	0.37	6.92
Day 2					
0.075	5	0.08	100.00	0.01	8.31
0.200	5	0.19	94.63	0.01	7.58
2.50	5	2.40	95.96	0.10	4.00
5.00	5	4.62	92.35	0.19	4.02
Day 3					
0.075	5	0.07	87.68	0.00	3.71
0.200	5	0.19	97.22	0.01	2.85
2.50	5	2.53	101.00	0.08	3.11
5.00	5	4.98	99.56	0.16	3.14
Total					
0.075	13	0.07	99.20	0.01	14.21
0.200	15	0.19	97.11	0.01	6.14
2.50	15	2.52	100.98	0.15	5.76
5.00	15	4.96	99.20	0.37	7.40

Table 2
Precision and accuracy of N-desmethyl xanomeline plasma controls in method validation

Concentration (ng/ml)	n	Concentration found (mean) (ng/ml)	Accuracy (%)	S.D.	C.V. (%)
Day 1					
0.200	5	0.22	109.66	0.02	8.42
2.50	5	2.52	100.92	0.20	8.04
5.00	5	5.38	107.69	0.30	5.49
Day 2					
0.200	5	0.16	80.10	0.01	6.48
2.50	5	2.61	104.43	0.14	5.44
5.00	5	5.52	110.49	0.32	5.85
Day 3					
0.200	5	0.17	84.48	0.02	12.57
2.50	5	2.16	86.59	0.15	6.76
5.00	5	4.34	86.73	0.47	10.85
Total					
0.200	15	0.18	91.41	0.03	17.73
2.50	15	2.43	97.31	0.25	10.36
5.00	15	5.08	101.64	0.65	12.74

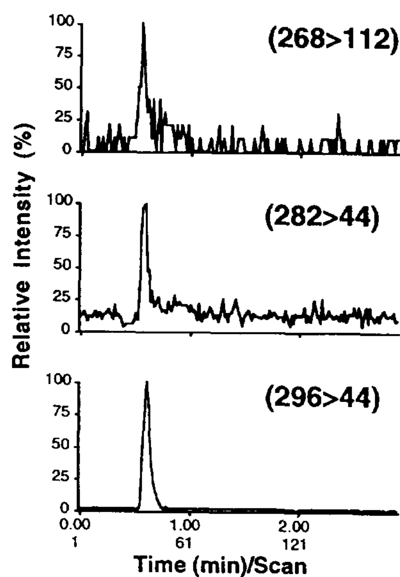

Fig. 4. Plasma standard (75 pg xanomeline and N-desmethylxanomeline/ml) showing ion traces for N-desmethylxanomeline (268 > 112), xanomeline (282 > 44), and the internal standard (296 > 44).

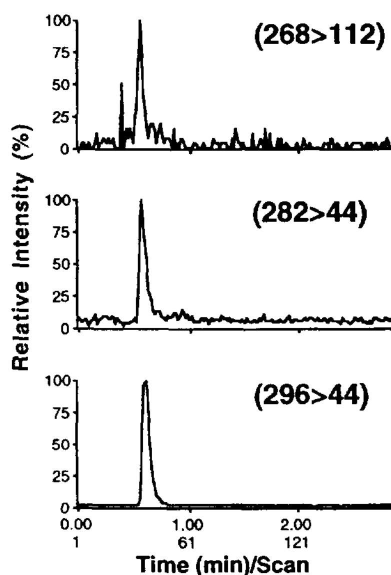

Fig. 5. Plasma standard (200 pg xanomeline and N-desmethylxanomeline/ml) showing ion traces for N-desmethylxanomeline (268 > 112), xanomeline (282 > 44), and the internal standard (296 > 44).

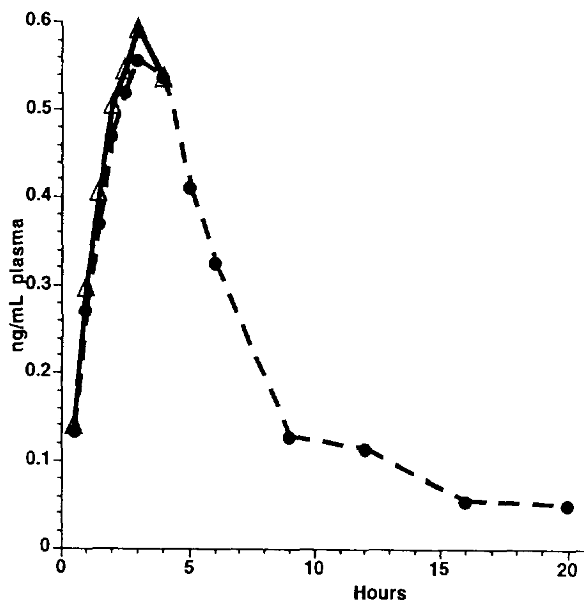

Fig. 6. Mean plasma concentration of xanomeline (●) and N-desmethylxanomeline (△) in a normal volunteer after receiving a single 75-mg oral dose of xanomeline.

4. Conclusions

We have described a method for the determination of xanomeline and its pharmacologically active N-desmethyl metabolite. The method has proven to be sensitive and specific, with quantitation limits of 0.075 and 0.200 ng/ml plasma for xanomeline and the N-desmethyl metabolite, respectively. The method has also proven to be fast and rugged, with each sample requiring less than 3 min of analysis time. A typical analysis batch of 130 samples, including standards and controls, could be processed in 6.5 h. We did not experience any sensitivity drift with the use of the heated nebulizer, even after 14 h of continuous operation.

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