Simultaneous Assay of Buprenorphine and Norbuprenorphine by Negative Chemical Ionization Tandem Mass Spectrometry*

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Abstract

A method for the simultaneous measurement of buprenorphine and its N-dealkylated metabolite, norbuprenorphine, in human plasma was developed with negative chemical ionization tandem mass spectrometry. Buprenorphine and norbuprenorphine were extracted from biological fluids by solid-phase extraction. The samples were derivatized with heptafluorobutyric anhydride and measured with negative chemical ionization tandem mass spectrometry. Buprenorphine formed a heptafluorobutyryl derivative and norbuprenorphine formed a bis-heptafluorobutyryl derivative; consequently, the sensitivity of norbuprenorphine was substantially higher than buprenorphine. The limit of quantitation (LOQ) for buprenorphine was 0.20 ng/mL, and the LOQ for norbuprenorphine was 0.03 ng/mL. Daily calibration curves were prepared. Buprenorphine was linear from 0.15 ng/mL to 20 ng/mL, and norbuprenorphine was linear between 0.016 ng/mL and 5 ng/mL. Between-run and within-run precision for buprenorphine at 0.5 ng/mL were 13.8% and 9.8%, respectively. Between-run and within-run precision for norbuprenorphine at 0.5 ng/mL were 23.1% and 17.9%, respectively. The molecular anion for buprenorphine was used as a precursor ion, whereas the [M-197]- was used as a precursor ion for norbuprenorphine in tandem mass spectrometry. Product ion spectra from collision-induced dissociation resulted principally from dissociations of the heptafluorobutyryl group. Monitoring select precursor to product ion reactions and using qualifier ion ratios increased the method's sensitivity and selectivity. The method was applied to samples collected from a patient who received oral and subcutaneous buprenorphine. Buprenorphine plasma concentrations ranged from less than 0.20 ng/mL to 8.7 ng/mL.

Introduction

Buprenorphine (Figure 1) is a semisynthetic opiate analgesic used in the treatment of moderate to severe pain. It is marketed in the United States under the trade name Buprenex and in Europe under the trade name Temgesic. As an analgesic, buprenorphine is 25–40 times more potent than morphine and possesses both agonist and antagonist properties (1). At low doses, buprenorphine acts as a partial agonist at the receptor with actions similar to morphine. At higher doses, it acts as a potent opioid antagonist with activity similar to naloxone (2,3). A unique property of buprenorphine's molecular pharmacology is its high affinity to opioid receptors and slow dissociation from the buprenorphine-receptor complex, which results in a long duration of action (4).

Buprenorphine is a highly lipophilic drug and has good intramuscular and sublingual bioavailability. Metabolism occurs by N-dealkylation to norbuprenorphine, and both buprenorphine and norbuprenorphine are conjugated with glucuronic acid. The elimination half life of buprenorphine is approximately 3 h; the half life for norbuprenorphine is not established but is considered to be longer than the half life of buprenorphine (5).

Buprenorphine has been shown to significantly suppress heroin administration and is being investigated for use in treating opioid addiction (6). Its effectiveness by the sublingual route, diminished physical dependence, mild withdrawal symptoms, suppression of heroin administration, and reduced possibility of overdose are desirable properties for use in treating opioid-dependent persons (7).

Buprenorphine's high potency results in low therapeutic blood concentrations. Consequently, a sensitive specific analytical method is necessary for measurement of the parent drug and metabolites in biological fluids. Radioimmunoassay has been used for the determination of buprenorphine, but the assay cross-reacts with norbuprenorphine and, to a lesser degree, with the glucuronide metabolites (8). Buprenorphine and norbuprenorphine have been measured using chromatographic methods. Cone et al. (9) developed an assay that employed a 63Ni electron capture detector for the measurement of pentafluoroproprionic anhydride (PFPA) derivatives of buprenorphine, norbuprenorphine, and its acid catalyzed rearrangement product, demethoxybuprenorphine. Blom and Bondesson (10) used electron-impact GC–MS and chemical degradation to demethoxybuprenorphine and demethoxynor-
buprenorphine to achieve detection limits of 0.15 ng/mL. A
detection limit of 0.20 ng/mL was attained by Ohtani et al. (11)
with the simultaneous measurement of PFPA derivatives of
buprenorphine and norbuprenorphine by positive chemical-
ionization GC–MS. Heptafluorobutyric anhydride (HFBA)
derivatives of buprenorphine and norbuprenorphine were pre-
pared by Martinez et al. (12) and detected with an electron cap-
ture detector at 0.50 ng/mL. Reversed-phase high-performance
liquid chromatography (HPLC) has been used in buprenor-
phine and norbuprenorphine quantitation with UV and elec-
trochemical detectors. Hackett et al. (13) used HPLC with a UV
detector to quantitate buprenorphine in urine at 7.5 ng/mL.
Electrochemical detection was used by Debrabandere et al. (14)
to quantitate buprenorphine in urine at 0.20 ng/mL, and
Schleyer and co-workers (15) were able to measure buprenor-
phine and norbuprenorphine at 0.04 ng/mL in plasma and
urine. HPLC and electrochemical detectors have also been used
to measure buprenorphine and norbuprenorphine in hair (16).

Buprenorphine kinetics have previously been studied pri-
marily by radioimmunoassay techniques (17–19). To better
understand buprenorphine and norbuprenorphine kinetics, a
sensitive, specific method capable of measuring both parent
drug and metabolite simultaneously in concentrations less
than 1 ng/mL was developed. The method combined the
increased sensitivity provided by negative chemical ionization
with the specificity of tandem mass spectrometry (MS–MS).

The tandem mass spectrometer consists of two quadrupole
mass filters and a radio frequency-only (RF) octapole collision
chamber that can be pressurized with an inert gas (Figure 2)
(20). The front quadrupole (Q1) provides a high degree of
selectivity by permitting the passage of a selected ion only.
Complex sample matrices can be analyzed with minimal
sample preparation by using Q1 to select the ion of interest and
reduce background interferences. The selected ion (precursor
ion) passes through Q1 and enters the RF-only octapole colli-
sion chamber (Q2) where the ion is fragmented by collisions
with inert gas atoms and an applied voltage. This fragmenta-
tion process is known as collision-induced dissociation (CID).
Fragment ions (product ions) are mass filtered by the rear
quadrupole (Q3). The product ions formed in CID are specific
and can be used to uniquely identify a compound.

Negative chemical ionization occurs by the capture of
thermal electrons and produces a soft ionization compared
with electron impact. The molecular anion is frequently the base peak and very little
fragmentation is observed. Ion source con-
ditions can be adjusted to maximize the pro-
duction of the molecular anion or a selected
precursor ion. Maximum production of the
precursor ion greatly increases the efficiency of
product ion formation. Sensitivity can be
increased by using the selected reaction
monitoring (SRM) mode. In SRM, selected
precursor ion to product ion dissociations
are monitored by Q3 rather than scanning
for all product ions within a specified mass
range. The SRM technique is analogous to
selected ion monitoring in GC–MS.

This study describes the solid-phase extraction (SPE) and
simultaneous identification and quantitation of buprenorphine
and norbuprenorphine in human plasma using a fluorinated
derivative, negative chemical ionization, and tandem mass
spectrometry. The method was applied to the analysis of plasma
samples collected from a patient who received subcutaneous
and oral doses of buprenorphine.

Materials and Methods

**Materials**

Buprenorphine HCl and norcodeine were purchased from
Sigma Chemical Company (St. Louis, MO). Buprenorphine-D₄
and buprenorphine were purchased from Radian Corporation
(Austin, TX). Buprenorphine from separate sources was used to
prepare calibrator and control samples. Norbuprenorphine
was obtained from the Research Technology Branch, NIDA
(Rockville, MD). Heptafluorobutyric anhydride (HFBA) was
purchased from Aldrich Chemical Company (Milwaukee, WI).
All solvents were obtained from Fisher Chemical (Fair Lawn, NJ) and were HPLC-grade. Clean Screen (ZCDAU020) SPE columns were purchased from World Wide Monitoring (Bristol, PA). Argon and ammonia gases from MG Industries (Valley Forge, PA) were used in chemical-ionization MS-MS.

**Extraction**

Buprenorphine-D₄ (100 ng/mL) and norcodeine (20 ng/mL) were prepared in aqueous solution for use as internal standards. Fifty microliters of each internal standard and 3 mL of pH 6 100 mM phosphate buffer were added to plasma or serum samples (1 mL). The samples were vortex mixed and centrifuged for 10 min at 3000 rpms. The supernatant was added to a Clean Screen Extraction column that was conditioned with 3 mL of methanol, 3 mL water, and 1 mL pH 6 100 mM phosphate buffer. Sample was applied and the columns were washed with 2 mL water, 2 mL pH 4.5 acetate buffer and 3 mL methanol. The drugs were eluted from the column with 4 mL of methylene chloride-isopropanol-ammonium hydroxide (78:20:2). Extracts were evaporated under nitrogen. The residue was placed in a 70°C dry bath for 3 min, removed, and allowed to come to room temperature. The sample was derivatized by adding 100 µL of toluene and vortex mixing. HFBA (30 µL) was added, and the mixture was vortex mixed again. The samples were capped and allowed to react at room temperature for 1 h. The derivatized extracts were evaporated and reconstituted with 20 µL of ethyl acetate. Four microliters were injected into the GC for MS-MS analysis.

**Instrumentation**

GC-MS-MS analyses were performed on a Finnigan MAT TSQ 700 tandem mass spectrometer equipped with a Varian 3400 gas chromatograph. A J&W Scientific (Folsom, CA) DB-5MS capillary column (15 m × 0.25 mm × 0.25 m) was employed with helium carrier gas at 10 psi column head pressure. Injections were made in the split–splitless mode. The injector temperature was 265°C, and the transfer line temperature was 300°C. The oven temperature program was held at 125°C for 1 min, then increased to 275°C at 30°C per min, followed by a second increase to 300°C at 5°C per min.

The tandem mass spectrometer was operated in the negative chemical ionization mode. Mass spectrometer conditions were as follows: electron energy, 95 eV; source temperature, 130°C; manifold temperature, 70°C; emission current, 400 µA; and conversion dynode, 18 kV. The quadrupoles were tuned for unit resolution. The reagent gas was ammonia adjusted to a source pressure of approximately 8000 millitorr as read on the Finnigan TSQ 700 source ion gauge.

The molecular anions (M⁻) of buprenorphine and buprenorphine-D₄ were used as precursor ions. The precursor ions selected for norbuprenorphine and norcodeine were M⁻-197 and M⁻-20, respectively. CID spectra were collected in the selected reaction monitoring mode with argon as the collision gas. Collision chamber conditions were as follows: argon cell pressure of approximately 8000 millitorr as read on the Finnigan TSQ 700 source ion gauge.

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![Figure 3. Unextracted full-scan product ion spectrum of HFBA-derivatized buprenorphine (10 ng).](https://example.com/figure3.png)
pressure, 2.0 millitorr; buprenorphine and buprenorphine-D₄ collision energy, 24 eV; norbuprenorphine collision energy, 20 eV; and norcodeine collision energy, 17 eV. Qualitative identification was determined by retention time and ion ratios. One qualifier ion ratio was monitored for each compound. Precursor ions, product ions, and ion ratios are listed in Table I.

Precursor ion production efficiency was optimized by measuring precursor ion abundance while varying ion source temperature and ion source electron energy. CID efficiency was measured by systematically varying argon pressure and collision energy. CID efficiency was defined as that argon gas pressure and collision energy where maximum reproducibility and abundance of the selected product ions occurred.

Calibration

Assay calibration was performed by addition of known concentrations of buprenorphine, norbuprenorphine, and internal standards to drug-free plasma, followed by extraction, derivatization, and assay. A calibration curve was prepared with six sample concentrations ranging from 0.15 to 10.0 ng/mL for buprenorphine and 0.05 to 5.0 ng/mL for norbuprenorphine. Peak areas of the quantitating ions were plotted versus concentration (ng/mL) for linear regression analysis. Two quality control samples were prepared at 0.5 and 2.5 ng/mL and analyzed in duplicate with each batch of plasma samples.

Limit of detection (LOD) and limit of quantitation (LOQ) were determined by analyzing drug-free plasma samples fortified with known drug concentrations. Each concentration was measured in triplicate. LOD was defined as the lowest concentration detected with at least two of three triplicates having acceptable qualifier ion ratios. The LOQ was defined as the lowest observed concentration within 20% of the theoretical concentration with acceptable qualifier ion ratios in all triplicates.

Clinical Samples

Blood samples were obtained from an adult male who provided written informed consent and was paid for participation in a research protocol involving the administration of buprenorphine by the oral (40 mg) and subcutaneous (1 and 2 mg) routes of administration. The research protocol was approved by the Francis Scott Key Institutional Review Board. The subject had a recent history of heroin abuse, but was drug-free at the time of the study. On the basis of physical examination, history, routine laboratory chemistries, and chest x-rays, the participant was judged to be in good health and without significant psychiatric disturbance other than drug abuse. The subject participated while residing on a secured clinical research ward at the Division of Intramural Research, National Institute on Drug Abuse (Baltimore, MD) for the duration of the study. Blood samples were collected in Vacutainer™ tubes before and periodically after buprenorphine administration. The samples were mixed, and plasma was collected after centrifugation. Plasma samples were frozen until time of analysis.

Results

Negative chemical ionization combined with tandem mass spectrometry and a deuterated internal standard provided high sensitivity, accuracy, and specificity. A full-scan product ion spectrum of buprenorphine is shown in Figure 3. The molecular anions of HFBA-derivatized buprenorphine (m/z 662) and buprenorphine-D₄ (m/z 666) were selected as precursor ions for MS-MS. The abundant product ion formed in CID at m/z 213 was not useful because it represented a derivative fragment and was common to both buprenorphine and buprenorphine-D₄. Product ions formed at m/z 364 and m/z 368 were not used because their intensity was too weak at concentrations less than 1 ng/mL. The precursor ions (m/z 662, m/z 666) and their major product
ions formed at m/z 464 and m/z 468 served for buprenorphine identification and quantitation. Optimum argon pressure (2.0 millitorr) and collision energy (24 eV) corresponded to approximately 60% transmission of the incident molecular anion (m/z 662). Changes in argon pressure and collision energy to increase the fragmentation of the precursor ions did not increase product ion formation and resulted in a decrease in the sensitivity of the assay. Greater ratios of m/z 464:662 and m/z 468:668 were produced, but the overall abundance of m/z 464 and m/z 468 decreased because the increased fragmentation was nonspecific.

The molecular anion of HFBA-derivatized norbuprenorphine (m/z 804) was too weak to use as a precursor ion. Ions at m/z 704 [M-100]- and m/z 607 [M-197]- were investigated. The m/z 607 was selected as the precursor ion because of its greater intensity and abundant product ion formation. The product ions monitored for norbuprenorphine were m/z 438 and m/z 409. The CID product spectra of norbuprenorphine is shown in Figure 4. Norbuprenorphine's incident precursor ion transmission at a collision energy of 20 eV was approximately 30%, but, unlike buprenorphine, most of the fragmentation formed the desired product ions. Deuterated analogues of norbuprenorphine were not available; consequently, norcodeine was used as an internal standard. The response for norcodeine in negative chemical ionization was somewhat similar to norbuprenorphine. The m/z 657 [M-20]- of norcodeine was selected as the precursor ion. Norcodeine's incident precursor ion transmission at a collision energy of 17 eV was less than 10%, and abundant product ions were formed (Figure 5). Product ions m/z 435 and m/z 478 were monitored.

The LOD for buprenorphine was 0.15 ng/mL, and the LOQ was 0.20 ng/mL. Optimal instrument conditions were needed to achieve these detection limits for buprenorphine. Injector port inserts and ion volumes were changed before each batch. The injector and detector end of the column was clipped frequently. The LOD for norbuprenorphine was 0.016 ng/mL, and the LOQ was 0.031 ng/mL. The assay was linear for buprenorphine from 0.15 ng/mL to 20 ng/mL and from 0.016 to 5.0 ng/mL for norbuprenorphine. Typical regression line equations and correlation coefficients were y = 0.206x - 0.004 (r = 0.9990) for buprenorphine and y = 4.577x + 0.165 (r = 0.9976) for norbuprenorphine. Within-run and between-run coefficients of variation were determined with the control samples. Within-run precision at 0.5 ng/mL was 9.8% for buprenorphine and 17.9% for norbuprenorphine. Between-run precision at 0.5 ng/mL was 13.8% (N = 29) for buprenorphine and 23.1% (N = 29) for norbuprenorphine. The assay was evaluated for interferences from a variety of drugs (Table II). Plasma samples were fortified with combinations of drugs at 250 ng/mL and analyzed for buprenorphine and norbuprenorphine. No interferences were found for any of the drugs tested.

Patient plasma samples were analyzed following the administration of buprenorphine by oral (40 mg) and subcutaneous routes (1 and 2 mg) to a single male subject. Approximately 14 plasma samples were collected over a 72-h period following each administration (Table III). The 40-mg oral buprenorphine dose had a peak buprenorphine concentration of 4.69 ng/mL at 3 h after dose administration, and the concentration dropped to 0.57 ng/mL at 72 h. Norbuprenorphine had a peak concentration of 3.26 ng/mL at 8 h, and the concentration dropped to less than 0.20 ng/mL. Norbuprenorphine had a peak concentration of 3.26 ng/mL at 8 h, and the concentration dropped to less than 0.20 ng/mL at 72 h. Norbuprenorphine had a peak concentration of 3.26 ng/mL at 8 h, and the concentration dropped to 1.32 ng/mL at 48 h. The subcutaneous 2-mg buprenorphine dose had a peak buprenorphine concentration of 8.74 ng/mL at 1 h after dose administration, and it declined to less than 0.20 ng/mL at 48 h. The norbuprenorphine had a peak concentration of 0.34 ng/mL at 4 h after dose administration, and it declined to 0.12 ng/mL at 48 h. Administration of a 1-mg dose by the subcutaneous route produced a peak buprenorphine concentration of 6.40 ng/mL at one half hour after dose administration. The concentration declined to less than 0.20 ng/mL at 24 h.
Discussion

The CID product ion spectra of buprenorphine and buprenorphine-D4 (m/z 464, m/z 468) demonstrated a common mass loss of 197 amu from the molecular anions. This loss most likely represented a loss of the heptafluorobutyryl group from the 3 position on the phenolic ring. The molecular anion of norbuprenorphine (m/z 804) underwent a similar mass loss of 197 amu in the ion source to form the m/z 607 precursor ion. The norbuprenorphine product ion (m/z 438) most likely involved a dissociation of the heptafluoropropyl group, CF₃CF₂CF₂-, from the 17 position nitrogen. The product ion m/z 409 resulted from complete loss of the heptafluorobutyryl group, CF₃CF₂CF₂CO-, from the nitrogen.

Different extraction systems for buprenorphine and norbuprenorphine were investigated during the development of the assay. Initial experiments using liquid-liquid extractions reported in the literature (9–12) were not as efficient, reproducible or rapid when compared with solid-phase extractions. Plasma samples were diluted with the pH 6 buffer used in the column conditioning steps to adjust the pH for optimum drug reducibility or rapid when compared with solid-phase extractions. Experiments with pretreating the plasma samples with methanol or acetonitrile for protein precipitation did not improve buprenorphine recovery compared with simple dilution with pH 6 buffer and extraction. Experiments in which the plasma pH was varied from pH 5.0 to pH 7.6 also did not further enhance recovery.

Fluorinated derivatives were evaluated to take advantage of the sensitivity offered by negative chemical ionization. Pentafluoropropropionic anhydride (PFPA), HFBA, and chlorodifluoroacetic anhydride were tested. Experiments with chlorodifluoroacetic anhydride were unsuccessful. The chlorodifluoroacetic anhydride derivative was not reliably formed for routine measurement. Both PFPA and HFBA derivatives of buprenorphine and norbuprenorphine were formed and detected by negative chemical ionization GC-MS-MS. HFBA was chosen over PFPA because of the higher molecular weight precursor and product ions it formed and the slightly better response HFBA obtained with negative chemical ionization. Bis(trimethylsilyl)trifluoroacetamide derivatization and positive chemical ionization were also attempted for buprenorphine. The [M-18]+ ion was selected as the precursor ion. The CID product ions (m/z 490, m/z 438) were less than 40% of the precursor ion in the CID spectra. Attempts to increase CID conditions did not increase product ion formation but increased nonspecific fragmentation.

The relative response of buprenorphine in negative chemical ionization was substantially lower than norbuprenorphine. Norbuprenorphine was approximately 10 times more reactive than buprenorphine. This difference in reactivity was likely due to formation of a di-derivative by norbuprenorphine rather than formation of a mono-derivative by buprenorphine. The formation of a single derivative by buprenorphine-D₄ made it unsuitable as an internal standard for norbuprenorphine. Nor-codeine was selected as an internal standard for norbuprenorphine because it contained a secondary amine suitable for derivative formation.

The large differences in LOD and LOQ between buprenorphine and norbuprenorphine can be attributed to the formation of the bis-heptafluorobutyryl derivative of norbuprenorphine. Buprenorphine's higher LOD and LOQ were also a result of buprenorphine's qualifier ion ratio containing the precursor ion (m/z 662). Buprenorphine's precursor ion had a greater abundance than the product ion (m/z 464), but the spectrum also contained significantly more noise, which caused the qualifier ion ratios to fail more often at low concentrations. The more desirable condition was to monitor two or more product ions as was done for norbuprenorphine. The product ions formed in CID contained significantly lower background noise.

The buprenorphine and norbuprenorphine standard curves were not sufficiently stable to permit the use of historical curves. Calibration curves were prepared with each assay. Analysis of the plasma samples by the previously described method demonstrated the need for a sensitive, specific analytical method capable of measuring buprenorphine and norbuprenorphine in subnanogram concentrations. Buprenorphine concentrations ranged from 8.74 to less than 0.20 ng/mL, and norbuprenorphine concentrations ranged from 3.26 to less than 0.03 ng/mL. Although these data were produced with specimens collected from only one subject, the plasma drug and metabolite concentrations indicated substantially different profiles of metabolism by the oral route.

### Table II. Drugs Tested for Assay Interference

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mepridine</th>
<th>Oxymorphine</th>
<th>Methadone</th>
<th>Pentazocine</th>
<th>Methamphetamine</th>
<th>Phencyclidine</th>
<th>Propoxyphene</th>
<th>Noraloxone</th>
<th>Oxycodeone</th>
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* Abbreviations: B = Buprenorphine; NB = Norbuprenorphine.

* Sample was not tested for this substance.

### Table III. Plasma Concentrations of Buprenorphine and Norbuprenorphine (ng/mL)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>40 mg oral</th>
<th>2 mg subcutaneous</th>
<th>1 mg subcutaneous</th>
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<tr>
<td></td>
<td>B* NB</td>
<td>B NB</td>
<td>B NB</td>
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<td>-0.50</td>
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<tr>
<td>0.08</td>
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<td>&lt;0.20 0.04</td>
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<td>&lt;0.20 0.04</td>
<td>0.26 &lt;0.03</td>
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<td>0.50</td>
<td>&lt;0.20 0.14</td>
<td>5.96 0.18</td>
<td>6.40 &lt;0.11</td>
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<td>1.00</td>
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<td>8.74 0.33</td>
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<td>72.00</td>
<td>0.57  n/a</td>
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</table>

* Abbreviations: B = Buprenorphine; NB = Norbuprenorphine.
versus the subcutaneous route. More extensive studies are planned with this assay to evaluate the pharmacokinetic properties of buprenorphine and norbuprenorphine following different routes of administration with multiple subjects.

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References


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