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Analysis of 2β-carbomethoxy-3β-(4-fluorophenyl)-*N*-(3-iodo-*E*-allyl)nortropane in rat plasma I. Method development and validation by capillary electrophoresis

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Abstract

Altropane, 2β-carbomethoxy-3β-(4-fluorophenyl)-*N*-(3-iodo-*E*-allyl)nortropane, is an imaging agent that was developed recently for early detection of Parkinson's disease. Its promise as a useful radiopharmaceutical for single-photon emission computed tomography or positron emission tomography imaging of the brain has been well demonstrated, and it is currently undergoing clinical trials. This paper presents methods development and validation of capillary electrophoresis (CE) techniques to analyze Altropane in aqueous environments as well as in rat plasma, using an internal standard, nicotinamide. *N*-Allylaltropane, 2β-carbomethoxy-3β-(4-fluorophenyl)-*N*-allylnortropane, which is a known degradation product of the Altropane precursor (tributyltinaltropane), was used to verify the method's specificity. A solid-phase extraction method for extraction of Altropane from rat plasma is also described. The results presented in this paper demonstrate the applicability of CE methods to study the pharmacokinetic properties of Altropane in animal models. The results of the pharmacokinetic study will be published later, as Part II.

Keywords: Method development; Validation; Altropane; Carbomethoxy (4-fluorophenyl) iodoallylnortropane; Allylaltropane; Nicotinamide

1. Introduction

This research was conducted as part of a study to evaluate the pharmacokinetic profile of Altropane (Altropane™, Boston Life Sciences Inc., MA, USA) in rat plasma. The objective of the work reported here was to develop capillary electrophoresis (CE) techniques to analyze the cocaine analog Altropane, 2β-carbomethoxy-3β-(4-fluorophenyl)-*N*-(3-iodo-*E*-allyl)nortropane, in an aqueous medium and in rat plasma. Other names for Altropane include (*E*)-*N*-iodoallyl-2β-carbomethoxy-3β-(4-fluorophenyl)-tropane, *E*-2β-carbomethoxy-3β-(4-fluorophenyl)-*N*-(1-iodoprop-1-en-3-yl)nortropane, and E-IACFT.

Altropane is being developed as a diagnostic product to detect the presence of dopamine-containing neurons and thus aid in detection of neuronal loss before clinical signs appear in patients with Parkinson's disease (PD), a chronic neurodegenerative disorder [1]. One in five, or 20%, of clinical

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diagnoses of PD are incorrect [2]. While the diagnosis is often straightforward in severely affected patients who display the disease's hallmark symptoms such as muscle tremor, stiffness, and weakness, early symptoms of the disease are difficult to distinguish, especially in young people [2]. Even for the experienced neurologist, a diagnosis of PD can be difficult to confirm, especially in the early stages of the disease. This brain disorder results from a gradual and progressive loss of neurons that contain the neurotransmitter dopamine. PD has no known cure [1]. New methods of treatment under investigation include gene therapy, implantable drug pumps, fetal cell transplantation, electrical brain stimulation and radiosurgery [3,4]. Apparently, about 60–80% of the brain's dopamine-containing neurons are lost before a patient starts showing clinical signs of the disease, which may appear as rigidity, bradykinesia, resting tremor, flexed posture and postural instability [1,4–6].

To detect neuronal loss before clinical signs appear in patients with PD, several substances such as 6-L-(^{18}F)-fluoro-dopa, which is directly transported into dopamine neurons [5] have been tested for their potential to indicate the presence of dopamine-containing neurons. Altropane, which specifically binds to dopamine transporters, is also being developed for this purpose. Recent literature on Altropane reports pre-clinical research in primates [6–8], postmortem normal and Parkinson's diseased brain [9], and healthy volunteers and patients with PD [10]. In these studies, Altropane has exhibited great promise as an imaging agent with SPECT (single-photon emission computed tomography) or PET (positron emission tomography), the two commonly used imaging methods. Effective detection of dopamine-containing neurons has been achieved using ^{123}I-labeled Altropane. This chemical was found to be a sensitive and selective marker of disease severity and it may effectively visualize the degree of nerve functioning at various stages of the disease.

The chemical synthesis and biological evaluation of radio-iodinated Altropane are discussed by Elmaleh et al. [11]. Prior to the development of Altropane, many other analogues of cocaine were synthesized and evaluated for their potency and potential applications as imaging agents [12]. A review of this earlier work is also included in Ref. [11].

The chemical structure of Altropane and, for easy comparison, that of cocaine are shown in Fig. 1, which also contains the chemical structures of *N*-allylaltropane, tributyltinaltropane (Altropane precursor), and nicotinamide. The former is a possible degradation by-product of Altropane, and the latter is the internal standard (I.S.) used in the work presented in this paper.

Until the early 1990s, high-performance liquid chromatography (HPLC), gas chromatography (GC) and gel electrophoresis were the mainstays among the separation-based analytical methods. The rapid development of CE in the 1990s added another powerful analytical tool for the analytical chemist. While no CE methods for the analysis of Altropane exist in the literature, there are classic examples of CE applications for cocaine analysis, such as those by Weinberger and Lurie [13] and Wernly and Thormann [14]. In addition to cocaine, many other drug substances were also included in the former study [13], and they were in the form of bulk drugs. The latter study [14] dealt with the analysis of common drugs of abuse and their metabolites in human urine. In both these investigations, efficient separations were achieved for cocaine and a few of its derivatives, including benzoyl ecgonine (a meta-

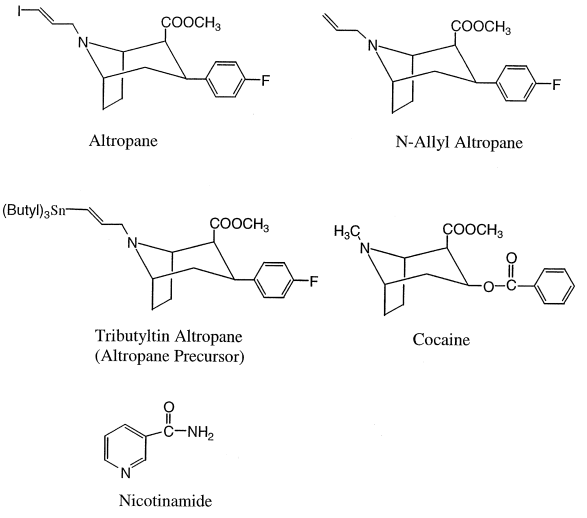

Fig. 1. Chemical structures of Altropane, *N*-allylaltropane, tributyltinaltropane, cocaine and nicotinamide.

bolite of cocaine), by using micellar electrokinetic capillary chromatography (MECC). The buffer solutions used in these MECC studies were 8.5 m*M* phosphate, 8.5 m*M* borate, 85 m*M* sodium dodecyl sulfate (SDS), and 15% acetonitrile at a pH of 8.5 [13] and 10 m*M* phosphate, 6 m*M* borate, and 75 m*M* SDS at a pH of 9.1 [14]. Capillary zone electrophoresis systems have also been successful for the analysis of cocaine. For example, in a study that involved investigation of cocaine and morphine in hair samples, determination of cocaine was accomplished using a 50 m*M* borate buffer at a pH of 9.2 [15]. The main tropane alkaloids from transformed *Hyoscyamus muticus* plants were analyzed using a 40 m*M* phosphate buffer at a pH of 7.8 [16]. These researchers reported that CE gave better peak symmetry and selectivity, shorter run times [13], and more efficient separations [15] than HPLC.

These potential advantages prompted us to develop CE techniques to analyze Altropane in an aqueous medium and in rat plasma. These CE methods were used successfully to analyze a dosing formulation of Altropane for its concentration and homogeneity. The same dosing formulation was used in a subsequent pharmacokinetic study of Altropane performed in male and female Sprague–Dawley rats. Plasma samples containing Altropane were extracted by solid-phase extraction (SPE) and analyzed by the same CE method to measure Altropane and its possible metabolites. This paper (Part I) presents the CE method development and validation. The results of the pharmacokinetic study will be published later, as Part II.

2. Experimental

2.1. Instrumentation

A BioFocus 3000 CE system with a programmable fast-scanning UV–Vis detector and BioFocus 3000 software for system control and data collection and analysis (Bio-Rad, Hercules, CA, USA) was used in this work. The CE capillaries were purchased from Bio-Rad and the C_{18} Sep-Pak cartridges were purchased from Waters (Milford, MA, USA).

2.2. Reagents

The buffer and the I.S. used were sodium acetate (99% pure) and nicotinamide (99+% pure), respectively. These two chemicals were purchased from Aldrich (Milwaukee, WI, USA). Methanol and ethyl acetate were HPLC grade, purchased from Mallinckrodt (St. Louis, MO, USA).

2.3. Samples

Altropane and *N*-allylaltropane samples investigated in this study were synthesized and supplied to us by Organix Inc. (Woburn, MA, USA).

All sample and buffer solutions were prepared in distilled, deionized (DD) water. Super-Q water was used for SPE.

3. Results and discussion

3.1. Altropane in aqueous medium

In the overall study to evaluate the pharmacokinetic profile of Altropane in rat plasma, a dosing formulation of Altropane that was slightly acidic (pH 4–5) also needed to be prepared and analyzed to determine its concentration and homogeneity. Therefore, prior to the methods development and validation for extraction of Altropane from rat plasma, a series of experiments were performed to analyze Altropane in aqueous medium under slightly acidic conditions.

3.1.1. Preliminary investigations

As discussed in the Introduction, effective separation of cocaine and a few of its derivatives, including its metabolite, benzoyl ecgonine, has previously been achieved using MECC systems. Because of the structural similarity of cocaine and Altropane with respect to the cocaine backbone, we first attempted to use MECC systems to study Altropane. However, reproducible electropherograms were not obtained when a MECC system (8.5 m*M* borate–8.5 m*M* phosphate–85 m*M* SDS, pH 8.5) was used. This could have been due to the instability of Altropane under basic conditions, although it is known to be very stable under acidic conditions. Subsequently, a

phosphate buffer of pH 6 and acetate buffers of pH 4–5 were found to be suitable for Altropane analysis. Further experiments revealed that the best conditions for the separation of Altropane, the I.S. (nicotinamide) and *N*-allylaltropane were obtained with a pH 4 acetate buffer. Thus, considering the stability of Altropane under acidic conditions, an acetate buffer of pH 4 was used throughout all subsequent studies. With regard to the capillary length, 24-, 30- and 36-cm capillaries were tested and the 30- and 36-cm lengths were found to be suitable for the efficient separation of all three constituents (Altropane, *N*-allylaltropane and the I.S.). The 24-cm capillary length was insufficient for separation of Altropane and *N*-allylaltropane and the sample volume that could be loaded.

3.1.2. Conditions

The CE experiments were performed using 30- and 36-cm×50 μm LPA (linear polyacrylamide) coated capillaries. The effective lengths (inlet to the detector) of these capillaries were 25.4 and 31.4 cm, respectively. The other conditions were 50 m*M* sodium acetate buffer of pH 4.1, ion migration from positive to negative polarity, run voltages of 20 or 18 kV, pressure injection of 10 p.s.i. s (1 p.s.i.=6894.76 Pa), a detection wavelength of 210 nm, and a capillary temperature of 20°C.

3.1.3. Precision

The precision was first evaluated using a single test solution of Altropane. A sample of Altropane (0.721 mg) was transferred to a 5-ml volumetric flask and 0.5 ml of 0.1 *M* HCl added to solubilize it. Then, DD water was added up to the 5-ml mark and mixed well. The I.S. solution was prepared by dissolving 1.052 mg nicotinamide in 10 ml DD water. The test solution was prepared by mixing 1.0 ml Altropane solution and 1.0 ml I.S. solution (after mixing with the I.S., the Altropane solution was 0.721 mg/10 ml). An aliquot of this test solution was electrophoresed seven times consecutively. Results from this set of experiments are presented in Table 1. The precision (instrument precision) calculated using the peak area ratio, expressed as relative standard deviation (RSD), was 0.9%. Furthermore, without the correction of I.S., the peak areas of Altropane alone yielded a satisfactory RSD value of 1.6%.

The precision was also evaluated by using test solutions prepared according to the above procedure but using six different weighings of Altropane. The results are presented in Table 2. The method precision (RSD) calculated using the peak area ratios in this case was 1.3%. Again, Altropane peak areas alone yielded a satisfactory RSD value of 1.8%.

3.1.4. Specificity

N-Allylaltropane and Altropane are structurally similar, the only difference being that the 3-iodo group is present in Altropane but absent in *N*-allylaltropane. Furthermore, *N*-allylaltropane has been shown to be a degradation product of the Altropane precursor (see Fig. 1), the tributyltin compound [11].

Table 1
Precision data (same solution, 36-cm capillary, 20 kV)

Altropane (mg/10 ml)	Altropane peak area	I.S. peak area	Peak area ratio	Peak area ratio/mg
0.721	720 715	2 972 874	0.242	0.336
0.721	737 250	3 018 632	0.244	0.339
0.721	716 085	2 965 944	0.241	0.335
0.721	715 533	2 978 836	0.240	0.333
0.721	745 569	3 049 052	0.245	0.339
0.721	726 699	2 963 596	0.245	0.340
0.721	738 534	2 984 900	0.247	0.343
Average	728 626			0.338
SD	11 935			0.003
RSD (%)	1.6			0.9

Table 2
Precision data (different solutions, 36-cm capillary, 20 kV)

Altropane (mg/10 ml)	Altropane peak area	Altropane peak area/mg	I.S. peak area	Peak area ratio	Peak area ratio/mg
0.919	898 370	977 552	2 577 258	0.349	0.379
0.807	809 123	1 002 631	2 630 149	0.308	0.381
0.906	912 908	1 007 625	2 598 205	0.351	0.388
0.986	994 792	1 008 917	2 688 585	0.370	0.375
0.813	834 633	1 026 609	2 733 982	0.305	0.375
0.883	906 512	1 026 627	2 749 787	0.330	0.373
Average		1 008 327			0.379
SD		18 163			0.005
RSD (%)		1.8			1.3

Therefore, *N*-allylaltropane is an ideal candidate to test the system's specificity. When aqueous solutions of the two compounds were individually electrophoresed, their migration times were very close, but a solution containing a mixture of the two compounds was found to be baseline resolved. Therefore, this CE method was considered sufficiently specific for pharmacokinetic or toxicokinetic studies of Altropane. A representative electropherogram of a mixture of Altropane, *N*-allylaltropane and the I.S. (in aqueous solution) is shown in Fig. 2. As evident from this figure, Altropane electrophoreses much earlier than nicotinamide (migration times 3.89 and 15.66 min, respectively), and this wide gap of migration times should allow a sufficient time window to detect major metabolites produced during the planned pharmacokinetic studies.

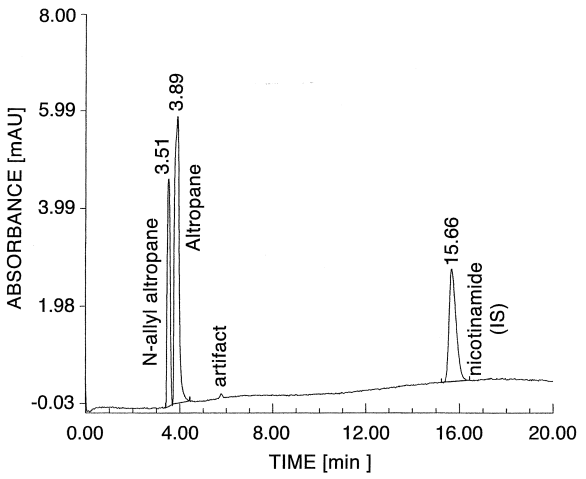

Fig. 2. A representative electropherogram of Altropane (1 mg/10 ml), *N*-allylaltropane (1 mg/10 ml) and nicotinamide, the I.S. (1 mg/100 ml) in aqueous solution.

3.1.5. Linearity and accuracy

By methods similar to those for the precision experiments, five test solutions covering a range of 0.3 to 2.2 mg Altropane were prepared and electrophoresed to determine the linearity and accuracy. After mixing with I.S., these solutions had concentrations ranging from 0.3 to 2.2 mg/10 ml. The results for linearity and accuracy are presented in Table 3. These results demonstrated linearity in the range of 0.3 to 2.2 mg Altropane/10 ml. The R^2 value was 0.9998. The method error was ±1.2%. Similar to the precision data (Tables 1 and 2), Altropane alone (without the correction of I.S.), yielded an R^2 value of 0.9995. However, the error was slightly higher (±2.4%, Table 3) in this case.

3.1.6. Linearity of Altropane at low concentrations

In anticipation that the levels of Altropane in plasma during pharmacokinetic studies would be lower than the concentrations of the neat standards described above, an experiment was designed to examine the linearity of Altropane neat standards at lower concentrations. Seven solutions were prepared and mixed 1:1 with an I.S. solution of 2.1 mg/ml to yield Altropane concentrations of 51.300, 25.650, 12.825, 5.130, 2.565, 0.513 and 0.257 μg/ml. The standard curve using the first five solutions yielded an R^2 value of 0.9999 (Table 4 and Fig. 3A). The method error was ±1.7%. The two solutions of lowest concentration (0.513 and 0.257 μg/ml) were

Table 3
Linearity and accuracy (36-cm capillary, 20 kV)

Altropane (mg/10 ml)	Altropane peak area	I.S. peak area	Peak area ratio	Back calculated mass	Deviation (absolute)	Deviation (%)
0.279	287 898	2 651 037	0.109	0.274	0.005	1.63
0.465	466 753	2 612 986	0.179	0.457	0.008	1.69
0.774	790 481	2 617 364	0.302	0.779	0.005	0.65
1.291	1 273 454	2 520 428	0.505	1.309	0.018	1.42
2.151	2 060 134	2 500 020	0.824	2.141	0.010	0.46
Average						1.2
Equation:	$y=0.3833x+0.0034$ (with the correction of I.S.) $R^2=0.9998$ Error=±1.2%					
	$y=946\,423x+36\,892$ (without the correction of I.S.) $R^2=0.9995$ Error=±2.4%					

near the minimum detection level and therefore were not used in the standard curve.

3.1.7. Linearity of N-allylaltropane at low concentrations

An experiment was also designed to examine the linearity of *N*-allylaltropane at lower concentrations. As for Altropane, seven test solutions of *N*-allylaltropane, 52.300, 26.150, 13.075, 5.230, 2.615, 0.523 and 0.262 μg/ml were prepared and electrophoresed. Results from the first five solutions are presented in Table 5. The observed R^2 value was 0.9996 (Fig. 3A) and the method error was ±2.2%. As for Altropane, the two solutions of lowest concentration (0.523 and 0.262 μg/ml) approached the minimum detection level and therefore, were not used in the standard curve.

3.2. Linearity of Altropane and N-allylaltropane when present in the same test mixture

As mentioned earlier, the only structural difference between Altropane and *N*-allylaltropane is that the 3-iodo group is present in Altropane but absent in *N*-allylaltropane. Moreover, *N*-allylaltropane has been shown to be a degradation by-product of the Altropane precursor, the tributyltin compound [11]. Thus, *N*-allylaltropane could be considered a po-

Table 4
Linearity experiments for Altropane at lower concentrations (30-cm capillary, 18 kV)

Solution no.	Altropane (μg/ml)	Altropane in test solution (μg/ml)	Altropane peak area	I.S. peak area	Peak area ratio	Migration time of Altropane (min)	Migration time of I.S. (min)
1	102.600	51.300	1 311 127	653 057	2.008	4.30	17.37
2	51.300	25.650	681 209	675 718	1.008	4.33	17.52
3	25.650	12.825	322 683	666 828	0.484	4.28	17.15
4	10.260	5.130	137 008	670 088	0.204	4.21	17.14
5	5.130	2.565	63 642	673 922	0.094	4.14	16.87
Average				667 923		4.25±0.06	17.21±0.25
Equation	$y=0.0393x-0.0056$, $R^2=0.9999$, error=±1.7%						

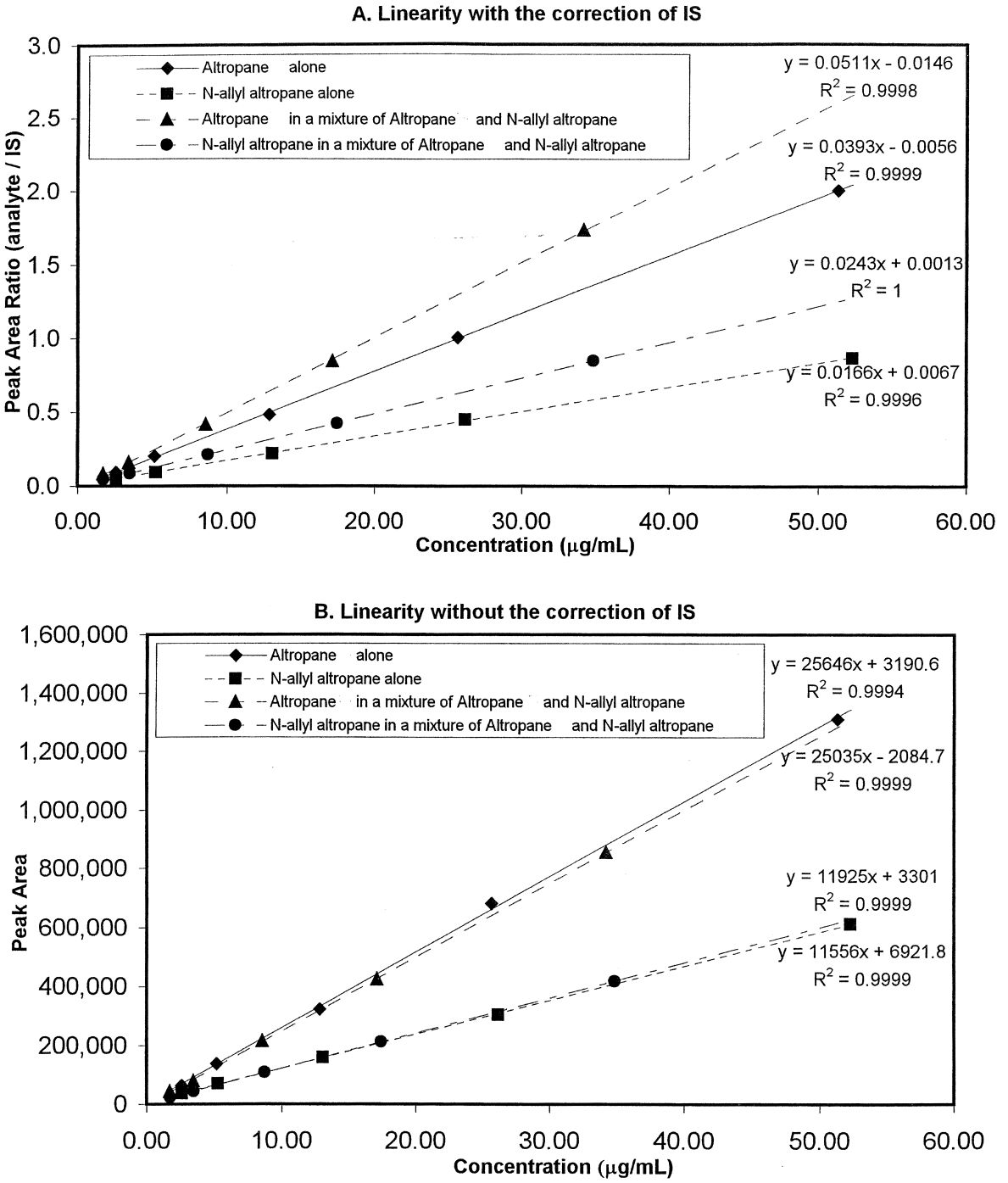

Fig. 3. Linearity curves for Altropane and *N*-allylaltropane at low concentrations alone and in mixture with the other component.

Table 5
Linearity experiments for *N*-allylaltropane at lower concentrations (30-cm capillary, 18 kV)

Solution no.	*N*-Allyaltropane (μg/ml)	*N*-Allyaltropane in test solution (μg/ml)	*N*-Allylaltropane peak area	I.S. peak area	Peak area ratio	Migration time of *N*-allylaltropane (min)	Migration time of I.S. (min)
1	104.600	52.300	612 753	703 614	0.871	3.72	16.79
2	52.300	26.150	305 191	675 159	0.452	3.66	16.55
3	26.150	13.075	159 247	727 187	0.219	3.70	16.58
4	10.460	5.230	69 714	742 333	0.094	3.73	16.54
5	5.230	2.615	35 992	752 292	0.048	3.77	16.72
Average				720 117		3.72±0.03	16.64±0.09
Equation	$y=0.0166x+0.0067$, $R^2=0.9996$, error=±2.2%						

tential product that may be detected along with Altropane in plasma extracts during pharmacokinetic studies. Therefore, an additional experiment was performed to examine whether any interference occurs when both compounds are present together.

Seven test mixtures ranging in concentration from 34.200 to 0.171 μg/ml Altropane and from 34.867 to 0.174 μg/ml *N*-allylaltropane were prepared and electrophoresed as before. The results from the first five mixtures are given in Table 6. The observed R^2 values were 0.9998 and 1.0, and the method errors were ±3.0% and ±0.8% for Altropane and *N*-allylaltropane, respectively (Fig. 3A). Under these conditions, the lowest detection limits were 171 ppb for Altropane and 174 ppb for *N*-allylaltropane. Although the I.S. concentration in the three sets of experiments (Tables 4–6) was the same, the corresponding I.S. peak areas were different. However within each set they agreed very closely. This behavior is attributable to the changes in the migration time and charge status of the I.S. in the electrophoretic medium. In capillary zone electrophoresis, the migration time of an analyte depends upon its mass and the charge. The charge status of the I.S. in the third experiment (Table 6) would have been much higher than in the first and the second experiments (Tables 4 and 5), which resulted in faster migration (15.68 min, Table 6) than the other two (17.21 and 16.64 min, Tables 4 and 5). Faster migration causes less peak broadening, and therefore the peak area of the I.S. in the third set (average 506 624) is smaller than that in the other two (averages 667 923 and 720 117). However, this argument does not hold true for the behavior of the I.S. within the first two sets. Although the average migration time in the first set is slightly higher (17.21 min) than in the second set (16.64 min), the average peak area of the former set (667 923) is smaller than that of the second set (720 117). One possible explanation of this ad hoc variation of the I.S. peak area could be that its UV properties are very sensitive to slight changes in the medium, such as the pH. However, additional experimental evidence is needed before such an assumption can be made. When the electrophoretic conditions including the sample matrix and electrophoretic buffer are kept the same, the migration times of an analyte are expected to remain more or less constant within each set of experiments. The CE peak areas derived under such circumstances will be reliable and acceptable.

The four standard curves represented in Fig. 3A were constructed with the correction of the I.S. Fig. 3B represents curves without the correction of the I.S. In Fig. 3B, the curves for Altropane alone and in the presence of *N*-allylaltropane came very close to each other (the two slopes were 25 646 and 25 035). This demonstrated that the Altropane peak area was not being affected by the presence of *N*-allylaltropane. Similarly, the curves for *N*-allylaltropane alone and in the presence of Altropane came very close to each other, the two slopes being 11 556 and 11 925. This demonstrated that *N*-allylaltropane is not being affected by Altropane either.

3.3. Plasma experiments

3.3.1. SPE of Altropane from rat plasma

Rat plasma samples were extracted by SPE using

Table 6
Linearity experiments at lower concentrations (30-cm capillary, 18 kV) for Altropane and *N*-allylaltropane in the same test mixture

Solution no.	Altropane (μg/ml)	*N*-Allylaltropane (μg/ml)	Altropane in test solution (μg/ml)	*N*-Allylaltropane in test solution (μg/ml)	Altropane peak area	*N*-Allylaltropane peak area	I.S. peak area	Altropane peak area ratio	*N*-Allylaltropane peak area ratio	Migration time of Altropane (min)	Migration time of *N*-allylaltropane (min)	Migration time of I.S. (min)
1	102.600	104.600	34.200	34.867	854 629	417 921	491 548	1.739	0.850	3.89	3.51	15.66
2	51.300	52.300	17.100	17.433	424 542	213 287	501 448	0.847	0.425	3.85	3.50	15.58
3	25.650	26.150	8.550	8.717	213 886	108 571	511 686	0.418	0.212	3.89	3.53	15.70
4	10.260	10.460	3.420	3.487	79 243	42 781	497 697	0.159	0.086	3.81	3.59	15.83
5	5.130	5.230	1.710	1.743	44 048	23 929	530 743	0.083	0.045	3.79	3.47	15.65
Average							506 624			3.85±0.04	3.53±0.03	15.68±0.06
Altropane equation	$y=0.0511x-0.0146$, $R^2=0.9998$, error=±3.0%											
N-Allylaltropane equation	$y=0.0243x-0.0013$, $R^2=1.0$, error=±0.8%											

C_{18} Sep-Pak cartridges (Waters). The cartridges were conditioned with approximately 4.4 ml of methanol followed by approximately 6.6 ml of Super-Q water. Aliquots (200 μl) of plasma samples and standards were loaded on the preconditioned SPE cartridges, and each cartridge was washed 20 min later with approximately 4.4 ml of water. The drug was eluted from the cartridge using 2.2 ml of ethyl acetate. A known amount (1.5 ml) of the extract was transferred to a 2-ml microcentrifuge tube and then evaporated to dryness at room temperature by means of a rotary evaporator. The samples were then reconstituted with 200 μl of 0.025 *M* HCl, filtered through Gelman GHP Acrodisc 0.45-μm, 13-mm syringe filters (Gelman Sciences, Ann Arbor, MI, USA), and a known volume (180–190 μl) transferred to a 500-μl microcentrifuge tube for CE analysis.

Neat and plasma spiked standards ranging from 5 to 75 μg/ml and plasma quality control (QC) standards at 25.6 μg/ml were used for quantifying the Altropane levels in the plasma samples. The neat standards, plasma spiked standards and QC standards were prepared and stored at −20°C until the day of analysis. Both the plasma spiked standards and the QC standards were thawed and extracted under the same conditions as the plasma samples. Nicotinamide at 12.5 μg/ml was added to the samples after extraction and just before CE analysis. Used in this way, the I.S. merely checks the variability of the injector rather than the extraction method [17].

When the structures and solubilities of Altropane and the I.S. were considered, the use of nicotinamide as the I.S. was not the ideal choice. The saturating solubility of Altropane in ethyl acetate was approximately 127.3 mg/ml and that of the I.S. was only 0.3 mg/ml. Because the I.S. had very different solubility properties from Altropane, the I.S. could not be added to the plasma samples before extraction. Furthermore, because the I.S. is extremely soluble in water, it could be washed away during the water washes of the SPE procedure. Therefore, the I.S. was added to the final extract immediately prior to the analysis.

3.3.2. Method validation for plasma analysis (evaluation of precision, linearity, accuracy, recovery, sensitivity and specificity)

The instrument precision was evaluated by electrophoresing a neat standard solution of Altropane, 75 μg/ml, six times consecutively. The resulting data are presented in Table 7. From these results, Altropane and nicotinamide peak areas alone yielded RSD values of 2.0% and 4.5%, respectively. When the average I.S. peak area was used to calculate the peak area ratios of Altropane/I.S., the resulting RSD value was 2.0%, whereas when the individual peak areas were used to calculate the peak area ratios, the

Table 7
Precision based on neat standards after SPE (30-cm capillary, 18 kV)

Neat standard no.	Altropane concentration (μg/ml)	Altropane peak area	Nicotinamide peak area	Altropane peak area/average nicotinamide peak area	Altropane/nicotinamide peak area
1	75	1 956 508	873 341	2.183	2.240
2	75	1 930 854	876 061	2.155	2.204
3	75	1 969 251	873 917	2.197	2.253
4	75	1 976 687	886 966	2.206	2.229
5	75	2 043 822	976 687	2.281	2.093
6	75	1 941 347	889 909	2.166	2.182
Average		1 969 745	896 147	2.198	2.200
SD		40 075	40 069	0.045	0.059
RSD (%)		2.0	4.5	2.0	2.7
Neglecting neat standard No. 5, RSD (%)		1.0	0.9	1.0	1.3

Table 8
Linearity of Altropane based on neat standards after SPE (30-cm capillary, 18 kV)

Neat standard no.	Altropane concentration (μg/ml)	Altropane peak area	Nicotinamide peak area	Altropane/average nicotinamide peak area	Altropane/nicotinamide peak area
1	0	0	743 690	0.000	0.000
2	0	0	839 993	0.000	0.000
3	5	105 338	804 604	0.127	0.131
4	5	120 982	840 824	0.146	0.144
5	15	369 204	829 685	0.447	0.445
6	15	373 987	846 910	0.453	0.442
7	25	610 990	814 228	0.739	0.750
8	25	599 689	829 156	0.726	0.723
9	50	1 267 547	838 954	1.534	1.511
10	50	1 267 143	842 346	1.533	1.504
11	75	1 975 453	832 033	2.390	2.374
12	75	1 919 419	854 616	2.323	2.246
Average			826 420		
Equations	$y=0.0317x-0.0357$ (with the correction of I.S., using average peak area) $R^2=0.9992$				
	$y=0.0308x-0.0171$ (with the correction of I.S., using individual peak areas) $R^2=0.9986$				
	$y=25\,990x-18\,917$ (without the correction of I.S.) $R^2=0.9992$				

resulting RSD value was 2.7%. However, when neat standard No. 5 was ignored, the RSD of the I.S. alone dropped from 4.5% to 0.9%, suggesting that it could be considered as an outlier. Then, the RSD of Altropane alone became 1.0%. This again illustrates (Table 1) that Altropane peak areas alone could have been used for the calculations.

The data from neat standard solutions (Table 8) yielded a linear equation of $y=0.0317x-0.0357$ with an R^2 value of 0.9992 when the average nicotinamide peak area was used. The corresponding linearity curve is shown in Fig. 4A. Furthermore, linear regression analysis using individual peak areas of the I.S. also yielded a satisfactory R^2 value of 0.9986 (Table 8). In particular, Altropane alone yielded an R^2 value of 0.9992, again suggesting the feasibility of eliminating the I.S. However, to check the instrument performance and to get a clear picture of the variation from one analysis to the next, the use of an I.S. is desirable.

The data from plasma standard solutions yielded a linear equation of $y=0.025x-0.0214$, and the corresponding R^2 value was 0.9868. The linearity curve for plasma standards is shown in Fig. 4B. A repre-

Table 9
Mean accuracy and precision of plasma calibration and plasma QC standards after SPE

Altropane concentration[a] (μg/ml)	Mean accuracy (%)	RSD (%)
5	106	6
15	100	NA
25	94	7
50	105	8
75	99	7
Quality control standards		
25.6	90	3

[a] $n=3$.
NA=Not applicable, $n=2$.

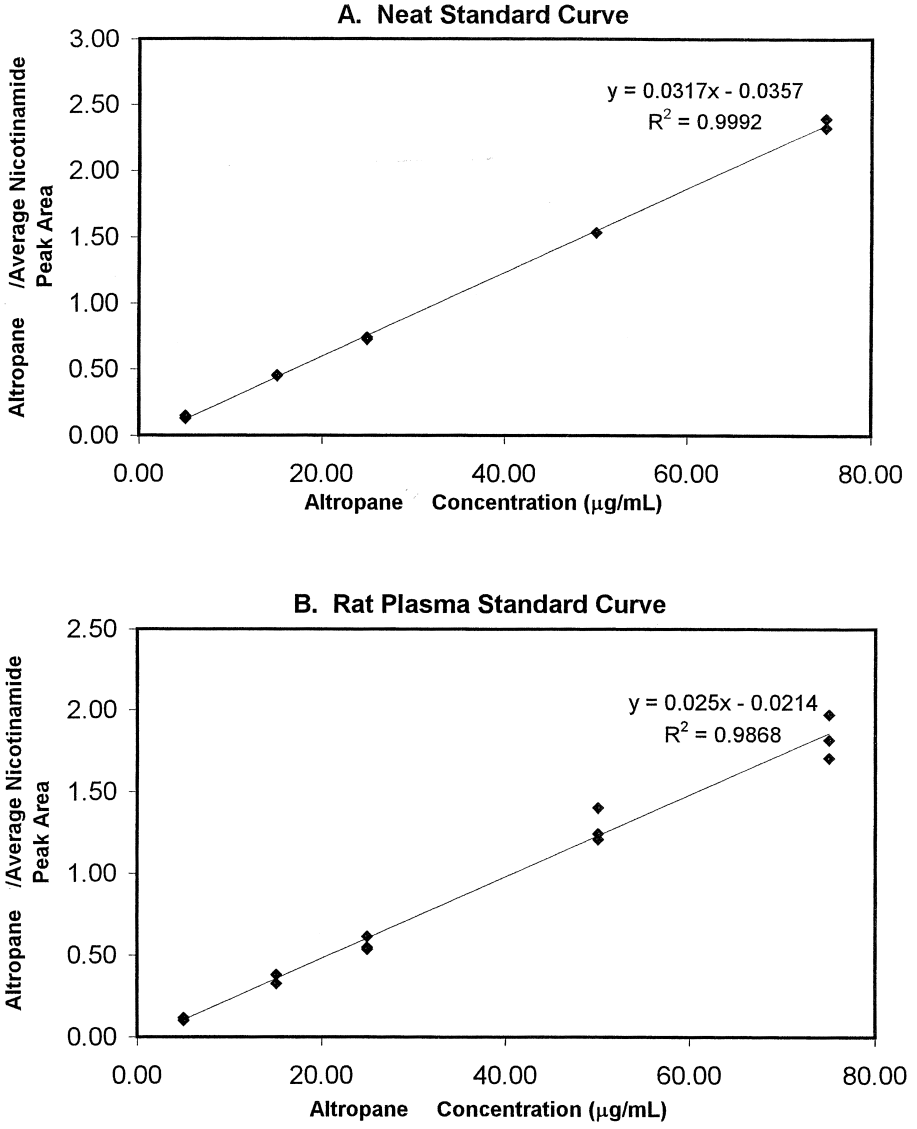

Fig. 4. Linearity curves for Altropane (A) neat standards and (B) rat plasma standards.

sentative CE profile of Altropane after SPE extraction and I.S. addition is shown in Fig. 5. The mean accuracy and method precision for the plasma standards ranged from 94% to 106% and 6% to 8% RSD, respectively (Table 9). The mean method precision for the QC standards was 3% RSD and the accuracy was 90% (Table 9). The accuracy and precision for this method is considered acceptable for the analysis of rat plasma samples from preliminary pharmacokinetic studies. A CE profile of an extracted rat plasma sample from the subsequent pharmacokinetic study is shown in Fig. 6.

The recovery, defined as the mean extraction efficiency, ranged from 75% to 90% (Table 10), which was considered acceptable. The limit of detection (LOD) and the limit of quantitation (LOQ) were found to be 0.17 and 0.3 μg/ml, respectively.

Rat plasma spiked with both Altropane and *N*-allylaltropane, when extracted under the same SPE procedure, yielded two peaks that were baseline

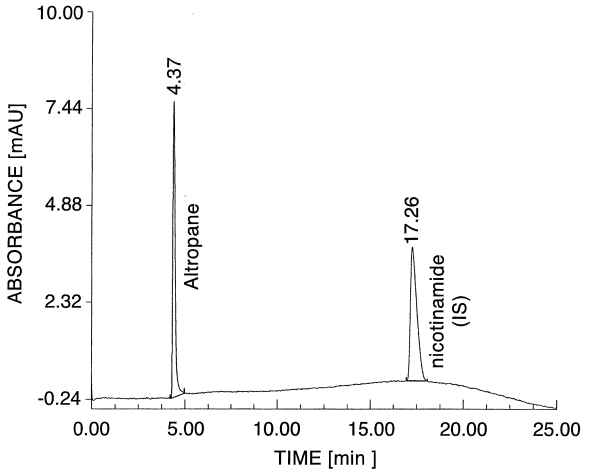

Fig. 5. CE profile of Altropane rat plasma standard after solid-phase extraction. Altropane concentration before extraction was 50 μg/ml. Nicotinamide, the I.S. (12.5 μg/ml) was added after extraction.

resolved (Fig. 7), demonstrating the method's specificity.

4. Conclusions

The CE and SPE methods validated above are suitable for analysis of Altropane in aqueous solutions and in rat plasma. CE is an inherently micro-analytical method, and therefore, the sample sizes that can be loaded into CE capillaries for analysis are extremely small. Despite this drawback of small sample load, the CE techniques presented in this paper offer an efficient means of analyzing for Altropane. The results also demonstrated the feasibility of eliminating nicotinamide as an I.S. in the CE study. The methods were also extended to analyze a dosing formulation of Altropane and extracts from rat plasma. A description of successful analysis of this dosing formulation of Altropane and an evaluation of its pharmacokinetic profile in Sprague–Dawley rats will be published later, as Part II of this study.

Table 10
Recovery of plasma standards after SPE

Altropane concentration (μg/ml)[a]	Mean recovery (%)
5	87
15	75
25	84
50	90
75	84
Average	84

[a] $n=3$.

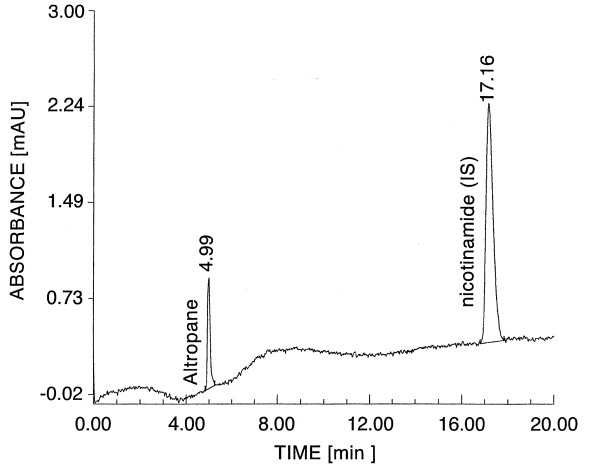

Fig. 6. CE profile of Altropane in rat plasma after solid-phase extraction and addition of nicotinamide as an I.S. Sample was collected 2 min after intravenous administration of an Altropane formulation to a female Sprague–Dawley rat.

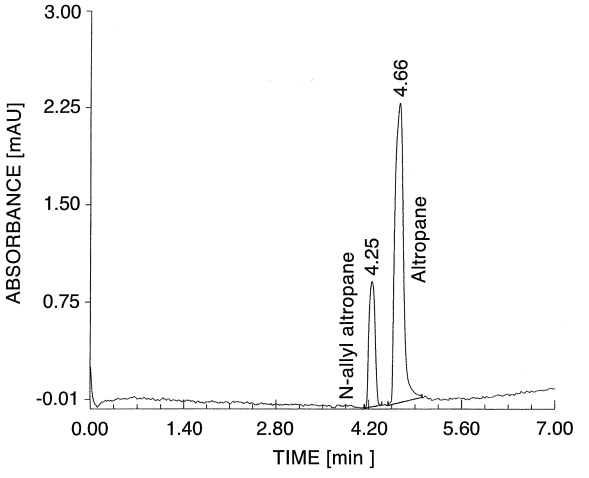

Fig. 7. CE profile of a rat plasma standard containing a mixture of Altropane (15 μg/ml) and *N*-allylaltropane (10 μg/ml) after solid-phase extraction.

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