Selective Quantification of Ambroxol in Human Plasma by HPLC

M. Nieder* and **H. Jaeger**
L.A.B. Gesellschaft für pharmakologische Studien mbH & Co, Neu-Ulm, FRG

Key Words:

Liquid chromatography, HPLC
Pharmacokinetics
Quantification in human plasma
Bronchosecretolyticum
Separation from metabolites
Ambroxol

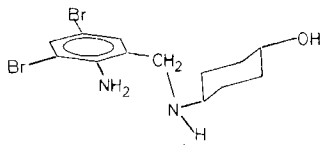
Summary

The bronchosecretolytic drug ambroxol can be reliably quantified in human plasma by high performance liquid chromatography. Plasma is buffered alkaline, extracted with ether, and the organic solvent back-extracted with diluted acid. An automatically sampled aliquot is separated by reversed phase HPLC; the analyte is well separated from two metabolites that interfered strongly in earlier methods. UV detection at 230 nm enables a lower limit of quantitation of 5 ng/ml. Internal standardization with propranolol allows accurate and precise quantification. Evaluation of the optimized combination of mobile and stationary phase is described, and application of the method to experimental and clinical pharmacokinetic studies is illustrated.

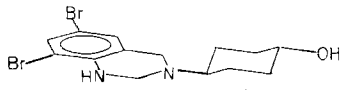
1 Introduction

trans-4-(2-Amino-3,5-dibrombenzyl)aminocyclohexanol (ambroxol) is intensively used in the treatment of chronic diseases of the respiratory tract as bronchosecretolyticum. It is supposedly the active principle of the parent drug bromhexin, a common mucolyticum. Earlier studies [1,2] showed that ambroxol as well as its metabolites had therapeutic concentrations in the low nanogram region. For quantification ambroxol was derivatized with ^{14}C-formaldehyde [2], separated by TLC, and quantified by radioactivity measurement. Alternatively, ^{14}C-labeled ambroxol was dosed and the radioactive fractions collected to determine the structure of the metabolites [3]. The main metabolites in human plasma are the product of condesation with formaldehyde, 6,8-dibromo-3-(*trans*-4-hydroxycyclohexyl)-1,2,3,4-tetrahydroquinazoline (NA 873), and dibromoanthranilic acid (DBAS). A recently published method [4] measured the formaldehyde condensation product (NA 873) after extraction from plasma by HPLC. As the measured concentrations are the sum of ambroxol and metabolite NA 873, each sample has to be reassayed for the concentration of the metabolite only. The concentration of ambroxol was calculated by subtraction of the metabolite concentration from the sum. This method is not only susceptible to errors of work-up and quantification, but is non-specific, non-selective, and laborious.

Ambroxol (*trans*-4-(2-amino-3,5-dibromobenzyl)aminocyclohexanol)



NA 873 (6,8-Dibromo-3-(*trans*-4-hydroxycyclohexyl)-1,2,3,4-tetrahydroquinazoline)



DBAS (3,5-Dibromoanthranilic acid)

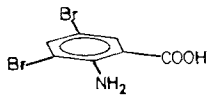


Figure 1

Chemical structure of ambroxol and its metabolites.

The present work describes a sensitive, accurate, and precise method for quantitating ambroxol selectively in human plasma.

2 Experimental

2.1 Materials

Pure standards of ambroxol hydrochloride and propranolol hydrochloride were provided by Sigma, (Munich, FRG). The metabolites NA 873 and DBAS were synthesized at L.A.B. The purity of all substances was > 99% by TLC. All other reagents, analytical or HPLC grade, were purchased from E. Merck (Darmstadt, FRG). PIC-B 5 reagent was delivered by Millipore-Waters (Eschborn, FRG). Mobile phase components (water, acetonitrile, methanol) were separately filtered prior to mixing, then degassed with helium after mixing.

2.2 Apparatus and Chromatographic Conditions

An automated HPLC chromatograph consisting of modular components was used. The components were a Merck-Hitachi 655A-11 solvent delivery pump, a Kratos SF 757 variable wavelength spectrophotometer operated at 230 nm, and a Gilson model 112 automatic sample processor. Detector output (peak height) was quantitated using a Shimadzu CR 1 B integrator. Data regression and concentration calculations were performed on a Commodore CBM 8032 Computer using LAB CAL software.

The column was an Alltech RoSil CPS, 5 µm, 150 × 4.6 mm (Alltech, Munich, FRG). The mobile phase consisted of water (1500 g), methanol (300 g), acetonitrile (300 g), and PIC B 5 (27 g). The flow rate was 1.6 ml/min. All analyses were performed at room temperature.

2.3 Stock Solutions

Standard solutions of ambroxol were prepared by dissolving 109.7 mg ambroxolhydrochloride in 100 ml water. Working solutions were prepared by appropriate dilution with water. Solutions are stable for at least three months when stored at 4°C in the freezer. The absence of carbonyl-active contaminants in the solvents must be verified.

An internal standard solution is prepared by dissolving 5.5 mg propranololhydrochloride in 1000 ml water.

2.4. Preparation of Samples

1.00 ml plasma, 25 µl internal standard solution, 250 µl 0.1 N sodium hydroxide and 6.00 ml ether were added to 10 ml round bottom culture tubes equipped with PTFE-lined screw-top caps. The samples were shaken in an overhead shaker for 30 minutes. After centrifuging at 5000 rpm, ca. 5.00 ml of the organic phase was transferred to a 10 ml tapered glass tube filled with 150 µl 0.1 N HCl. The tubes were treated for 30 min in the overhead shaker, centrifuged at 5000 rpm, and stored at −20°C. After thawing, the organic phase is discarded, and the samples are transferred to autosampler vials, and 100 µl of each injected for chromatographic analysis. Samples could be stored for at least 48 hours.

3 Results and Discussion

3.1 Evaluation of the Chromatographic System

Because ambroxol is active at very low plasma levels, a sensitive method of detection is required. Ambroxol has three UV maxima, one at 310 nm which has a low specific extinction coefficient, a second one at 230 nm, which is more intensive by a factor of 2.5, and a third one at 207 nm, which is the most intensive but the least selective. Detection at 230 nm was sufficiently sensitive to detect less than 250 pg ambroxol injected directly on column. Ambroxol can be assayed by electrochemical detection at an oxidation potential of +800 mV or by fluorescence detection after derivatization with o-phthalaldehyde/mercaptoethanol; both alternatives require more effort and are less applicable to routine analysis than UV detection.

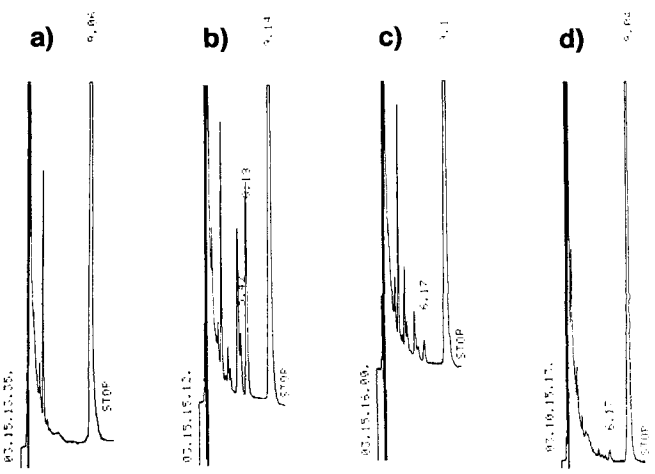


Figure 2

Typical chromatograms from a pharmacokinetic study with an administred single dose of 60 mg ambroxol in drops.

a) Predose plasma of a subject; peak at 9.06 is internal standard propranolol.
b) Plasma 4 h after administration; peak at 6.18 is about 71 ng/ml ambroxol, at 5.42 about 12 ng/ml DBAS (estimated); peak eluted just before is about 60 ng/ml NA 873.
c) Plasma 24 h after administration; peak at 6.17 is about 7 ng/ml ambroxol.
d) 5 ng/ml ambroxol calibration spike.

The original chromatographic system, an acidified water/tetrahydrofuran mixture with an octadecylsilyl-silica as stationary phase, performed well for spiked samples, but in real samples a group of peaks occurred at the site of the ambroxol peak. The interfering peaks were later attributed to metabolites. Changing the organic modifier to methanol or acetonitrile resulted in a complete loss of separation between ambroxol and its metabolites. The concentrations of these metabolites equaled or exceeded the ambroxol concentrations in several subjects, leading to inaccuracies in the measurement of ambroxol concentrations with such chromatographic systems.

The combination of cyanopropyl-silica as stationary phase with a ternary mobile phase containing ion-pairing reagent gave complete separation of ambroxol from its metabolites. The metabolites were only partially resolved, NA 873 is eluted just before DBAS (**Figure 2**). Variations in amount or kind of the organic modifiers as well as of the ion-pairing reagent improved the resolution between the metabolites and impaired the separation from ambroxol.

3.2 Evaluation of the Extraction Method

In contradiction to [4] and verification of [1] and [2], ambroxol can be quantitatively extracted from alkaline plasma by ether, and back extracted into diluted acid. This

Table 1a

Results of the ambroxol calibrations after 1/x weighted regression.

Cal-No.	Concentration spiked in ng/ml					
	5.0	10.0	25.0	50.0	100	250
	Concentration found in ng/ml					
1	5.18	9.10	26.4	50.5	98.7	250
2	4.85	10.2	–	50.6	99.9	250
3	4.77	9.70	26.6	52.2	96.9	250
4	4.80	9.97	26.6	49.5	98.6	251
5	5.08	9.81	25.6	49.5	98.2	252
6	5.10	9.88	25.5	49.2	98.1	252
N	6	6	5	6	6	6
MEAN	4.96	9.78	26.1	50.2	98.4	251
SDEV	0.18	0.38	0.56	1.13	0.97	1.10
Accuracy	−0.8%	−2.2%	4.58%	0.49%	−1.6%	0.27%
Precision	3.6%	3.8%	2.2%	2.2%	1.0%	0.4%
Mean Slope:		319 ± 11				
Mean intercept:		−138 ± 117		(−0.3 ng/ml ± 0.3 ng/ml)		
Mean R^2:	0.9995 ± 0.0002					

Table 1b

Results of the ambroxol quality controls after 1/x weighted regression.

Cal-No.	Concentration spiked in ng/ml		
	5.0	50.0	250
	Concentration found in ng/ml		
1	4.93	51.5	242
2	5.56	46.7	242
3	4.85	49.4	245
4	3.85	47.5	240
5	4.22	48.4	248
6	4.33	47.9	227
N	6	6	6
MEAN	4.62	48.6	241
SDEV	0.61	1.71	7.29
Accuracy	−7.5%	−2.9%	−3.8%
Precision	13.2%	3.53%	3.03%

Table 2

Results of the recovery from plasma.

Spiked concentration in ng/ml	Recovery from plasma in %	Number of determinations
5.0	104	3
10.0	99	3
25.0	98	3
50.0	101	3
100	99	3
250	100	3

procedure is also valid for both metabolites. The back-extraction procedure gave clean, interference-free extracts. Special care was necessary to exclude traces of formaldehyde and other carbonyl-active compounds. Ambroxol reacts instantly via condensation and cyclization to produce tetrahydroquinazolines. Methanol, which produces formaldehyde on exposure to air, was particularly troublesome. Traces of acetaldehyde in acetonitrile reacted somewhat more slowly. While the presence of formaldehyde during work-up resulted in formation of NA 873, contact in mobile phase during chromatography had no effect. Ambroxol spiked in fresh plasma at 37°C showed no degradation within 1 hour, and in frozen plasma it is stable for at least 2 months.

3.3 Method Validation

The method was validated in terms of sensitivity, linearity, inaccuracy, imprecision, recovery, and selectivity. For this purpose pooled plasma from a local blood-bank was spiked with ambroxol in concentrations from a lower limit of quantification (LLQ) of 5.0 ng/ml to an upper limit of quantification (ULQ) of 250 ng/ml. On six consecutive days a calibration curve was extracted together with a control blank and three freshly prepared quality controls (QC's) at 5.0, 50.0, and 250 ng/ml.

Table 1a shows the results of six calibration sequences with spiked ambroxol concentrations from 5 ng/ml to 250 ng/ml. The peak height ratios were processed by 1/x weighting; from the weighted results the slope and intercept of the calibration curve was determined.

Table 1b shows the results for the quality controls. The inaccuracy for calibration samples at the LLQ was −0.8% with an imprecision of 3.55% and decreased to 0.27% with an imprecision of 0.44%. The QC's at the LLQ had an inaccuracy of −7.5% with an imprecision of 13.2%, which decreased at the ULQ to −3.8% with an imprecision of 3.03%. The calibration curves were linear with a mean $R^2 > 0.9998$, constant slopes, and an intercept near zero.

The recovery was checked between both limits of quantification; recovery from plasma is almost quantitative. **Table 2** presents the results.

The specificity of the assay was tested with nicotine, caffeine, salicylic acid, and bromhexin. Except for bromhexin, none interfered with the assay. Bromhexin is eluted after the internal standard with a capacity factor of about 13.

3.4 Application to Clinical Studies

With this improved method, more than 1000 samples from several different studies with different formulations have been measured. Figure 2 shows typical chromatograms of real samples. Ambroxol eluted between 6.0 and 6.2 minutes, and the internal standard between 9.0 and 9.2

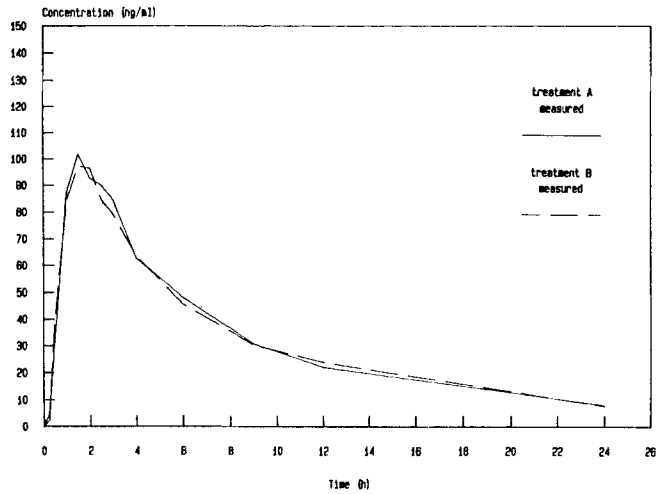


Figure 3

Plasma levels of ambroxol (mean over 12 subjects) after single dose of 60 mg as drops.

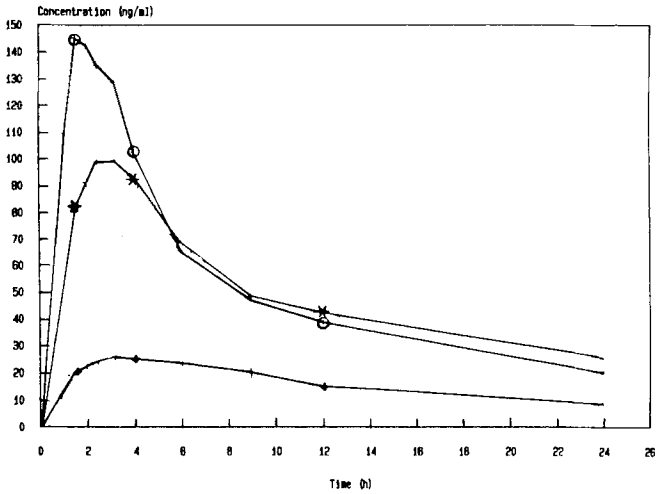


Figure 4

Plasma levels of ambroxol, NA 873, and DBAS for one subject after single dose of 60 mg as drops. ○ ambroxol; * NA 873; + DBAS.

Table 3

Summary of mean plasma concentrations and pharmacokinetic parameters after single dose of 60 mg ambroxol in two drop formulations.

Ambroxol (free base) in ng/ml				
Sampling time (h)	Treatment A mean	SD	Treatment B mean	SD
0	0	0	0	0
0.25	4.78	8.55	2.44	4.21
0.5	32.18	22.02	39.89	28.63
1	87.57	50.35	84.38	29.81
1.5	101.78	47.06	97.36	40.51
2	96.67	36.22	96.49	39.25
2.5	90.03	34.97	84.61	30.16
3	84.44	28.82	79.11	33.15
4	62.64	22.16	63.41	20.65
6	48.1	15.08	45.65	16.37
9	30.92	11.49	30.5	10.67
12	22.17	9.72	24.06	11.23
24	8.04	6.46	7.74	6.08
AUC-1 (hxng/ml)	764.53	340.39	760.24	336.58
AUC-3 (hxng/ml)	914.58	374.08	956.89	357.58
Cmax (m) (ng/ml)	106.11	47.28	99.81	40.22
Tmax (m) (h)	1.68	0.51	1.64	0.45
t 1/2 (h)	6.37	1.38	6.92	0.91

mean = mean value SD = standard deviation.

minutes. Metabolite NA 873 eluted at 5.2 minutes, followed by DBAS at 5.4 minutes. In the chromatograms presented, both metabolites reach concentrations comparable to ambroxol. **Figure 3** shows the mean plasma ambroxol concentration/time profile over 12 subjects, which were dosed with 60 mg ambroxol hydrochloride in two commercial drop formulations. **Table 3** summarizes the pharmacokinetic parameters with mean values and standard deviations. **Figure 4** presents a typical plasma concentration/time profile of ambroxol, NA 873, and DBAS for one subject.

A general observation over the studies was that liquid formulations like drops or syrup led to NA 873 concentrations similar to ambroxol, whereas after administration of tablets the metabolite reached about half of the ambroxol concentrations. The mean maximal concentrations of ambroxol in plasma with the present method were lower than those previously reported [4] for the respective formulations, indicating the selectivity of our method as well as selectivity problems of others.

4 Conclusion

This work describes for the first time a rapid, selective, and sensitive HPLC method for quantification of ambroxol in human plasma. An optimized combination of polar stationary phase and ternary mobile phase containing ion-pairing reagent is used to separate the analyte from the metabolites NA 873 and DBAS. UV detection at 230 nm allows a LLQ of 5.0 ng/ml, which is adequate to monitor the low plasma concentrations after single doses of several formulations. The method was applied to samples from pharmacokinetic studies, with excellent results. The metabolite concentrations are comparable to ambroxol and seriously influence the results, unless selective methods are used.

Acknowledgment

The skillful and careful work of *Mr. R. Otto* during method development and routine measurement is gratefully acknowledged.

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Ms received: June 11, 1986
Accepted by WJ: July 14, 1986