Journal of Chromatography, 163 (1979) 373—382
Biomedical Applications

CHROMBIO. 356

EVALUATION OF A MODIFIED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR ACEBUTOLOL AND ITS MAJOR METABOLITE

THEODOR W. GUENTERT, GUILLAUME M. WIENTJES, ROBERT A. UPTON, DANIEL L. COMBS and SIDNEY RIEGELMAN*

School of Pharmacy, University of California, Medical Center, San Francisco, Calif. 94143 (U.S.A.)

(Received March 12th, 1979)

SUMMARY

Extensive modification of an existing high-performance liquid chromatography assay for acebutolol and its major metabolite has markedly improved chromatographic stability eliminating the previous need for frequent adjustment of the eluent composition to accommodate continuous loss of column retention. The eluents now used and avoidance of the requirement for elevated column temperature may be significant factors in the ability to maintain column life over 8 months of continuous use with little decrease in retention As a result of the improved chromatographic stability full advantage can now be taken of automatic injection devices for the unattended processing of large numbers of samples. A significant modification of the work-up of blood samples has improved precision of the assay in whole blood. Nevertheless, it is recommended that plasma samples rather than whole blood be analyzed, since the plasma assay is faster and still more precise.

INTRODUCTION

Acebutolol (Fig. 1) is a new beta-adrenergic receptor antagonist [1, 2]. In contrast to propranolol, its cardioselectivity allows treatment of cardiac arrhythmias in asthmatics [3]. A major metabolite of the drug, (±)-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylaminopropane (metabolite I; Fig. 1), has been isolated and identified [4]. This metabolite may exceed the plasma concentrations of the parent drug several-fold, but its contribution to the antiarrhythmic effect of acebutolol in man is unclear. Several specific assays which allow a comparison of activity of acebutolol with other β-blockers and a test for possible contribution of metabolites to the pharmacodynamic response have been developed. Meffin et al. [4] reported on a gas chromatographic procedure suitable for simultaneous but separate quantitation of the

*To whom correspondence should be addressed.

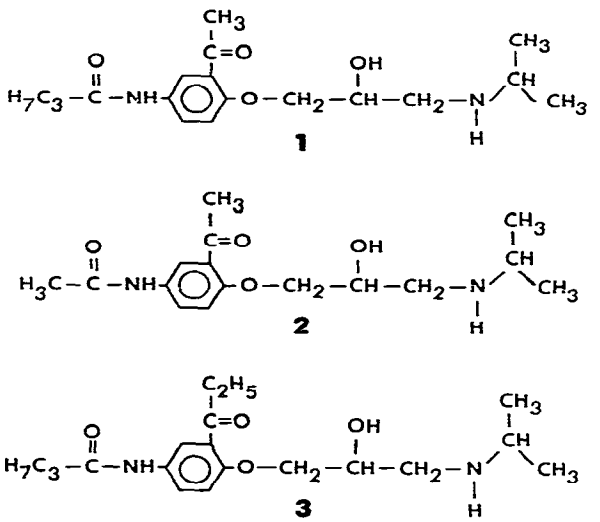

Fig. 1. Molecular structures of (±)-1-(2-acetyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane (acebutolol; 1), (±)-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylaminopropane (metabolite I; 2) and (±)-1-(2-propionyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane (internal standard; 3).

drug and its major metabolite. The sample work-up procedure in this assay is, however, tedious. Three extraction and two derivatization steps are necessary to provide a sample suitable for injection. The thin-layer chromatographic procedure described by Steyn [5] uses quinidine, another antiarrhythmic drug, as internal standard and will give inaccurate results in patients where this drug is co-administered. The high-performance liquid chromatographic (HPLC) assay reported by Meffin et al. [6] uses an ion-pair reversed-phase system at slightly elevated column temperature (30°). When applying this method we noted periodic changes in retention times of the compounds necessitating frequent readjustment of the solvent composition. Rapid deterioration and a short life of the column were observed. This seemed to be due to the ion-pairing reagent used (sodium dodecyl sulfate) and its high surfactant activity. Changing the counter-ion in the system resulted in insufficient retention and/or resolution of the drug, its metabolite and the internal standard used. With the possibility of processing large numbers of samples with the aid of an automatic injector we have developed a reversed-phase chromatographic system not using ion-pairs.

EXPERIMENTAL

Materials

Acetonitrile and methanol were obtained from Fisher (Pittsburgh, Pa., U.S.A.) (HPLC grade) and ethyl acetate from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Sodium hydroxide pellets (USP), phosphoric acid (N.F. 85% wt/wt), sulfuric acid (95—98%) and potassium phosphate monobasic, all of analytical reagent grade, were supplied by Mallinckrodt (St. Louis Mo., U.S.A.)

Water was demineralized, distilled in glass and filtered before use. The reference substances, (±)-1-(2-acetyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane (acebutolol), (±)-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylaminopropane (metabolite I) and (±)-1-(2-propionyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane (internal standard) (Fig. 1) were kindly supplied by May & Baker (Dagenham, Great Britain).

Instrumentation and chromatographic conditions

A Varian (Palo Alto, Calif., U.S.A.) Model 8500 high-performance liquid chromatograph was used equipped with a Varichrom (Varian) UV detector set at λ = 240 nm. The Altex (Berkeley, Calif., U.S.A.) Spherisorb ODS 5-μm HPLC column (25 cm × 3.9 mm I.D.) was eluted with acetonitrile—0.1 *M* phosphate buffer (pH 3.3)—water (55:6:39) at a flow-rate of 60 ml/h.

Injections were made with a Varian automated injector (Model 8000) through a Valco sweepflow injector valve CV-6-UHPa-N60 with a 50-μl loop. A dual pen recorder (Linear, Irvine, Calif., U.S.A.) enabled an eighty-fold range to be covered.

Assay procedure

Plasma. A 1.0-ml volume of methanolic internal standard solution containing 2.0 μg/ml of the internal standard is measured into a test-tube (18 × 150 mm with PTFE-lined screw cap) and evaporated to dryness at 35° under a stream of nitrogen. A 1.0-ml quantity of the plasma to be assayed is added and vortexed for a few seconds. After addition of 1.0 ml of distilled water and 200 μl of 2 *N* aqueous sodium hydroxide solution, the sample is vortexed for 90 sec with 10 ml of ethyl acetate. After centrifugation for 5 min at 600—1200 *g* the organic phase is transferred with a disposable pipette into an evaporation tube*, 150 μl of 0.01 *N* aqueous sulfuric acid added and the mixture vortexed for 90 sec. The tube is then placed into a dry-ice—acetone bath for 1 min to cause the aqueous component to separate and is then centrifuged for 3 min. The aqueous phase (150—250 μl) is sampled with a syringe through the ethyl acetate layer, transferred into vials and injected onto the column with an automatic sampler.

Blood. A 1.0-ml volume of the blood to be assayed is spiked in a disposable test-tube with 2.0 μg of internal standard as described in the plasma assay above. To precipitate proteins 2 ml of acetonitrile are added. After vortexing the sample for 1 min and centrifugation for 3—5 min at 600—1200 *g* the supernatant is decanted into another test-tube (18 × 150 mm with PTFE-lined screw cap), the acetonitrile evaporated at 35° under a stream of nitrogen and the sample further processed as described for plasma samples.

Calibration and interpretation of chromatograms

Quantitation of acebutolol and its metabolite was achieved on the basis of a standard curve. To accommodate small variations in the chromatographic system which may occur from one day to another, standard curves were prepared daily by spiking 1.0 ml of blank, drug-free plasma or blood with 2.0

*Centrifuge tube with a glass capillary tube (capacity ≈ 150 μl) fused to the base.

μg internal standard and 0, 50, 100, 200, 500, 1000, 2000 or 3000 ng of both acebutolol and metabolite I and assaying them together with the patients' samples. An unweighted least squares regression relationship between concentration and the peak height ratio of each compound to the internal standard, was calculated.

RESULTS AND DISCUSSION

Using the conditions described, the chromatographic system could be maintained for months provided the column was washed periodically with acetonitrile—water or pure acetonitrile and column frits were replaced as soon as significant increase in back-pressure was observed. Adjustment of neither the solvent composition nor the flow-rate was necessary, nor did the column show any sign of deterioration in spite of the large sample volumes injected. After an eight-month period of use of the column the retention times of acebutolol, metabolite I and internal standard had decreased slightly but the column was still usable.

Typical chromatograms from patient samples are shown in Figs. 2 and 3 for plasma and blood, respectively. Retention times for acebutolol, metabolite I

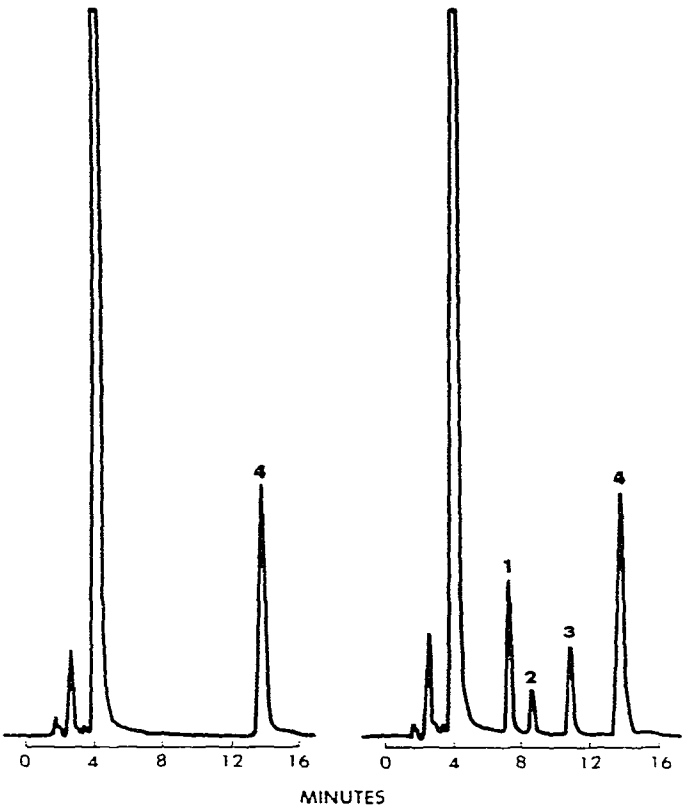

Fig. 2. Chromatograms of plasma extract prior to (left) and after (right) administration of acebutolol. Peaks: 1, metabolite I; 2, unknown substance; 3, acebutolol; 4, internal standard.

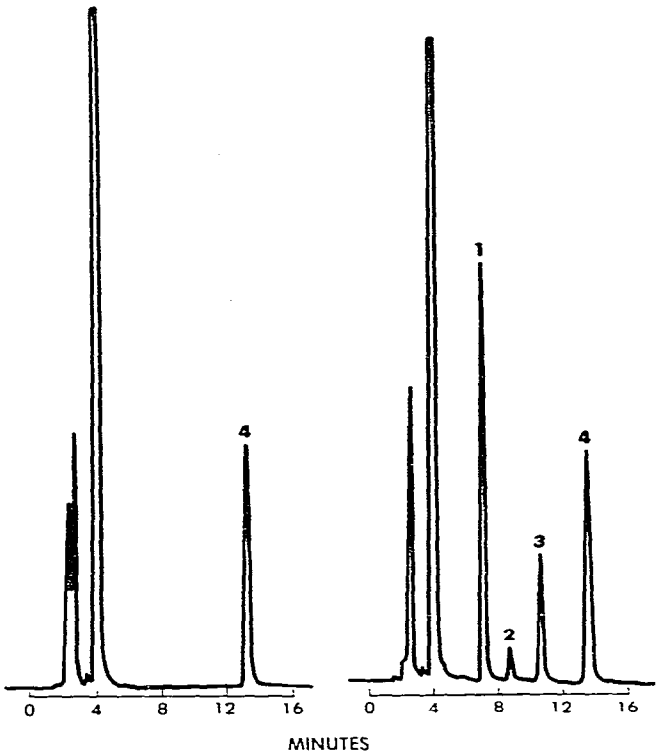

Fig. 3. Chromatograms of blood extract prior to (left) and after (right) administration of acebutolol. Peaks: 1, metabolite I; 2, unknown substance; 3, acebutolol; 4, internal standard.

and internal standard are 10.6, 7.0, 13.4 min, respectively. As can be seen, neither plasma nor blood obtained from the subject prior to administration of the drug gave rise to interfering peaks. Quinidine and oxprenolol, also antiarrhythmic drugs, did not interfere with the assay. Although propranolol did not itself interfere, one of its metabolites (N-desisopropyl-propranolol) had the same retention time as acebutolol. In some patients taking acebutolol, a small unidentified peak with a retention time of 8.7 min could be found (cf. Figs. 2 and 3). Since these patients took no other drugs and since the additional peak was not present in the sample taken prior to administration of acebutolol, it is likely that the additional substance found in our assay is a previously unknown minor metabolite. It could be quantitated with no changes in the procedure if a reference sample were available.

The assay procedures include extraction of the drug and its major metabolite from the biological sample into ethyl acetate and back-extraction into sulfuric acid. The acid is then injected directly onto the column. Since the column material is the most variable factor in a chromatographic system and may deteriorate under strongly acidic conditions, we investigated the minimum acid strength required to reproducibly extract the bases from the organic phase. It was shown that 0.01 *N* sulfuric acid was sufficient for extraction of the basic compounds and no increased peak height could be obtained by extracting identical samples with higher acidic strengths. Addition of 150 µl of acid

yielded, after chilling, sufficient aqueous phase for two injections with the automatic injector.

Since with automatic sampling the drug and its metabolite were kept in the sulfuric acid for up to 12 h prior to chromatography, the stability of the compounds in 0.01 *N* sulfuric acid was tested. In 0.01 *N* and even in 0.1 *N* sulfuric acid no degradation of either compound could be detected even after 24 h.

Using the same sample work-up procedure for both plasma and whole blood as suggested by Meffin et al. [6], there was distinct nonlinearity in the standard curves obtained for blood. We therefore altered the sample work-up for blood to precipitate proteins by addition of acetonitrile. After centrifugation of the precipitated proteins acetonitrile had to be evaporated from the supernatant to ensure complete extraction of the drug from the aqueous phase with ethyl acetate.

The sensitivity of the method described is dependent not only on the sample size taken for assay but also on the injection mode, since the automatic injector used requires excess sample to rinse away residue from the preceding sample. Taking 1 ml of biological sample and using the automated injector device the limit is about 50 ng/ml. Increasing the sample size to 2 ml, using only 50 μl of sulfuric acid for back-extraction of the bases from the ethyl acetate and injecting the sample manually with a Hamilton syringe onto the column, a sensitivity of 10 ng/ml can easily be achieved. The variability of the assay over the whole concentration range (50—3000 ng/ml) as reflected in the coefficient of variation for normalized peak height ratios in typical standard curves was 4.6% and 6.0% for acebutolol and metabolite, respectively, in plasma and 6.1% and 9.7% for these compounds in the blood.

Results from precision studies in patient samples with the plasma assay are shown in Tables I and II. Assaying the same plasma sample six times on one day, a coefficient of variation of 0.9% and 2.3% for acebutolol and metabolite determinations respectively was found. Repeating the determination of five plasma samples on different days yielded coefficients of variation from 2.3% to 6.8% (mean: 4.3%) for acebutolol and from 2.1% to 5.5% (mean: 3.8%) for the metabolite. Tables III and IV show the corresponding precision values for the blood assay. The slightly higher coefficients of variation for the blood

TABLE I

INTRADAY PRECISION OF PLASMA ASSAY (PATIENT SAMPLE)

Replicate No.	Concentration (ng/ml)	
	Acebutolol	Metabolite I
1	1425	3023
2	1389	2862
3	1400	2875
4	1389	2887
5	1397	2875
6	1397	2975
Mean	1400	2916
C.V.	0.9%	2.3%

TABLE II

INTERDAY PRECISION OF PLASMA ASSAY (PATIENT SAMPLES)

Subject	Concentration of acebutolol/metabolite (ng/ml)				C.V. (%)	
	1	2	3	4	Acebutolol	Metabolite
A	319/937	312/917	300/898		3.1	2.1
B	439/1696	440/1744	388/1587	394/1672	6.8	3.9
C	779/1908	853/2057	783/1883	764/1807	5.0	5.5
D	273/523	294/554	286/508	267/528	4.4	3.6
E	1147/2782	1130/2702	1153/2626	1193/2872	2.3	3.8
				Mean	4.3	3.8

assay, both within-day (4.0%, 4.5%) and between-day (7.1%, 5.4%), are probably due to the additional sample clean-up step necessary for blood. An additional possible source for the increased variability is the pipetting of the aliquot of sample (increased viscosity of blood) taken for assay.

The assay procedure was checked not only for precision but also for a consistent trend (bias) by evaluation of replicate determinations of plasma or blood samples spiked with a quantity of compound unknown to the analyst. As can be seen from Table V the coefficient of variation in repeated measurements in the low and high range of parent drug and metabolite concentrations was less than 4% and there appears to be no significant bias introduced in the assay. Precision and bias in spiked blood samples is shown in Table VI. Although the variability is higher than for the plasma assay the accuracy of the analytical procedure is still very acceptable.

To determine whether there are differences in plasma and whole blood concentrations of acebutolol and metabolite, whole blood samples and the plasma derived from them were both assayed. As can be seen from Table VII the blood: plasma ratio for acebutolol was very close to 1.0 for all the seven samples tested while metabolite levels determined in blood tended to be higher than in plasma (mean: +11%). This might be due to binding of the metabolite to red

TABLE III

INTRADAY PRECISION OF BLOOD ASSAY (PATIENT SAMPLE)

Replicate No.	Concentration (ng/ml)	
	Acebutolol	Metabolite I
1	517	1188
2	568	1333
3	525	1235
4	533	1242
5	529	1268
6	564	1341
Mean	539	1268
C.V.	4.0%	4.5%

TABLE IV

INTERDAY PRECISION OF BLOOD ASSAY (PATIENT SAMPLES)

Subject	Concentration of acebutolol/metabolite (ng/ml)						C.V. (%)	
	1	2	3	4	5	6	Acebutolol	Metabolite
F	(0)* /377	(28)/380	(26)/369	(5)/410			—	4.7
G	181/682	185/698	210/678	186/632	185/641	152/700	10.1	4.3
H	283/881	279/852	244/750	281/777	262/893		6.2	7.7
I	778/1215	721/1185	812/1274				6.0	3.7
K	1606/2959	1551/2975	1430/2671	1419/2615			6.1	6.7
						Mean	7.1	5.4

*Figures in parentheses denote estimates below assay sensitivity.

TABLE V

PRECISION AND BIAS IN PLASMA ASSAY (SPIKED SAMPLES)

Replicate No.	Measured concentration of acebutolol/metabolite (ng/ml)	
	Sample 1	Sample 2
1	284/276	1735/1720
2	269/290	1730/1689
3	268/284	1716/1695
4	263/277	1699/1696
5	280/279	1746/1718
Mean	273/281	1725/1704
C.V.	3.2%/2.1%	1.0%/0.84%
Added amount (ng) per ml	290/290	1690/1693
Bias	−5.8%/−3.1%	+2.1%/+0.7%

TABLE VI

PRECISION AND BIAS IN BLOOD ASSAY (SPIKED SAMPLES)

Replicate No.	Measured concentration of acebutolol/metabolite (ng/ml)	
	Sample 1	Sample 2
1	469/103	1681/341
2	488/101	1657/344
3	457/108	1646/323
4	517/117	1651/343
5	511/ 95	
Mean	488/105	1659/338
C.V.	5.3%/7.9%	0.9%/2.9%
Added amount (ng) per ml	507/102	1775/356
Bias	−3.7%/+2.7%	−6.5/−5.1%

TABLE VII

COMPARISON OF BLOOD AND PLASMA LEVELS OBTAINED IN THE SAME SAMPLE

Subject No.	Concentration of acebutolol/metabolite (ng/ml)		Blood:Plasma ratio for acebutolol/metabolite
	in blood	in plasma	
1	698/1223	706/1042	0.99/1.17
2	1859/1230	1836/1006	1.01/1.22
3	1100/1798	1067/1817	1.03/0.99
4	515/684	489/620	1.05/1.10
5	546/1285	517/1288	1.05/1.0
6	171/402	168/312	1.02/1.29
7	2218/3526	2295/3650	0.97/0.97
Mean			1.02/1.11

blood cells. The significance of such a finding is, however, unclear since there are no definitive studies correlating plasma or blood levels of acebutolol and metabolite to the pharmacodynamic response. Since the plasma assay, however, is faster and more accurate, analysis of plasma, where possible, appears to be preferable to determinations of whole blood.

ACKNOWLEDGEMENT

T.W. Guentert is grateful for support received from the Swiss National Science Foundation.

REFERENCES

1 B.S. Lewis, A.S. Mitha and M.S. Gotsman, S. Afr. Med. J., 48 (1974) 821.
2 A.H. Gradman, R.A. Winkle, J.W. Fitzgerald, P.J. Meffin, J. Stoner, R.A. Bell and D.C. Harrison, Circulation, 55 (1977) 785.
3 W.P. Leary, A.J. Coleman and A.C. Asmal, S. Afr. Med. J., 47 (1973) 1245.
4 P.J. Meffin, S.R. Harapat and D.C. Harrison, Res. Commun. Chem. Pathol. Pharmacol., 15 (1976) 31.
5 J.M. Steyn, J. Chromatogr., 120 (1976) 465.
6 P.J. Meffin, S.R. Harapat, Y.G. Yee and D.C. Harrison, J. Chromatogr., 138 (1977) 183.

Journal of Chromatography, 163 (1979) 383—389
Biomedical Applications

CHROMBIO. 357

TRACE ANALYSIS OF THE MIF* ANALOGUE PAREPTIDE IN BLOOD PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SHORT-WAVELENGTH EXCITATION FLUOROMETRY

G.J. KROL**, J.M. BANOVSKY, C.A. MANNAN, R.E. PICKERING and B.T. KHO

Ayerst Laboratories Inc., Rouses Point, N.Y. 12979 (U.S.A.)

(First received March 28th, 1978; revised manuscript received April 20th, 1979)

SUMMARY

A high-performance liquid chromatographic procedure was developed and applied to analysis of the pharmacologically active MIF* analogue pareptide in human plasma. The procedure involves formation of a fluorescent 7-chloro-4-nitrobenzyl-2-oxa-1,3-diazole (NBD-Cl) pareptide derivative followed by separation of the NBD derivative from plasma components on a 30-cm microparticle octadecylsilane bonded column. The separated derivative was quantitated using a short-wavelength excitation fluorometric detector. The detection limit of pareptide in plasma samples was 5 ng or 17 pmoles per ml of plasma. In the absence of plasma, the corresponding on-column detection limit was 0.5 pmoles.

INTRODUCTION

The analysis of a pharmacologically active peptide compound in body tissues presents a challenge to the analytical chemist; the relatively low concentrations involved and the presence of other peptides, proteins and amino acids complicate the problem. Furthermore, the peptide of interest in this study has no natural fluorescence or strong UV absorption and the only readily derivatized functional group is the secondary amine of the proline moiety. The peptide investigated was L-prolyl-N-methyl-D-leucyl-glycinamide, a synthetic analogue of MIF* [1—5]. The USAN*** name of this MIF analogue tripeptide is pareptide.

In view of the above complications, high-performance liquid chromatography(HPLC) of the fluorescent pareptide derivative was investigated. The reagent used for this purpose was 7-chloro-4-nitrobenzyl-2-oxa-1,3-oxadiazole (NBD-Cl) [6], which reacts with the prolyl secondary amino group. The

*MIF = melanocyte stimulating hormone release-inhibiting factor.
**Present address: Miles Laboratories, West Haven, Conn. 06516, U.S.A.
***USAN = United States Adopted Name.