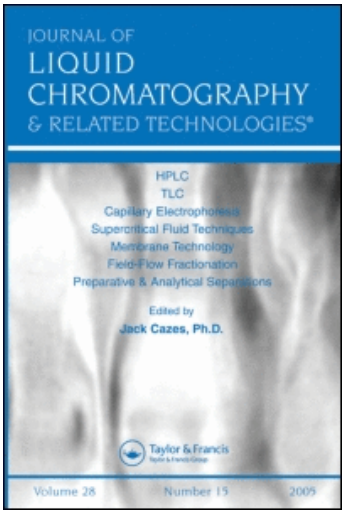

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A Validated High-Performance Liquid Chromatographic Method for the Determination of Acebutolol and Diacetolol in Human Plasma

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A VALIDATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ACEBUTOLOL AND DIACETOLOL IN HUMAN PLASMA

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ABSTRACT

A validated high-performance liquid chromatographic (HPLC) procedure employing ultra-violet (UV) detection for the analysis of acebutolol (Monitan) and its major metabolite, diacetolol, in human plasma in reported. The method is rapid and coupled with standard HPLC procedures leads to a sensitive, accurate, and reproducible assay. The retention times of diacetolol, acebutolol and internal standard, celiprolol, were 4.1, 6.7 and 9.7 minutes, respectively. The peak height *versus* plasma concentration is linear over the range of 20.0 to 1000 ng/mL for each analyte, with a detection limit of 10.0 ng/mL. The mean absolute recovery of acebutolol and diacetolol using the described assay is 74.6 and 88.8%, respectively. The inter- and intra-day accuracy and precision are within 14.1% of the actual values for all concentrations investigated. Furthermore, this procedure is applied to assess the pharmacokinetics of a single 200 mg oral dose of Monitan.

INTRODUCTION

Acebutolol, N-[3-acetyl-4-[2-hydroxy-3-[(1-methylethyl)amino]proproxy]-phenyl]-butamine, is a cardioselective β_1-adrenoreceptor blocking agent agent that

is used alone and in conjunction with other drug therapy in the treatment of hypertension. It is also used in the treatment of angina pectoris and ventricular arrhythmias [1]. Due to its cardioselective action, acebutolol HCl is often prescribed to individuals with concomitant bronchospastic disease [2].

Acebutolol HCl is well absorbed from the gastrointestinal tract and is extensively metabolized in the hepatic system. The major metabolite, diacetolol, is pharmacologically active and equipotent to the parent drug, minimizing any loss of drug effectiveness due to the first-pass effect [3]. Few HPLC assays exist for the simultaneous determination of acebutolol and diacetolol in human plasma [4-6]. Consequently, this prompted the development of a sensitive, specific and robust analytical method, which could follow the pharmacokinetics of both analytes in human plasma.

The method reported herein for the determination of acebutolol and diacetolol is linear over the range of 20.0-1000 ng/mL in human plasma. This range was predicted on the basis of pharmacokinetic characteristics reported for other strengths of acebutolol and diacetolol [7,8]. Furthermore, this procedure was successfully applied to ascertain the pharmacokinetics of a single 200 mg oral dose of Monitan in humans.

EXPERIMENTAL

Materials

Acebutolol hydrochloride was purchased from Sigma (St. Louis, MO, USA). Diacetolol was prepared by Boz Chem Engr. (Dollard des Ormeaux, QC, Canada). Celiprolol hydrochloride was obtained from Rorer Central Research (Horsham, PA, USA). HPLC grade potassium phosphate monobasic, sodium

carbonate, sulfuric acid and phosphoric acid were purchased from Fisher Scientific (Montréal, QC, Canada). HPLC grade acetonitrile and ethyl ether were purchased from Caledon (Georgetown, ON, Canada). Glass distilled methanol was purchased from BDH (Ville St. Pierre, QC, Canada). The water was deionized Type 1, reagent grade (Millipore, Ville St. Laurent, QC, Canada). All reagents were used without further purification.

Instrumentation

The chromatographic system consisted of a Waters model 590 pump, a WISP 710B autosampler, and a Lambda Max model 481 UV detector (Waters Associates, Milford, MA, USA). A stainless-steel column (15 cm x 4.6 mm i.d.) was packed with Nucleosil C-18, particle size 5 micron (prepared in-house). The column was maintained at ambient temperature. The UV detector was set at 245 nm to monitor the analytes. The mobile phase consisted of 7.5 mM potassium phosphate monobasic, pH 3.5/acetonitrile (1:1, v/v), and was delivered at a flow rate of 1.8 mL/minute. Under these conditions, the retention times for diacetolol, acebutolol and the internal standard were 4.1, 6.7 and 9.7 minutes, respectively.

Biological Samples

Blood samples were collected from healthy male volunteers after receiving a single 200 mg oral dose of Monitan. Blood was drawn into evacuated EDTA collection tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA), cooled in an ice bath and centrifuged at **ca.** 1500 g under refrigeration (4°C) as soon as possible after collection. The plasma was stored at -20°C until analyzed.

Preparation of Standards

Stock solutions of acebutolol (free base) and diacetolol were prepared at 1.00 mg/mL in methanol. Appropriate dilutions of the stocks were made with

deionized water to prepared plasma standards of both acebutolol and diacetolol at concentrations of 20.0, 50.0, 100, 250, 500, 750 and 1000 ng/mL. Spiked plasma quality control samples (QCs) were prepared in pools of 30.0 mL at final concentrations of 30.0, 400 and 900 ng/mL for each analyte. Individual aliquots of 250 μL were stored in 16x100 mm screw cap glass culture tubes and stored at -20°C until analyzed. A stock internal standard solution of celiprolol (free base) was prepared at 1.00 mg/mL in methanol and diluted to 1.5 μg/mL with deionized water. All stock solutions were stable for at least one month when stored at -20°C.

Sample Preparation

Aliquots of plasma (250 μL) were added to 16x100 mm screw cap glass culture tubes. Plasma samples were treated with 200 μL of 1.5 μg/mL celiprolol in deionized water (working internal standard) then basified by the addition of 200 μL of 1.0 **M** sodium carbonate. The analytes were extracted with ethyl acetate (5 mL) on a reciprocating shaker (150 ± 20 oscillations/minute) for 15 minutes. After centrifugation for 10 minutes at **ca.** 1500 g, the organic layer was transferred into a clean 16x125 mm conical tube containing 400 μL of 25 **mM** sulfuric acid. The tubes were placed on a reciprocating shaker for 15 minutes (150 ± 20 oscillations/minute), centrifuged at **ca.** 1500 g for 10 minutes and the organic layer was discarded. The remaining aqueous layer was transferred into an injection vial and 100 μL was injected onto the liquid chromatograph under the previously stated conditions. The back-extracted samples were stable at room temperature for at least 24 hours.

Data Acquisition

The peak heights of acebutolol, diacetolol and the internal standard were measured with a Spectra-Physics model 4270 integrator and down-loaded to

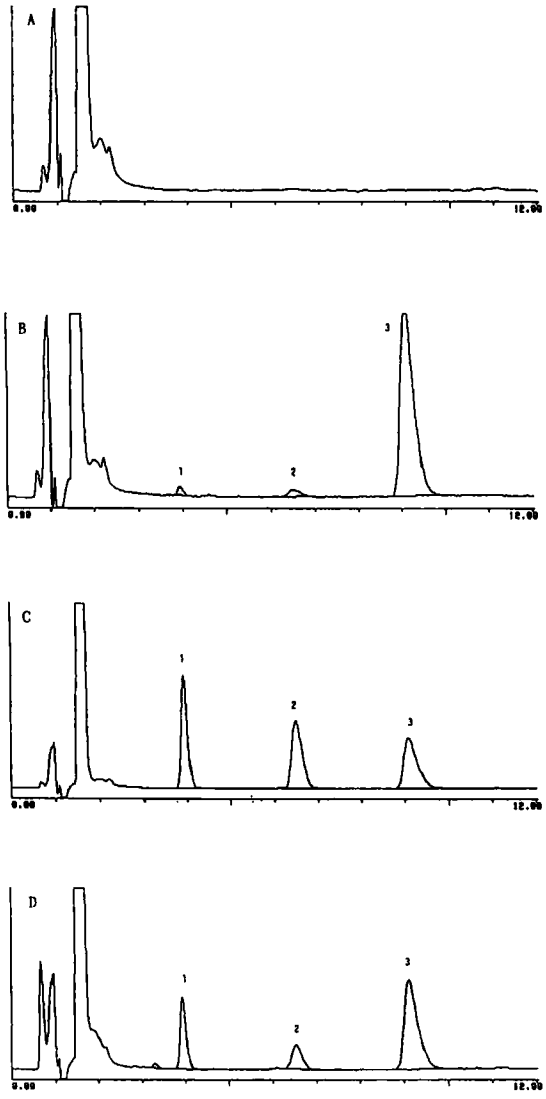

Figure 1. Samples prepared according to the described procedure (a) plasma blank, attenuation=2, (b) plasma spiked at 20.0 ng/mL for acebutolol and diacetolol, attenuation=8, (c) plasma spiked at 1000 ng/mL for acebutolol and diacetolol, attenuation=8, and (d) plasma of a subject 4.0 hr after a 200 mg oral dose of Monitan, attenuation=4. Peak identification is as follows: diacetolol (1), acebutolol (2), and internal standard (3).

Chrom-Station (Spectra-Physics Inc., Mountain View, CA, USA). The chromatographic data were automatically processed for peak height ratios for each drug and fitted to a weighted (1/C) linear regression.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms obtained from back-extracted plasma samples are illustrated in Figures 1(a-d). Figure 1(a) shows a representative chromatogram of a processed plasma blank. This chromatogram indicates that no endogenous compounds exist at the retention times of acebutolol, diacetolol and internal standard. Figure 1(b) is a chromatogram amplified to the same degree as the blank showing the limit of quantification (LOQ, 20.0 ng/mL). Figure 1(c) is a plasma sample at the upper limit (1000 ng/mL) of the calibration range. Figure 1(d) is a processed plasma sample obtained from a subject 4.0 hours after a single 200 mg oral dose of Monitan. The retention times of diacetolol, acebutolol and the internal standard were 4.1, 6.7 and 9.7 minutes, respectively. The overall chromatographic run time was 12.0 minutes.

Linearity and Quantification Limit

A linear response in the peak height ratios for both acebutolol and diacetolol to internal standard over the range of 20.0 to 1000 ng/mL was observed with a minimum signal-to-noise ratio of 4:1. The correlation coefficients were 0.9989 or better (n=5).

Recovery

The absolute recoveries of acebutolol and diacetolol were evaluated by comparing the concentrations found in plasma samples spiked with known amounts

TABLE 1

Recovery of Acebutolol, Diacetolol and Internal Standard From Human Plasma*

Drug	Conc. (ng/mL)	% Recovery	% RSD
Acebutolol	30.0	74.7	10.4
	900.0	74.5	1.9
Diacetolol	30.0	88.0	3.1
	900.0	89.6	1.3
Internal Standard	666.0	101.4	4.5

*n=6

of each analyte to the concentrations found in solution (adjusted for concentration effects in the extraction). Spiked human plasma at two concentrations; one at 1.5 times the LOQ and the other at 90% of the upper limit of the assay, in replicates of six, were extracted as described previously except the internal standard was not added. The absolute peak heights from the extracted samples were compared to unextracted standard solutions prepared in the mobile phase. Similarly, the recovery of the internal standard was determined at the final recommended concentration. These results are provided in Table 1.

Specificity

Human plasma was collected from 10 healthy donors and screened for interference at the retention times of acebutolol, diacetolol and the internal standard. No significant interference had been observed in drug free plasma samples.

Precision and Accuracy

The inter-day precision and accuracy was assessed by the repeated analyses of plasma specimens containing different concentrations of acebutolol and diacetolol (Table 2). The precision of the assay was based on the relative standard deviation (% RSD). An indication of accuracy was based on the calculation of the relative error (% RE) of the mean found concentration as compared to the actual concentration. Two samples at each QC concentration (low, medium, and high) together with a calibration curve were run as a single batch. To be regarded as a separate batch, the entire sample processing must take place in a time domain completely separate from one another. At spiked QC plasma concentrations of 30.0, 400 and 900 ng/mL for acebutolol the method yields % RSD of 12.6, 2.9 and 1.9%, respectively. The % RE obtained from the calibration curve ranged from -3.2 to 3.0% of the nominal concentrations for acebutolol. At spiked QC plasma concentrations of 30.0, 400 and 900 ng/mL for diacetolol the method yields % RSD of 5.9, 3.4 and 4.6%, respectively. The % RE obtained from the calibration curve ranged from -4.0 to 5.6% of the nominal concentrations for diacetolol.

The intra-day precision and accuracy was determined by the evaluation of a typical production run. Plasma samples spiked with acebutolol and diacetolol at concentrations of 20.0, 30.0, 400 and 900 ng/mL were evaluated. The % RSD for all samples analyzed were within 14.1% and the % RE ranged from 0.7 to 4.7% of the nominal concentrations. These results are presented in Table 3.

Application

Plasma samples were obtained prior to dosing and at 13 subsequent time points following a 200 mg oral dose of Monitan. Following collection, the samples were stored at -20°C until analyzed. All samples were analyzed by the method presented here. The plasma profiles of acebutolol and its metabolite, diacetolol, are depicted in Figure 2.

TABLE 2

(a) Inter-day Precision and Accuracy of Acebutolol in Human Plasma

Actual Conc. (ng/mL)	n	Mean Found Conc. (ng/mL)	SD	% RSD	% RE
Std 20.0	5	20.6	1.14	5.5	3.0
Std 50.0	5	49.0	2.55	5.2	-2.0
Std 100	5	98.8	3.27	3.3	-1.2
Std 250	5	242.0	4.18	1.7	-3.2
Std 500	5	512.0	17.78	3.5	2.4
Std 750	5	755.6	15.85	2.1	0.7
Std 1000	5	991.6	17.74	1.8	-0.8
QC 30.0	8	28.9	3.64	12.6	-3.7
QC 400	10	397.4	11.49	2.9	-0.6
QC 900	10	913.0	17.23	1.9	1.4

(b) Inter-day Precision and Accuracy of Diacetolol in Human Plasma

Actual Conc. (ng/mL)	n	Mean Found Conc. (ng/mL)	SD	% RSD	% RE
Std 20.0	5	19.2	1.10	5.7	-4.0
Std 50.0	5	52.8	1.50	2.8	5.6
Std 100	5	98.8	5.72	5.8	-2.2
Std 250	5	249.0	5.05	2.0	-0.4
Std 500	5	501.0	3.87	0.8	0.2
Std 750	5	754.4	8.82	1.2	0.6
Std 1000	5	995.4	6.69	0.7	-0.5
QC 30.0	8	29.2	1.72	5.9	-2.7
QC 400	10	390.7	13.40	3.4	-2.3
QC 900	10	920.4	41.93	4.6	2.3

TABLE 3

(a) Intra-day Precision and Accuracy of Acebutolol in Human Plasma

Actual Conc. (ng/mL)	n	Mean Found Conc. (ng/mL)	SD	% RSD	% RE
Std 20.0	12	20.8	2.93	14.1	4.0
QC 30.0	12	30.9	2.75	8.9	3.0
QC 400	12	418.8	11.79	2.8	4.7
QC 900	12	922.7	20.07	2.2	2.5

(b) Intra-day Precision and Accuracy of Diacetolol in Human Plasma

Actual Conc. (ng/mL)	n	Mean Found Conc. (ng/mL)	SD	% RSD	% RE
Std 20.0	12	20.9	1.16	5.6	4.5
QC 30.0	12	30.2	1.66	5.5	0.7
QC 400	12	405.5	9.11	2.2	1.4
QC 900	12	914.5	20.55	2.2	1.6

Conclusion

The described method for the analysis of acebutolol and diacetolol in human plasma is specific, sensitive, and robust. The intra- and inter-assay precision of the method was below 14.1%, while the accuracy of the method was within 5.6% even at the LOQ. Furthermore, the method is fast and requires a relatively simple sample preparation, resulting in **ca.** 90 samples being processed daily. This method has been used to monitor plasma levels in clinical trials generating over 900 samples.

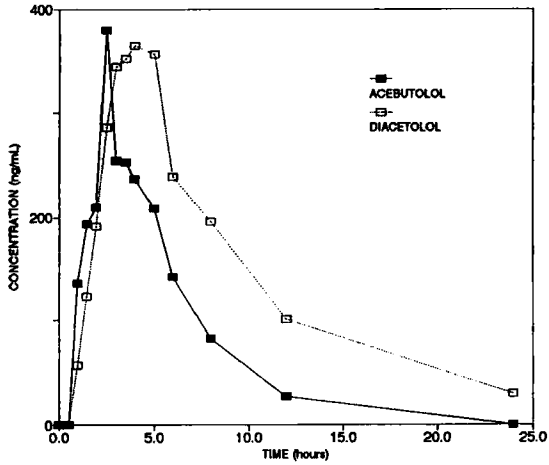

Figure 2. Representative concentration-time profile of a subject following a single 200 mg oral dose of Monitan.

More than 750 injections of the back-extracted plasma samples have been made on a single analytical column with minimal loss of chromatographic integrity.

The extractibility of acebutolol and diacetolol with various organic solvents including ethyl acetate, dichloromethane, ether, ethyl acetate/isopropanol, and hexane was investigated following alkalinization with sodium carbonate, sodium hydroxide, or ammonium hydroxide. The base and solvent of choice was sodium carbonate and ethyl acetate, with extraction yields (mean ± SD, n=4) of 77.3 ± 3.2 and 90.4 ± 2.2 for acebutolol and diacetolol, respectively. However, trace contaminants were observed at the retention time of acebutolol with all basic extractions and this prompted the use of a back-extraction with dilute acid. Various β-blockers were investigated as a potential internal standard. Most compounds eluted prior to acebutolol with the exception of propranolol, which exhibited severe tailing, and celiprolol, which proved to be adequate. Consequently, celiprolol was chosen as the internal standard.

This procedure allows the quantification of acebutolol and diacetolol in plasma for at least 24 hours following a single 200 mg oral dose of Monitan, and permits the complete characterization of the resulting plasma profile.

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