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A sensitive assay of metoprolol and its major metabolite α-hydroxy metoprolol in human plasma and determination of dextromethorphan and its metabolite dextrorphan in urine with high performance liquid chromatography and fluorometric detection[1]

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Abstract

A reverse-phase High Performance Liquid Chromatographic (HPLC) method was developed for the analysis of metoprolol in the large number of human plasma samples obtained in in vitro–in vivo correlations (IVIVC) and bioavailability studies of extended release formulations of metoprolol tartrate. The metabolite, α-hydroxy metoprolol (OH-met), could also be quantified. The analytes were extracted from the plasma using solid phase columns, separated on a C-4 analytical column followed by fluorimetric detection. The linearity, precision, accuracy, stability, selectivity and ruggedness were validated for the concentration ranges of 1–400 ng ml^{-1} for metoprolol and 0.5–200 ng ml^{-1} for OH-met. The same chromatographic conditions were slightly modified to quantify dextromethorphan and its metabolite dextrorphan in urine in the concentration range 0.052–0.05 μg ml^{-1} as a method for screening for fast metabolizers.

Keywords: Metoprolol; α-Hydroxymetoprolol; Dextromethorphan; Dextrorphan; HPLC assay; Fluorescence detection

1. Introduction

Metoprolol is a β-1 selective aryloxypropanolamine used in treatment of cardiovascular disorders such as hypertension and coronary heart disease. The drug is a lipophilic adrenoceptor antagonist (β-blocker) with a short half life (3–4 h). It undergoes extensive first pass

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metabolism with about 95% of the dose being metabolized in humans. The hydroxylated metabolite α-hydroxy metoprolol (OH-met) results from oxidation of the benzylic carbon atom of metoprolol by the cytochrome P-450 isozyme system in liver. There are marked interindividual differences in the pharmacokinetics partially due to the rate of drug oxidation by cytochrome P-450 isozymes. This metabolic variation is determined genetically [1,2].

Commonly used markers for phenotyping patients with fast or slow metabolic activity are sparteine, debrisoquine, and dextromethorphan. The 4-hydroxylated metabolite of debrisoquine and O-demethylated metabolite of dextromethorphan are mediated by the same cytochrome P-450 isozyme system. Dextromethorphan is preferred over sparteine and debrisoquine for phenotyping because of its ready availability and apparent lack of side effects [1,2,18,19].

Several methods for assaying metoprolol, with and without metabolites, in biological fluids have been published [3–17]. The utility of previously published methods is limited by laborious sample clean up procedures, lack of sensitivity, need for derivatization to improve sensitivity, inability to simultaneously measure parent and metabolite and the use of less readily available internal standards. Chromatographic methods for analyzing dextromethorphan and its metabolite dextrorphan in urine have been published [18–24] involving Gas Liquid Chromatography (GLC), High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatographic (TLC) techniques.

The HPLC method described in this communication was developed and validated for the analysis of metoprolol in a large number of human plasma samples obtained in in vitro-in vivo correlation (IVIVC) and bioavailability studies of extended release formulations of metoprolol tartrate. OH-met could also be measured in the assay. Since it was desired that only fast metabolizers be admitted into the studies a method was needed for the determination of dextromethorphan and its metabolite dextrorphan in urine. As dextrorphan was being used as an internal standard in the metoprolol assay, it was convenient to use essentially the same conditions for the measurement of dextromethorphan and its metabolite. Both HPLC methods used fluorescence detection after solid phase extraction (SPE) of the analytes from the biological matrices. The assays for metoprolol and OH-met had lower limits of quantification of 1.00 and 0.504 ng ml^{-1}, respectively, with good precision, accuracy, ruggedness and reproducibility. The assays for dextromethorphan and dextrorphan had lower limits of quantification of 0.05 $\mu g\ ml^{-1}$.

2. Experimental

2.1. Chemicals and supplies

Metoprolol tartrate, obtained from Sigma Chemical Company, St. Louis, MO was certified against USP reference compound. OH-met was a gift from Ciba-Geigy. Dextromethorphan, dextrorphan-D-tartrate and pentazocin were obtained from Research Biochemicals International, Natick, MA. Mobile phase components were of HPLC grade and other chemicals were at least certified ACS quality. C-2 solid phase extraction columns, 1 ml, were obtained from Varian (Harbor City CA).

2.2. Chromatographic system

The HPLC system consisted of a C4/E, 150 × 4.6 mm, 5 μ column (MetaChem Technology, CA) fitted with a Supelguard™ 5 μ LC-8, 2 cm cartridge guard column (Supelco, Belfont, PA). Initially a LC-4 guard column was used but it was subsequently found that the LC-8 guard column provided greater protection and longer life for the analytical column. The injection volume of 50 μl was delivered by a Perkin Elmer (Norwalk, CT) ISS 100 autosampler. The mobile phase for the metoprolol plasma assay was prepared by mixing 850 ml of 0.02 M phosphate buffer, pH 3.0, with 130 ml of acetonitrile, 20.0 ml of tetrahydrofuran and 0.24 g of 1-octane sulfonic acid. The mobile phase for dextromethorphan urine assay was prepared by mixing 825 ml of 0.05 M phosphate buffer pH 5.0, with 87.5 ml of methanol and tetrahydrofuran each. The mobile phases were

filtered through a 0.45 μm nylon filter and degassed by vacuum and sonication. The mobile phase was pumped at 1.9 and 1.6 ml min^{-1} for metoprolol plasma assay and dextromethorphan urine assays, respectively, using a Waters Model 6000 delivery system (Milford, MA). The effluent in both chromatographic systems was monitored by a Perkin Elmer LC 240 fluorescence detector with excitation and emission wavelengths of 225 and 310 nm, respectively (Response 5, Fix Factor 0.25–0.35). The output from the detector was collected using a Waters Millenium® system. Representative chromatograms showing peaks for metroprolol, OH-met and the internal standard are shown in Fig. 1 and Fig. 2.

2.3. Sample preparation

Metoprolol plasma assay: C-2 solid phase extraction columns were fitted into a Vac Elut® (Varian, Harbor City, CA) vacuum manifold. Each column was conditioned with 2 ml of acetonitrile (ACN), followed by 2 ml of water. Plasma standard, 1 ml, sample or control sample was drawn through the columns followed by 0.3 ml of internal standard solution (250 ng ml^{-1} dextrorphan). Each column was washed with 3 ml of deionized water and then by 3 ml of ACN/water (50/50, v/v). The analytes and internal standard were eluted with two 0.2 ml portions of 0.1 M HCl/ACN (50/50, v/v) and the eluent evaporated to dryness at 45°C in a water bath with a gentle flow of nitrogen (25–30 psi). Each residue was dissolved in 0.2 ml of 0.02 M phosphate buffer pH 3.0/ACN (90/10) and transferred to 0.3 ml glass inserts (SRI, NJ), which were placed in HPLC vials (SRI, NJ) and 50 μl portions were injected into the equilibrated HPLC system.

For the dextromethorphan in urine assay C-2 solid phase extraction columns were conditioned as described for the metoprolol assay and 1.0 ml of incubated urine standard, sample or control sample was drawn through the columns followed by 0.3 ml of internal standard solution (840 ng ml^{-1} pentazocin in water). Each column was washed with 3 ml of deionized water and then by 3 ml of ACN/water (50/50, v/v). The analytes and internal standard were eluted with two 0.25 ml portions of 0.1 M HCl/ACN (50/50, v/v) and the eluant was diluted with 0.5 ml of deionized water and 15 μl portions injected into the equilibrated HPLC system.

3. Validation of assay for metoprolol and OH-met

Standards of metroprolol and OH-met were prepared in human heparinized plasma (Biological Specialty, Colmar PA) at concentrations of 400, 200, 80.0, 40.0, 20.0, 8.00, 4.00, 2.00 and 1.00 ng ml^{-1} for metoprolol and 200, 100, 40.0, 20.0, 4.00, 1.00 and 0.500 ng ml^{-1} for the metabolite. The internal standard was a 250 ng ml^{-1} solution of dextrorphan-D-tartrate in deionized water. It was stored at 4°C. Three 1-day runs and related experiments were performed to assess linearity, specificity, accuracy, stability, recovery, sensitivity and precision. Each daily run, designed to simulate an assay run, consisted of processing the set of standards in the metoprolol concentration range 1.00–400 ng ml^{-1} described above, two sets of standards at concentrations of 400, 80.0, 4.00, 2.00 and 1.00 ng ml^{-1}, another set of the standards, two more sets of the 400, 80.0, 4.00, 2.00 and 1.00 ng ml^{-1} standards, another complete set of the standards followed by another set of the 400, 80.0, 4.00, 2.00 and 1.00 ng ml^{-1}. As noted above the concentrations of metabolite in the all standards were half those of metoprolol. The ratio of the peak height of the analyte peaks to internal standard was calculated. As would be done in an actual assay run the slope and intercept of peak height ratio versus concentration for the first standard line were determined by linear least squares with a weighting factor of (1/concentration^2) and the slope and intercept then used to calculate the concentrations of the other standard samples in the run. The results were used to assess linearity, accuracy, precision and the lower limit of quantification (LLOQ).

3.1. Linearity

Linearity was assessed by visual inspection of a plot of concentration versus the peak height ratio

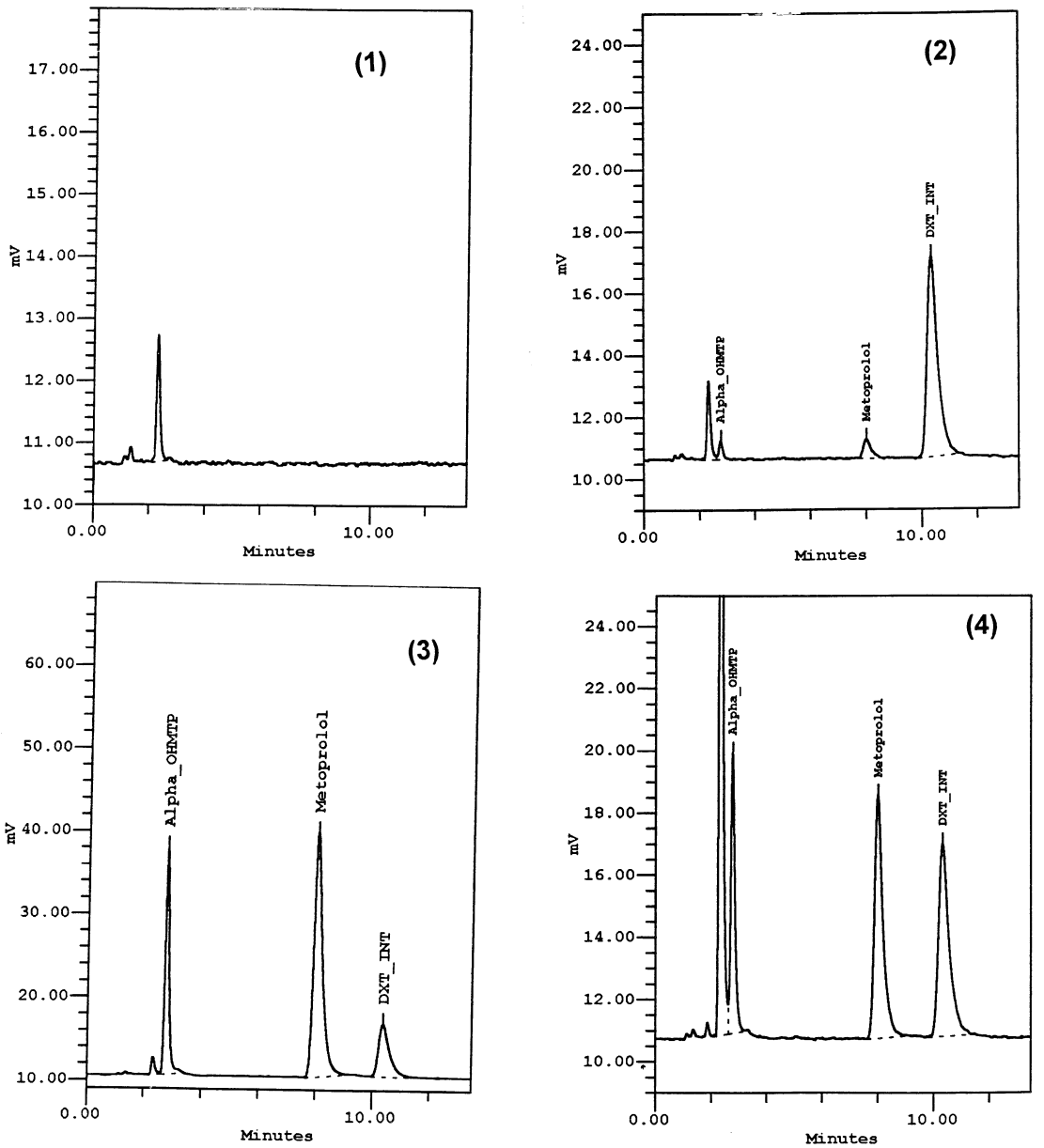

Fig. 1. Typical chromatograms of standards and unknown for the metoprolol plasma assay: (1) blank plasma; (2) blank plasma spiked with 4.00/2.01 ng ml^{-1} metoprolol/OH-met; (3) blank plasma spiked with 200/101 ng ml^{-1} metoprolol/OH-met; (4) 2 h post dose plasma sample from a subject after administration of 100 mg metoprolol tartrate.

for each run. The correlation coefficients obtained from linear least squares regression with 1/concentration^2 weighting were greater than 0.999. It is concluded from these data and the values obtained back calculated concentrations of the standards that the calibration line conforms to a

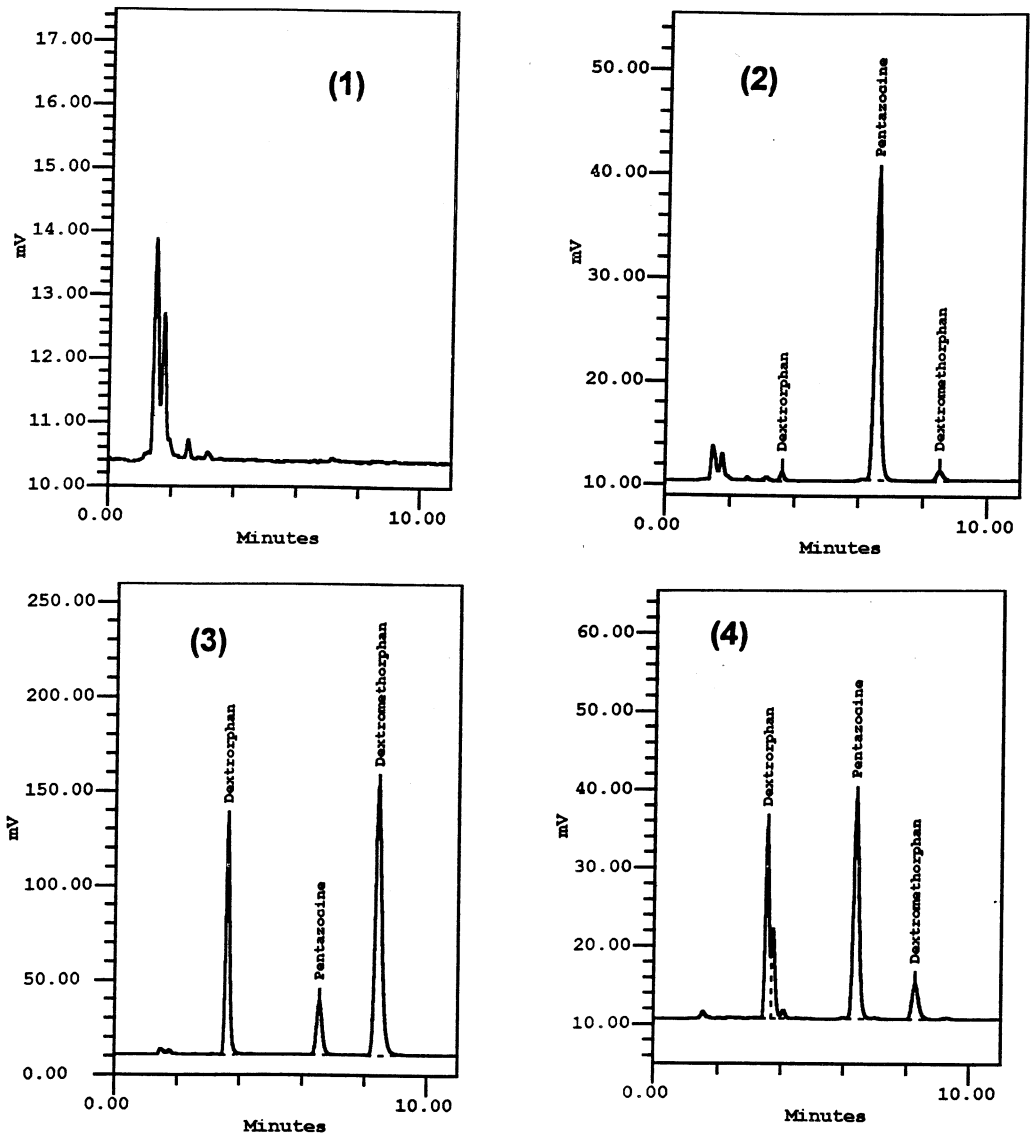

Fig. 2. Typical chromatograms of standards and unknown for the dextromethorphan urine assay: (1) blank urine; (2) a standard containing 0.1 μg ml^{-1} of dextromethorphan and 0.1 μg of dextrorphan; (3) a standard containing 15.0 μg ml^{-1} of dextromethorphan and 15.0 μg of dextrorphan; (4) a 24 h pooled urine of a subject after administration of dextrmethorphan.

linear model over the range of 1.00–400 ng ml^{-1} for metoprolol and 0.504–201 ng ml^{-1} for OH-met.

3.2. Accuracy

Accuracy is defined in this study as the ratio of the mean assayed concentration to that of the spiked concentration, expressed as a percentage. The mean accuracies for metoprolol and OH-met were calculated for the standards in each daily validation run. All these mean intrarun accuracies were in the range of 92.0–113%. When the accuracies from the individual daily validation runs were combined the overall mean accuracies, measures of

interrun accuracy, were in the range of 98.6–101%, as shown in Table 1(a).

3.3. Precision

Intrarun precision was assessed by calculating the relative standard deviations (%RSD) of the concentration values obtained in each validation run. The mean %RSD was calculated for the standards in each daily validation run. All these mean intrarun %RSDs were less than ten except for OH-met at a concentration of 0.5 μg ml^{-1}, when the RSD was 15.7%. When the RSDs from the individual daily validation runs were combined the overall mean precision, a measure of interrun precision, were in the range of 1.1–9.1% for metoprolol and 5.5–13.9% for OH-met, as shown in Table 1(a).

Table 1
Precision and accuracy of metoprolol and OH-met assay

(a) In validation					
Metoprolol, ng ml^{-1}					
Conc	400	80.0	4.00	2.00	1.00
Mean	395	79.2	3.97	2.02	0.957
RSD%	1.1	1.2	3.9	4.7	9.1
Accuracy %	98.6	99.0	99.1	101	95.7
N	24	24	23	25	21
OH-met, ng ml^{-1}					
Conc	201	40.3	2.01	1.01	0.504
Mean	208	41.4	2.08	1.01	0.481
RSD%	5.5	5.6	8.1	10.5	13.9
Accuracy%	103	103	103	100	95.4
N	24	24	23	26	18
(b) In studies					
Metoprolol, ng ml^{-1}					
Conc	300	60.0	12.0		
Mean	303	59.8	12.2		
RSD%	3.2	3.0	3.7		
Accuracy%	101	99.6	101		
N	30	31	30		
OH-met, ng ml^{-1}					
Conc	153	30.6	6.12		
Mean	159	30.0	6.27		
RSD%	6.3	6.1	6.8		
Accuracy%	104	98.0	102		
N	27	28	26		

3.4. Lower limit of quantification (LLOQ)

The lower limit of quantification is the lowest concentration that can be assayed with an accuracy of 85–115% and a relative standard deviation of no greater than 15%. The lowest standard processed in the 3 day validation meeting these requirements had a concentration of 1.00 ng ml^{-1}, accuracy 95.7% and a RSD of 9.1% for metoprolol and accuracy of 95.4% RSD of 13.9% for OH-met at a concentration of 0.504 ng ml^{-1}. The LLOQ for this assay is considered to be 1.00 and 0.504 ng ml^{-1} for metoprolol and OH-met, respectively.

3.5. Selectivity

Selectivity was assessed by processing four different blank plasma matrices in duplicate and assaying in triplicate the same plasma matrices spiked with 40.0 ng ml^{-1} of metoprolol and 20.1 ng ml^{-1} OH-met. No endogenous peaks at the retention times of metoprolol, OH-met or internal standard (dextrorphan) were observed in any of the four matrices. The mean of the assayed concentrations of the four lots of plasma was found to be between 38.4 and 39.1 ng ml^{-1} for metoprolol and 19.3 and 19.6 ng ml^{-1} for OH-met.

OH-met eluted at about 2.8 min compared with about 8 min for metoprolol itself. The internal standard eluted at about 11 min.

3.6. Stability

Three different concentrations, 400, 80.0 and 4.00 ng ml^{-1} of metoprolol and 201, 40.3 and 2.01 ng ml^{-1} of OH-met were prepared in plasma and subjected to four freeze (-25°C) and thaw cycles. They were analyzed in triplicate using freshly prepared standards. The mean percent recovery of metoprolol and OH-met calculated from all concentrations was 98.3% with 3.9% RSD and 107% with 9.7% RSD, respectively.

The stability of the extract of processed samples dissolved in mobile phase was determined by reinjecting such samples after standing for ≈ 23 h at ambient room temperature. No significant changes in the measured peak height ratios were

observed showing that the analytes are stable at room temperature in the extracted samples for at least 23 h. The mean change in peak height ratios after injection was less than 2%.

3.7. Ruggedness

Ruggedness was assessed by determining the retention times of metoprolol, OH-met and internal standard using four different analytical columns with different batches of mobile phases. No significant differences in retention times was observed. Three different lots of solid phase extraction columns showed no significant differences in results when used to assay the metoprolol in a plasma sample containing 400 ng ml^{-1} metoprolol and 201 ng ml^{-1} OH-met. The mean assayed concentrations for the 400 ng ml^{-1} metoprolol and 201 ng ml^{-1} OH-met standard assayed in triplicate for the three batches of C2 solid phase extraction column was 391 ng ml^{-1} with 1.4% RSD for metoprolol and 199 ng ml^{-1} with 2.3% RSD for OH-met.

3.8. Recovery

The absolute recovery of metoprolol, OH-met and internal standard were assessed by comparing the peak areas of extracted plasma standards to those of unextracted standards. The recoveries of metoprolol were 108% ($n = 12$) at 400 ng ml^{-1}, 110% ($n = 12$) at 80.0 ng ml^{-1} and 112% ($n = 12$) at 8.00 ng ml^{-1}. The recoveries of OH-met were 93.5% ($n = 12$) at 201 ng ml^{-1}, 94.7% ($n = 12$) at 40.3 ng ml^{-1} and 95.2% ($n = 12$) at 4.03 ng ml^{-1}. The absolute recovery of internal standard (dextrorphan) was 94.5% ($n = 10$).

4. Assay for dextromethorphan and dextrorphan

The assay for dextromethorphan and dextrorphan in urine is used as a screening assay to select fast or slow metabolizers for a study. The selection is made on the basis of the ratio of metabolite to parent drug and only subjects who are clearly fast or slow metabolizers are selected. Thus, the assay requirements for accuracy and precision are less stringent that those to obtain concentrations from which pharmcokinetics parameters are to be obtained.

4.1. Accuracy, linearity

As described in Section 2.3, sample preparation using solid phase extraction was similar to the procedure used for metoprolol plasma samples except that glucuronidase was used to hydrolyze the conjugates prior to solid phase extraction. The retention times were 3.6, 6.4 and 8.3 min for dextrorphan, pentazocine (internal standard) and dextromethorphan, respectively.

To test the method, a set of standards in the concentration range 0.052–15.2 μg ml^{-1} and sets of four control samples of concentrations of 15, 3.0, 0.10 and 0.05 μg ml^{-1} were processed in the order: standard set, three control sets, standard set, three control sets and a standard set. The peak height ratio of each analyte was divided by the peak height of the internal standard. Linear least squares with a weighting factor of $1/(\text{concentration})^2$ was used to calculated the parameters of the line of peak height ratio versus concentration for the first standard line, to simulate an actual analytical run. The slope and intercept of this line were used to calculate the concentrations of the analytes in all the other standard sets and control samples. The mean and standard deviation of all the samples in the run were used to assess accuracy and precision. The standard line was observed to be linear. The correlation coefficients were greater than 0.999. The observed mean values were within 5% of the spiked concentrations and the relative standard deviations were less than 6%. The data are summarized in Table 2(a).

4.2. Enzyme incubation

Urine was collected at hourly intervals from a subject who had been administered dextromorphan and the samples used to determine the time needed for glucuronidase hydrolysis of the metabolite conjugate. It was found that after 0, 1 and 3 h of incubation the total metabolite amount was 0.31, 5.29 and 7.27 mg, respectively. The concentration after 15 h incubation was 7.63 mg.

Table 2
Precision and accuracy of dextromethorphan and dextrorphan assay

(a) In validation				
Dextromethorphan, mg ml^{-1}				
Conc	15.5	3.09	0.103	0.052
Mean	15.0	3.14	0.098	0.052
RSD%	1.1	1.7	1.7	1.7
Accuracy %	96.7	102	95.1	100
N	7	7	7	7
Dextrorphan, mg ml^{-1}				
Conc	15.2	3.03	1.101	0.051
Mean	15.0	3.02	0.100	0.052
RSD%	2.3	1.2	2.5	5.6
Accuracy%	98.6	99.6	99.0	102
N	8	8	8	8
(b) In studies				
Dextromethorphan, mg ml^{-1}				
Conc	10.0	2.00	0.200	
Mean	10.2	2.03	0.199	
RSD%	2.3	1.1	1.0	
Accuracy%	102	102	99.5	
N	7	7	7	
Dextrorphan, mg ml^{-1}				
Conc	10.0	2.00	0.200	
Mean	9.72	2.04	0.202	
RSD%	9.1	2.7	2.8	
Accuracy%	97.2	102	101	
N	8	8	8	

It was therefore decided to incubate samples with the enzyme for at least 15 h.

4.3. Stability

Urine samples spiked with dextromethorphan and dextrorphan at concentrations of 15.0, 3.0 and 0.30 μg ml^{-1} were subjected to two freeze/thaw cycles and analyzed using freshly prepared standards. No degradation of either analyte occurred during the freeze/thaw cycles. Similar standards were allowed to stand for 24 h at ambient room temperature and reanalyzed and again no degradation of either analyte occurred. The processed sample extracts were injected into the HPLC system immediately after processing and then at 22, 42 h and 12 days after standing at ambient room temperature. The results showed no instability of the processed samples after 12 days.

4.4. Ruggedness

It was ascertained that the recovery was close to 100% from three different batches of solid phase columns and that changing the analytical column to a new one of a similar type showed no differences in retention time and peak width.

4.5. Selectivity

The chromatograms from predose urine samples from the subjects showed no peaks at the retention times of dextromethorphan, dextrorphan and pentazocin. In some of the postdose samples, but not in the standards, a peak of unknown origin eluted close to that of dextromethorphan but was sufficiently resolved to obtain an accurate peak height measurement.

5. Results and conclusions

An assay for the determination of metoprolol and OH-met in human plasma has been developed, validated and used successfully in three IVIVC and bioavailability studies in which 100 mg doses of various formulations of metoprolol tartrate were administered to normal human volunteers. A typical pharmacokinetic profile is

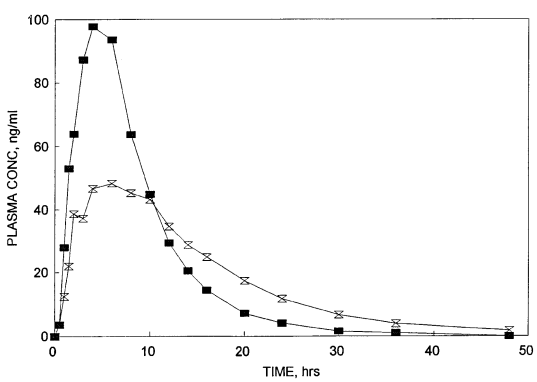

Fig. 3. Typical plasma pharmacokinetic profile from a subject after administration of 100 mg sustained release metoprolol tartrate. Squares—metoprolol; Hourglasses—OH-met.

shown in Fig. 3. In these studies quality control samples containing metoprolol at concentrations of 300, 60 and 12 ng ml^{-1} and OH-met at concentrations of 153, 30.6 and 6.12 ng ml^{-1} were processed along with the subjects' samples with the results summarized Table 1(b). The dextromethorphan assay performed equally well as can be seen from the quality control sample data summarized in Table 2(b). Accuracy and precision are of the same magnitude as those obtained during the validation runs.

The method presented here represents an improvement over several previously published methods to provide a rugged, sensitive and efficient method to analyze the metoprolol and OH-met in a large number of plasma samples. The method employs reverse phase HPLC which has become the method of choice in drug analysis. The solid phase extraction procedure used provides improved sample clean up permitting greater sensitivity to be achieved. The greater sensitivity was needed to assay the plasma concentrations expected in the extended release formulations studies. High reproducible recovery was achieved in a single step and did not require the use of large volumes of organic solvents used in liquid–liquid extractions. The natural fluorescence of metoprolol was used to attain the required sensitivity without derivatization and did not require a large volume of plasma for assay. It was also possible to use the assay procedure with slight modification for the assay of dextromethorphan and dextrorphan in urine to screen volunteers for fast and slow metabolizers.

In the actual studies the assayed performed to the specifications obtained during validation and no difficulties were encountered in the analysis of a large number of samples. Reliable pharmacokinetic profiles were obtained that were used in the IVIVC studies, the results of which are being prepared for publication.

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