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Estimation of the analgesic muscle relaxants chlorzoxazone and diazepam in human plasma by reversed-phase liquid chromatography

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SUMMARY

A rapid and sensitive method of extraction of human plasma containing acetaminophen, chlorzoxazone, oxyphenbutazone and diazepam along with their active metabolites was developed. The plasma samples were extracted by a solid-phase extraction procedure with theophylline as an internal standard for accurate quantitation. The reversed-phase liquid chromatographic method provided a linear ultraviolet detector response in the range 100.0–700.0 ng/ml. Recoveries greater than 95% were achieved from human plasma samples. Limits of detection of 100, 200, 150 and 250 ng/ml for acetaminophen, chlorzoxazone, oxyphenbutazone and diazepam, respectively, were obtained.

INTRODUCTION

Analgesic muscle relaxants, either as mixtures or as single drugs, are very helpful in the treatment of painful muscle spasms. The centrally acting skeletal muscle relaxants, such as chlorzoxazone and diazepam, have long been used in pharmaceutical dosage forms. Chlorzoxazone in combination with analgesic and antipyretic agents, such as dimethylphenyl derivative of pyrazolone (Analgin), oxyphenbutazone and acetaminophen, was found to have long-acting muscle relaxant activity [1]. Many thin-layer chromatographic and gas chromatographic methods have been reported for the estimation of chlorzoxazone in pharmaceuticals [2–4], but they have not been adopted to the analysis of biological samples. Spectrophotometric and fluorometric assays via chemical derivatization were also developed for the samples of chlorzoxazone from

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biological fluids [5,6]. Reversed-phase liquid chromatographic (RP-LC) methods for the determination of muscle relaxant analgesic mixtures [7], and chlorzoxazone and its metabolites in serum and plasma, have already been reported [8,9]. Estimation of acetaminophen and its metabolites in rat plasma [10] and of diazepam and its metabolites in serum [11,12] has been developed. The extraction procedures for these methods are often laborious, tedious and time-consuming. Moreover, no method has yet been developed for complete separation of the potent muscle relaxant chlorzoxazone in plasma in the presence of anti-inflammatory analgesic agents, such as oxyphenbutazone and acetaminophen.

This paper describes a simple RP-LC method for the determination of chlorzoxazone and its metabolite in plasma, together with acetaminophen, oxyphenbutazone and diazepam. A mixture of acetaminophen and chlorzoxazone was used, whereas oxyphenbutazone and diazepam were administered in the individual pharmaceutical dosage forms commonly marketed in India. The plasma samples containing these drugs were extracted on a maxi-clean C_{18} cartridge. Extraction and elution of all the components was completed in less than 7 min with 0.05 *M* ammonium acetate–methanol (60:40, v/v). The active metabolites of chlorzoxazone and diazepam do not interfere with the assay and can be easily chromatographed with a simple mobile phase.

EXPERIMENTAL

Reagents and materials

Reference standards of acetaminophen, oxyphenbutazone, chlorzoxazone, diazepam, theophylline, 6-hydroxychlorzoxazone and oxazepam were obtained from the Central Drug Laboratory (Calcutta, India). Acetonitrile and methanol (S.D. Fine Chemicals, Boisar, Tarapur, India) were HPLC grade and used without further purification, except for filtration through a 0.45-μm Millipore filter. Ammonium acetate and other reagents used were analytical grade and obtained from E. Merck India (Worli, Bombay, India). Distilled and deionized water prepared in our laboratory was used to prepare reagents.

Drug disposition procedure

Acetaminophen (300 mg) and chlorzoxazone (250 mg) (Parafon, Ethnor, Bombay, India), oxyphenbutazone (200 mg) (Suganril, SG Pharmaceuticals, Baroda, India) and diazepam (5 mg) (Calmpose, Ranbaxy Labs., New Delhi, India) were orally given to two healthy male and two healthy female volunteers (average age 26 years, average weight 55 kg). Blood (10 ml) was withdrawn after 4 h when peak plasma levels had been reached for all the components and placed in bulbs containing oxalate. The plasma was separated immediately and stored frozen until analysis.

Extraction of drugs from human plasma

Plasma (3 ml) and 2 ml of an internal standard solution (500 ng theophylline) prepared in methanol were mixed in a 5-ml glass test-tube containing 1 ml of trichloroacetic acid (10%). The tube was vortex-mixed for 10 s. The spiked plasma was quantitatively transferred with rinses to a 5-ml glass syringe fitted with a C_{18} maxi-clean disposable cartridge (Alltech Assoc., Deerfield, IL, U.S.A.). The cartridge was previously conditioned with 15 ml of 0.05 *M* ammonium acetate–methanol (60:40, v/v) containing 1 ml of 10% trichloroacetic acid for a period of 20 min. A vacuum was applied to the cartridge assembly, which selectively eluted a protein-free extract in 5 min. This was used directly for the chromatographic analysis. Control plasma samples were prepared in the same manner, but without the addition of the internal standard theophylline.

Chromatography

A modular LC system from LDC/Milton Roy (Riviera Beach, FL, U.S.A.) was used. It consisted of a ConstaMetric III dual-piston reciprocating pump, a SpectroMonitor III Model 1204D UV–visible detector and a Spherisorb S5 ODS2 column (250 mm × 4.6 mm I.D., 5 μm particle size). A 7125 Rheodyne valve injector with a 20-μl loop was used. Data acquisition was accomplished with a CI-10 computing integrator from LDC/Milton Roy with a printer–plotter (Sekonics, Tokyo, Japan). The mobile phase was methanol–water (58:42, v/v) at a flow-rate of 1.60 ml/min and an inlet pressure of 103.4 bar. The system was operated at ambient temperature, with UV detection at 280 nm. Reference stock standard solutions were prepared in the mobile phase.

Calibration and reproducibility

Known amounts of acetaminophen, chlorzoxazone, oxyphenbutazone and diazepam in the range 100–700 ng/ml were added to control plasma samples. The samples prepared in the mobile phase were then assayed for each of the components. Calibration curves were constructed by plotting the peak-area ratios between the components and the internal standard, theophylline, versus the amount of individual component added. To check the reproducibility of the analytical procedure two different calibration curves were constructed on two different days using human plasma samples from the same healthy male and female volunteers.

Recovery

The recoveries of acetaminophen, chlorzoxazone, oxyphenbutazone and diazepam were assessed by comparing peak-area ratios obtained from the standard stock solutions of the drugs and control plasma spiked with the respective drugs. The recovery of the added drug was determined at four concentrations:

400, 500, 600 and 700 ng/ml. Each measurement was repeated five times. The detection wavelength was 280 nm.

RESULTS AND DISCUSSION

The major problems encountered in the development of this method involved devising a suitable solid-phase extraction procedure and setting the operating parameters of the chromatographic system so that the drugs and their metabolites would be separated without peak overlap.

The pharmacological responses of chlorzoxazone, diazepam and oxyphenbutazone from samples of plasma were studied. Several organic modifiers, such as methanol, acetonitrile, and tetrahydrofuran, were tested in the mobile phase, together with different buffers and ion-pairing agents. However, a good resolution of acetaminophen and chlorzoxazone, along with oxyphenbutazone and diazepam in the presence of their metabolites, was achieved with a relatively simple mobile phase, methanol–water (58:42, v/v).

Fig. 1 shows a chromatogram of a plasma sample containing chlorzoxazone with acetaminophen, oxyphenbutazone and diazepam. The plasma metabolites of chlorzoxazone (6-hydroxychlorzoxazone) and of diazepam (oxazepam) were also detected at 280 nm and resolved from all other components,

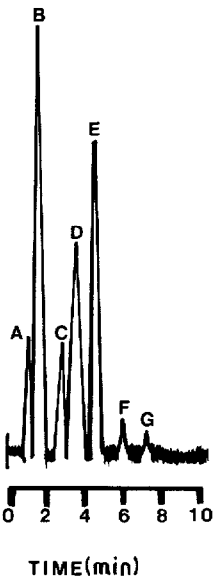

Fig. 1. Chromatogram of human plasma after extraction. Metabolites A and F were identified by comparison with their respective standards under identical chromatographic conditions. Peaks: A = 6-hydroxychlorzoxazone; B = acetaminophen; C = chlorzoxazone; D = oxyphenbutazone; E = theophylline (internal standard); F = oxazepam; G = diazepam.

including the internal standard theophylline. Other expected metabolites of diazepam, including N-desmethyldiazepam (retention time=6.4 min) and temazepam (retention time=6.9 min) did not interfere with the assay, although they were converted into oxazepam (retention time=5.9 min) by demethylation during the extraction process.

No significant peaks were observed after the retention time of diazepam, and the baseline was not affected during the analysis. In an attempt to confirm the homogeneity of the plasma sample and the compatibility of the extraction procedure, a control plasma sample was chromatographed (Fig. 2i). This was compared with a control plasma spiked with acetaminophen (700 ng/ml), chlorzoxazone (1000 ng/ml), oxyphenbutazone (500 ng/ml), theophylline (500 ng/ml) and diazepam (250 ng/ml) as demonstrated in Fig. 2ii. No interference from control plasma was noted.

The calibration curve was linear over the range 100–700 ng/ml (Fig. 3). Each point represents an average of triplicate injections monitored at 280 nm from the control plasma.

Fig. 4 indicates that the limit of detection at a signal-to-noise ratio of at least 5.5 at 280 nm for acetaminophen and chlorzoxazone was ca. 100 and 200 ng/

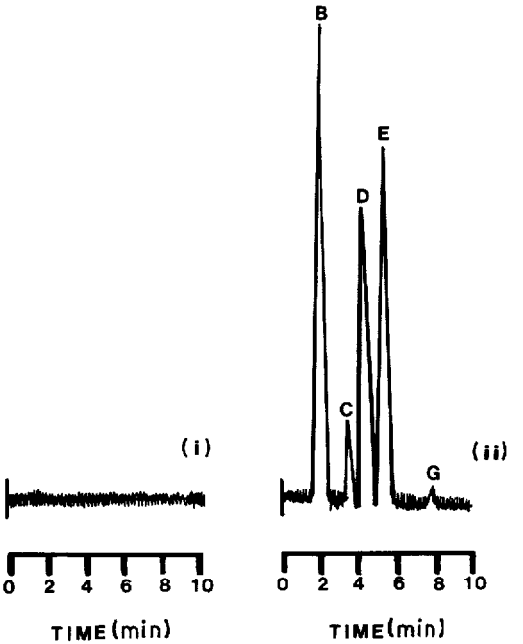

Fig. 2. (i) Chromatogram of control human plasma. (ii) Chromatogram of control human plasma spiked with (B) acetaminophen (700 ng/ml), (C) chlorzoxazone (1000 ng/ml), (D) oxyphenbutazone (500 ng/ml), (E) theophylline (500 ng/ml) and (G) diazepam (250 ng/ml). Detection wavelength, 280 nm.

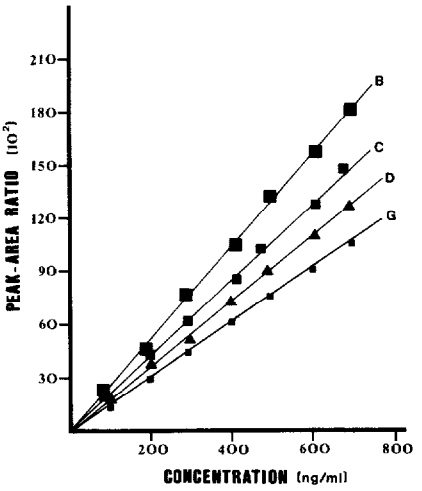

Fig. 3. Calibration curves for human plasma. (B) Acetaminophen; (C) chlorzoxazone; (D) oxyphenbutazone; (G) diazepam. Detection wavelength, 280 nm.

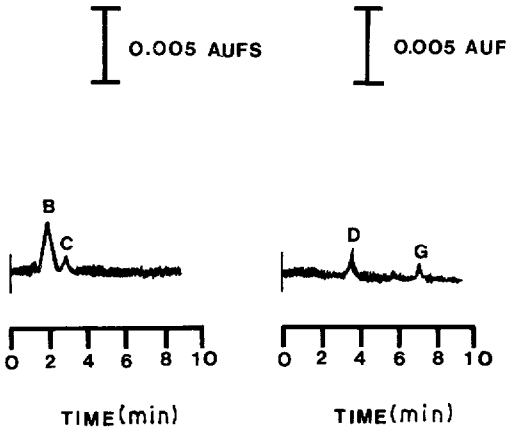

Fig. 4. Limits of detection (0.005 a.u.f.s., 280 nm). (B) Acetaminophen (100 ng/ml); (C) chlorzoxazone (200 ng/ml); (D) oxyphenbutazone (150 ng/ml); (G) diazepam (250 ng/ml).

ml, respectively, that for oxyphenbutazone was 150 ng/ml and that for diazepam 250 ng/ml.

The data in Fig. 5 were obtained from a plasma sample from a healthy male volunteer who received an oral dose containing acetaminophen (300 mg) and chlorzoxazone (250 mg), together with oxyphenbutazone (200 mg) and diazepam (5 mg) in a bioavailability study. These plasma concentration–time profiles were amenable to pharmacokinetic studies and were determined after dosage administration. Recovery studies were performed by adding individual

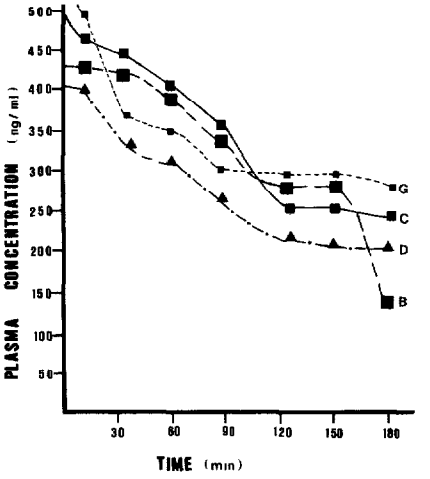

Fig. 5. Plasma concentration-time profile of a human volunteer given an oral dose of 300 mg of acetaminophen (B), 250 mg of chlorzoxazone (C), 200 mg of oxyphenbutazone (D) and 5 mg of diazepam (G).

TABLE I

EXTRACTION FROM HUMAN PLASMA

Detection wavelength 280 nm.

Drug	Concentration added (ng/ml)	Concentration found (ng/ml)	Recovery (mean ± S.D., $n=5$) (%)	C.V. (%)
Acetaminophen	700	710	101.4 ± 1.27	1.2
	600	605	100.8 ± 1.35	1.3
	500	508	100.6 ± 1.38	1.3
	400	415	103.7 ± 1.04	1.0
Oxyphenbutazone	700	700	100.0 ± 0.68	0.6
	600	605	100.8 ± 0.71	0.7
	500	495	99.0 ± 0.74	0.7
	400	400	100.0 ± 0.79	0.7
Chlorzoxazone	700	695	99.2 ± 1.72	1.7
	600	605	100.8 ± 1.91	1.8
	500	493	98.5 ± 1.45	1.4
	400	410	102.5 ± 1.89	1.8
Diazepam	700	695	99.2 ± 0.80	0.8
	600	595	99.1 ± 0.73	0.7
	500	500	100.0 ± 0.91	0 9
	400	399	99.7 ± 0.68	0.6

components to the controls prior to extraction. The results are indicated in Table I.

CONCLUSION

The method is simple and selective and enables the reliable quantitation of all the components studied. If different reagents are used for extraction, the possibility that the metabolites of acetaminophen and diazepam would co-chromatograph cannot be ruled out. All the chromatograms obtained were free from interferences and endogenous components. There was no deterioration of the column packing observed during repeated analysis of plasma samples. This indicates the specificity and compatibility of the solid-phase extraction assay.

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