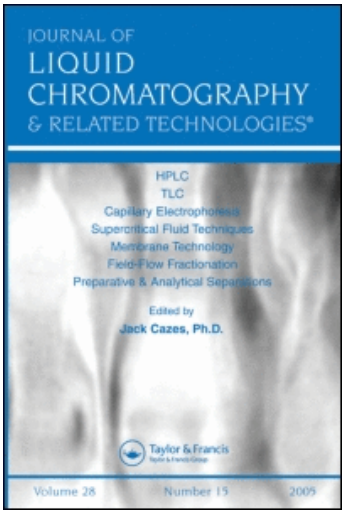

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Determination of Chlormezanone in Plasma and Urine by High Performance Liquid Chromatography

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DETERMINATION OF CHLORMEZANONE IN PLASMA AND URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (HPLC) method with UV detection was developed for quantifying chlormezanone in plasma and urine. An extraction step was needed to avoid endogenous interferences especially in urines and led to an average recovery of 90%. The sensitivity limit was 20 ng/ml in both plasma and urine. The method was reproducible with intra- and inter-assay coefficients of variations below 5%. This method was applied to the determination of plasma and urine levels during a pharmacokinetic study in the elderly. It was found suitable to follow the concentrations as long as 120 h after a single oral administration of 400 mg chlormezanone.

INTRODUCTION

Chlormezanone, 2-(4-chlorophenyl)-4-metathiazanone -1-dioxide(CM) has been widely used since 30 years in

various musculoskeletal disorders. CM has been reported to releive muscle spasm, and secondarily pain by depressing spinal and supra-spinal polysynaptic pathways. Until McChesney's study(1) little has been known in the field of metabolism(2-3) or pharmacokinetic(4-5). The aim of this study was to provide an analytical method sensitive and specific enough to follow in humans the plasma and urine levels of CM after a single oral administration of 400 mg CM in capsule. Previous analytical methods based upon decomposition of CM into 4-chlorobenzaldehyde(1) did not distinguish CM from possible metabolites. An HPLC technique with enhanced sensitivity was described by Dixon and Furey(6) and was recently applied to plasma determinations(5) but in our hands it was found insufficiently specific for urine medium and was not able to reveal eventual metabolites. The present report describes an HPLC technique with improved sensitivity and specificity for the simultaneous determination of CM in plasma and urine.

MATERIALS AND METHODS

Chemicals

CM was supplied by Winthrop Laboratories. Internal standard (EI) 3,5-dichlorobenzoic acid was obtained from Merck, Darmstadt, RFA. Diethylether, acetonitrile and methanol were purchased from Carlo Erba, Milano, Italy. Phosphoric and hydrochloric acids and potassium dihydrogenphosphate were purchased from Merck. Sodium heptane sulfonate was purchased from Fluka, Buchs, Switzerland. All chemicals were of analytical grade. Standard solutions were prepared by dissolving 10 mg of each compound in methanol and stored at 4°C in darkness. These solutions were used (100 or 50 µl) for spiking the plasma and urine standards and the quality controls.

Chromatographic Conditions

Reversed phase HPLC was performed at 22°C (air conditioned) using a Kontron 810 pump (Kontron, Zürich, Switzerland), a Model 7125 injector (Rheodine, Cotati, USA) with a 100 µl loop and a Nucleosil C18 column (250 x 4.6 mm, 10µm particle size, Macherey-Nägel, Düren, RFA). The absorbance of the eluent was monitored by use of a model Uvikon 720 LC, (Kontron) spectrophotometric detector set at 228 nm and equipped with a 8 µl flow cell. The absorbance was recorded on a model W+W 600 Tarkan recorder (W+W Electronic INC, Basel, Switzerland), 1000 mV full scale.

Optimal separation of compounds was obtained using a mobile phase consisting of acetonitrile, phosphate buffer 0.05 M (80/20, v/v) and sodium heptane sulfonate 1g/l as counterion, in which the pH of aqueous phase was adjusted at pH = 4.3 with concentrated phosphoric acid.

Extraction Procedure

A 1ml biological sample (urine or plasma) was introduced in a glass centrifuge tube containing 100 µl of internal standard (100 µg/ml), 2 ml hydrochloric acid (1N) and 8 ml diethyl ether. The tube was shaken on a rotating agitator for 15 min, then centrifuged at 1200 g for 15 min. The organic layer was transferred to another conical tube and evaporated until dryness under a stream of nitrogen at 37°C. The residue was dissolved in 1 ml mobile phase and 100 µl injected onto the chromatographic column.

Calculations

Standard curves were determined from peak height ratios obtained with biological samples supplemented for

CM in concentrations varying from 0.1 to 10 µg/ml and constant concentration of internal standard (10 µg/ml).

During routine analysis, two standards of plasma and urine extemporaneously spiked with CM and EI (10 µg each) were used to calculate unknown concentrations. Two quality controls of plasma and urine spiked with 5 µg/ml of CM at the beginning of the study and stored frozen in the same conditions as experimental clinical samples were introduced in each running batch to check the drift during long-term utilisation.

RESULTS AND DISCUSSION

The present technique is adapted from that proposed by Dixon and Furey(6). Several modifications were made to improve its specificity and sensitivity. Indeed the method set up by these authors could be considered satisfactory when applied to the plasma of healthy volunteers(5). However the chromatographic conditions proposed led to retention times too short (less than 6 minutes) to allow a good separation from endogenous interferences especially in urine or even in plasma. Moreover, elderly volunteers included in pharmacokinetic studies usually receive several comedications and exhibit reduced renal functions. Therefore exogenous and endogenous substances are at higher levels in plasma and in urine of elderly than in healthy volunteers. Finally as the presence of metabolites could be expected (1-2-3) the separation appeared largely insufficient. In addition current kinetic studies require long time follow-up to ascertain the terminal part of the curve when concentrations are near the minimum quantity level. For these reasons specificity and sensitivity were enhanced by modification of mobile phase and of the extraction procedure.

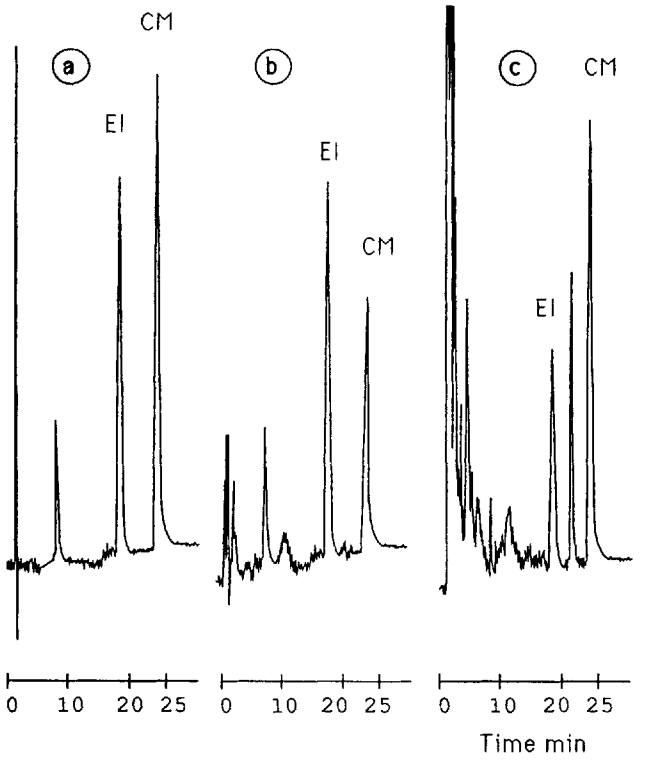

FIGURE 1. Chromatograms obtained with CM and EI in methanolic solutions (a) and in plasma (b) or urine extracts (c) from patients 4 hours after oral administration of a 400 mg dose of CM. (Full scale of the ordinate is 0.2 absorbance units).

Chromatographic Conditions

Retention times of CM and metabolites were increased by the following modifications of mobile phase composition : stabilization of pH of aqueous phase by addition of buffer, improvement of sharpness of peaks by addition of a counterion and by replacement of methanol by acetonitrile. Consequently, internal standard retention time was out of range (> 60 min) and

TABLE 1

Chromatographic Characteristics of a Mixture of Chlormezanone (5 µg/ml) and Internal Standard (10 µg/ml) in Mobile Phase.

Parameters	Compound	
	Chlormezanone	Internal standard
Retention time (min)	19	23
Capacity factor (k')	8.5	10.5
Theoretical plates (m^{-1})	14220	16226
Height of a plate (µm)	70.3	61.6
Asymmetry factor	1.03	1.01
Resolution factor	1.70	

another compound had to be proposed with retention time closer to that of chlormezanone. Among numerous compounds, 3,5-dichloro benzoic acid appeared the most suitable. Figure 1 shows a typical chromatogram and table 1 the parameters obtained with final chromatographic conditions.

Extraction

The extraction procedure proposed for plasma samples was not suitable for the urine medium, moreover the new internal standard was not extracted by the same process. In order to keep an homogeneous system for both plasma and urine a new procedure was developed using a one-step diethylether extraction in acidic medium. The procedure was highly efficient (see table 2).

Characteristics of the Method

The linearity of the method was verified in both plasma and urine spiked with CM in the range 0.1 to 10

Table 2

Overall Recovery Expressed as Percentage (x ± sem, n = 10) in Plasma and Urine.

	Chlormezanone		Internal standard	
	1 µg/ml	10 µg/ml	1 µg/ml	10 µg/ml
Plasma	90.1 ±1.4	97.5 ±0.9	93.1 ±1.7	97.3 ±1.3
Urine	88.9 ±3.1	93.7 ±2.5	87.8 ±3.1	95.0 ±1.6

µg/ml. The calibration curves between the peak height ratios CM/EI and CM concentrations obtained for plasma were : $y = 0.157 x + 0.005$, $r^2 = 0.998$ and for urine : $y = 0.156 x + 0.007$, $r^2 = 0.989$. In both cases the very low intercept attests the purity of measured peaks.

The intra-assay coefficients of variation determined over 10 samples at the concentration of 1 µg/ml and 10 µg/ml were found respectively at 4.8% and 0.7% in plasma and at 2.0% and 2.1% in urine.

The day-to-day coefficients of variations (over 10 days) were 4.1% and 2.5% in plasma, 5.0% and 3.7% in urine at 1 µg/ml and 10 µg/ml respectively.

Plasma and urine quality controls stored at -20°C for up to 9 months showed no signs of decomposition suggesting that CM is stable under these storage conditions. However the recovery of CM was constantly estimated by default until it was first modified by freezing process. The spiked concentration (5 µg/ml) was found stable at 4.67 ± 0.06 µg/ml in plasma and 4.39 ± 0.04 µg/ml in urine.

Using the signal to noise ratio of 2 as a criterion, the detection limit was 0.02 µg/ml in both plasma and urine samples.

This method was successfully applied to the determination of CM levels in plasma and urine of healthy and elderly volunteers after a single oral administration of 400 mg of CM. Neither endogenous nor exogenous compounds finally interfered with the detection of CM in plasma or urine even in patients. No metabolites could be identified contrarily to previous findings(1-3) and unchanged CM was the main compound observed in plasma as well as in urine of healthy volunteers and patients. These results are in accordance with more recent pharmacokinetic studies(4-5) involving a large number of subjects.

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