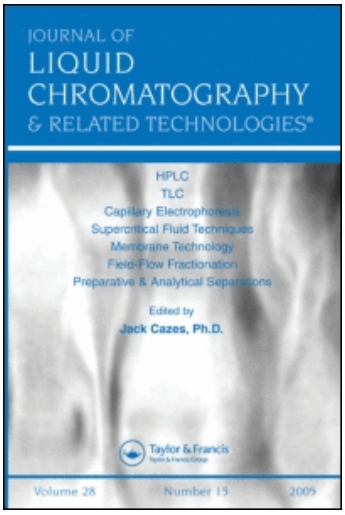

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Simultaneous Determination of Clobutinol Together with Some Anti-inflammatory Drugs in Urine by HPLC

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ABSTRACT

An isocratic high performance liquid chromatography method is described for simultaneous determination of clobutinol hydrochloride together with some anti-inflammatory drugs, such as diclofenac, meloxicam, and nimesulide in urine. For the development and optimization of the system, three different buffers containing ammonium acetate, tetraethylammonium hydrogen sulfate (THAS), and tetrabutylammonium hydrogen sulfate (THBS) were investigated, because it has been proven that different salts added in the mobile phases considerably affect solute retention and selectivity. The effect of salt content in the aqueous portion

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of the mobile phase together with pH over a wide range, was investigated. A response surface method based on non-linear multiple regression analysis was employed to illustrate the changes in k' values as a function of a range of pH values and different salts contents. On the basis of the chromatographic behavior of clobutinol together with the other drugs determined, optimum chromatographic conditions were achieved with good peak symmetry, reasonable retention time, and noticeable separation. The intra- and inter-day accuracy and precision at low, medium, and high concentration(s) for the compounds were in the range %error 5.40–11.50 and %RSD 1.76–5.06, respectively.

Key Words: Clobutinol; Diclofenac; Meloxicam; Tolmetin; Nimesulide; Non-steroidal anti-inflammatory drugs; RP-HPLC; Body fluids.

INTRODUCTION

Clobutinol hydrochloride *p*-chloro-α-[2-(dimethylamino)-1-methylethyl]-α-methylphenethyl alcohol[1–3] is widely used as a centrally acting cough suppressant. Although, different formulations of the compound are available in the free-market, it is not described officially in any Pharmacopoeia. The drug can be co-administered with some widely used anti-inflammatory drugs[1–3] (Diclofenac Sodium—Voltaren®, Nimesulide—Mesulid®, Meloxicam—Movatec®, and Tolmetin—Tolectin®). A literature survey reveals that the number of the reported methods referring to clobutinol[4,5] is rather limited. The compound has been determined in biological fluids by gas chromatography combined with surface ionisation detection.[4] Recently, a comparative study was carried out between a chromatographic assay and a derivative spectrophotometric method for the determination of the compound in different pharmaceutical formulations.[5] However, the already described HPLC procedure proved unsuitable for the simultaneous determination of clobutinol hydrochloride together with different anti-inflammatory drugs, which possibly co-exist in biological fluids. On the other hand, different analytical methods have been described to quantify the individual anti-inflammatory drugs in various matrixes, but the methods are inadequate for their simultaneous determination.[6–23]

The presented work includes an extensive investigation of the presence of different salts in the mobile phase that led to optimized chromatographic conditions. The developed analytical method was applied for separation and quantitation of clobutinol hydrochloride together with the anti-inflammatory drugs.

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EXPERIMENTAL

Reagents and Chemicals

Clobutinol hydrochloride, meloxicam, and nimesulide were kindly donated by Boehringer Ingelheim International GmbH. Diclofenac sodium salt, tolmetin sodium salt dehydrate, tetraethylammonium hydrogen sulfate (THAS), tetrabutylammonium hydrogen sulfate (THBS), and ammonium acetate were purchased from Sigma Chemicals Co. The molecular formulae of the analytes are shown in Fig. 1. Also, HPLC-grade methanol,

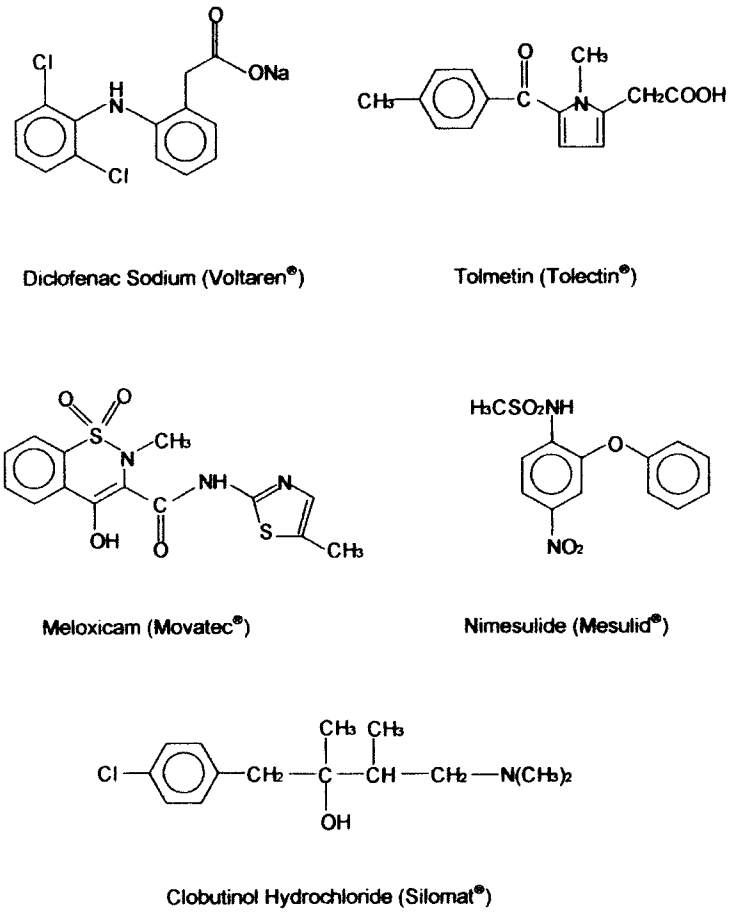

Figure 1. Chemical structures of the compounds investigated.

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acetonitrile, and water were bought from Riedel-de Haën, Germany. The extraction buffer consisted of 0.2550 g Na_2HPO_4 and 0.1505 g KH_2PO_4 dissolved in 500 mL HPLC grade water, pH 7. All chemicals were of analytical grade and used as received.

Instrumentation

The HPLC apparatus consisted of a Shimadzu Series LC-6A, a SPD-6AV UV spectrophotometric detector, controlled by a SCL-6B system programmed module, and was operated at 258 nm. Chromatograms were recorded on chart paper with a Shimadzu Chromatopac Model C-R6A thermal printer-plotter, at a speed of 2 mm min^{-1}. The samples were analyzed using two different reversed phase columns equipped with a C-18 guard column: (a) Rosil C-18, 5 μm, 150 × 4.6 mm i.d.; (b) Lichrosorb RP-18, 5 μm, 250 × 4.6 mm i.d. They were placed in a model CTO-6A column oven (40°C) and equipped with a Rheodyne 7161 injector fitted with a 20 μL loop. The mobile phase was delivered to the columns isocratically.

Chromatographic Conditions

Three chromatographic systems were developed, the mobile phase of which consisted of a mixture of aqueous solution ammonium acetate or THAS or THBS, methanol, and acetonitrile, 40 : 30 : 30, v/v/v. The pH of the aqueous mobile phase portion of ammonium acetate buffer was adjusted with glacial acetic acid, whereas, the other buffers with ammonium hydroxide, pH 4.33. The mobile phase was filtered, degassed, and pumped isocratically at a flow rate of 0.6 mL min^{-1}.

Preparation of Standard Solution

Stock solutions were prepared by accurately weighing the appropriate amounts of clobutinol hydrochloride and other analytes. Then they were dissolved in methanol in 50 mL different volumetric flasks to prepare standard stock solutions. Working standard solutions of all drugs were prepared from the stock solutions by sequential dilutions with mobile phase, to give concentrations in the range as indicated in Table 1. A standard graph for each component was prepared by plotting concentration(s) vs. peak height(s). Quantification was carried out from peak heights of the samples and corresponding standard graphs.

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Table 1. Concentration range, linear regression and correlation data of calibration curves for the compounds (standards) determined at 258 nm, using column (a) Rosil C-18, 5 μm, 150 × 4.6 mm i.d.

Compound	Concentration range (μg/mL)	t_R (min)	k'	Slope	Intercept	r	LOD (μg/mL)	LOQ (μg/mL)
Clobutinol	60.00–378.00	9.077	2.549	0.243 ± 0.004	0.225 ± 1.075	0.9993	14.39	47.96
Diclofenac	0.51–1.53	8.182	2.211	52.311 ± 0.846	3.571 ± 0.909	0.9993	0.04	0.15
Meloxicam	0.36–2.49	3.497	0.367	39.182 ± 0.753	5.893 ± 1.198	0.9991	0.11	0.36
Nimesulide	3.15–22.10	6.963	1.682	3.906 ± 0.021	2.857 ± 0.301	0.9999	0.27	0.91
Tolmetin	0.07–0.49	3.995	0.568	74.745 ± 0.782	0.286 ± 0.245	0.9997	—	—

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Extraction and Clean-up Procedure

Both extraction and clean-up steps were carried out in 15 mL screw-capped centrifuge tubes, pretreated with 5% (v/v) dichlorodimethylsilane in toluene, in order to minimize drug adsorption. Spiked urine samples (2 mL) were transferred into the centrifuge tubes and 6 mL of acetone were added for protein and other endogenous component precipitation. The mixture was vortexed for 10 sec and centrifuged at 3000 g for 10–15 min. Then, the supernatant clear liquid layer was transferred into another tube, leaving behind the protein matrix, whereas, the acetone was eliminated in a stream of nitrogen at 40°C. Extraction buffer (0.5 mL) and drops of 4 M NaOH were added, vortexed, and extracted with an organic solvent (2 mL × 3). The choice of extraction conditions is frequently based on compromise between extraction yield and selectivity of extraction. In the preliminary studies, three extraction solvents were chosen: *n*-pentane, chloroform, and toluene. Toluene afforded cleaner and rather higher yield of drug extracts from spiked urine samples, hence, it was used to further experiments. In the last step of the extraction procedure, the upper organic layers were transferred into another tube and evaporated to dryness at 40°C under a stream of nitrogen for almost 20 min. The residues were dissolved by the addition of 2 mL of mobile phase and an aliquot of 20 μL was injected onto the HPLC column(s) for analysis. The described extraction procedure gave a high yield of clobutinol, diclofenac, meloxicam, and nimesulide (~90%). On the contrary, the extraction results for tolmetin were extremely low.

Assay Validation

The calibration curves of the working standards showed good linearity in the range indicated in Table 1 for clobutinol, diclofenac, tolmetin, meloxicam, and nimesulide. The correlation coefficients (r) of calibration curves of each drug were higher than 0.999. The limit of detection ($\mu g\,mL^{-1}$) and the limit of quantitation ($\mu g\,mL^{-1}$) were estimated from the standard solution regression line, using the equation $y = bx + a$ where $y = a + 3s_{y/x}$ or $y = a + 10s_{y/x}$, a is the intercept, b is the slope, and $s_{y/x}$ is the residual standard deviation (standard deviation about regression).[24,25] In standard spiked urine samples, the LOD for each analyte was demonstrated by a gradual decrease in concentration, at which the signal-to-noise ratio was about 3. Therefore, the LOD ($ng\,mL^{-1}$) for clobutinol was 16350.1 $ng\,mL^{-1}$, diclofenac 44.7 $ng\,mL^{-1}$, meloxicam 123.2 $ng\,mL^{-1}$, and nimesulide 307.5 $ng\,mL^{-1}$.

The efficiency of the extraction procedure of clobutinol (%total recovery) was determined by plotting "added" amount (from six standard spiked urine

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samples, concentration range 63.0–378.1 $\mu g\,mL^{-1}$) vs. "found". The slope value × 100 indicate the %total recovery, 90.70 ± 1.34%. The intra-day (repeatability) accuracy and precision of the assay were determined by assaying three spiked urine samples at low (60, 2, 2, 5 $\mu g\,mL^{-1}$), medium (150, 10, 10, 15 $\mu g\,mL^{-1}$), and high (200, 20, 20, 30 $\mu g\,mL^{-1}$) concentrations for clobutinol, diclofenac, meloxicam, and nimesulide, respectively, in three analytical runs within the same day. For inter-day (reproducibility) accuracy and precision, urine samples were analyzed on three different days (Table 2).[26,27] The stability of all five analytes present in standard and spiked urine standard extracts was assessed by injected samples at spaced intervals over a 24 hr period.

RESULTS AND DISCUSSION

The goal of the present study was to develop an isocratic HPLC assay for the determination of clobutinol together with some compounds with widely different physical and chemical properties. Initial attempts to develop a reversed phase chromatographic system to separate clobutinol from some anti-inflammatory drugs based on methanol–acetonitrile–phosphate buffer as mobile phase, yielded less satisfactory chromatograms. The ammonium acetate buffer was easily miscible with different organic solvents and improved peak symmetry of clobutinol hydrochloride, but proved insufficient to separate the compound from other anti-inflammatory drugs. THAS and THBS are ion pair reagents for acidic compounds. In the present work, when these two salts were employed as buffers, we were led to two valuable chromatographic systems of which solute retention and selectivity were noticeably affected. These were proved suitable for the simultaneous determination of the compounds mainly in biological fluids. In an attempt to access the chromatographic behavior of clobutinol hydrochloride, the relationship between capacity factor, salt content in the aqueous portion of the mobile phase, pH, and solute properties, were investigated. It is well known that basic ingredients are difficult to elute and determine in their un-ionized form. On the contrary, at low pH, the proportion of protonated species is increased and, as a consequence, the charged molecules were distributed preferentially into the aqueous phase or more polar phases. In the case of clobutinol hydrochloride, low pH values considerably affect the chromatographic behavior of the compounds. Moreover, different salts added in mobile phases affect solute retention and selectivity, considerably. Figure 2 illustrates predicted retention behavior of clobutinol as functions of pH, and different concentrations of salt content in mobile phase by employing non linear regression analysis. Experiments showed that the effective amounts of

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Table 2. The intra- and inter-day precision (%RSD) and accuracy (%error) of clobutinol, diclofenac, meloxicam, and nimesulide in human urine.

		Intra-day ($n = 3$)			Inter-day ($n = 9$)		
Analyte	Concentration added (μg/mL)	Concentration found (μg/mL)	RSD (%)	Error (%)	Concentration found (μg/mL)	RSD (%)	Error (%)
Clobutinol	60	54.18	4.88	9.70	55.08	3.95	8.20
	150	136.80	3.12	8.80	134.61	3.28	10.26
	200	184.20	2.76	7.90	187.04	3.17	6.48
Diclofenac	2	1.79	5.06	10.50	1.77	5.01	11.50
	10	9.08	3.28	9.20	9.12	3.12	8.80
	20	18.54	2.41	7.30	18.92	2.54	5.40
Meloxicam	2	1.85	3.16	7.50	1.83	3.64	8.50
	10	9.34	2.11	6.50	9.70	2.86	3.00
	20	18.75	1.96	6.25	18.24	2.44	8.80
Nimesulide	5	4.66	2.14	6.80	4.62	2.54	7.60
	15	14.16	1.76	5.60	13.96	1.91	6.93
	30	28.31	1.98	5.60	27.97	2.37	7.77

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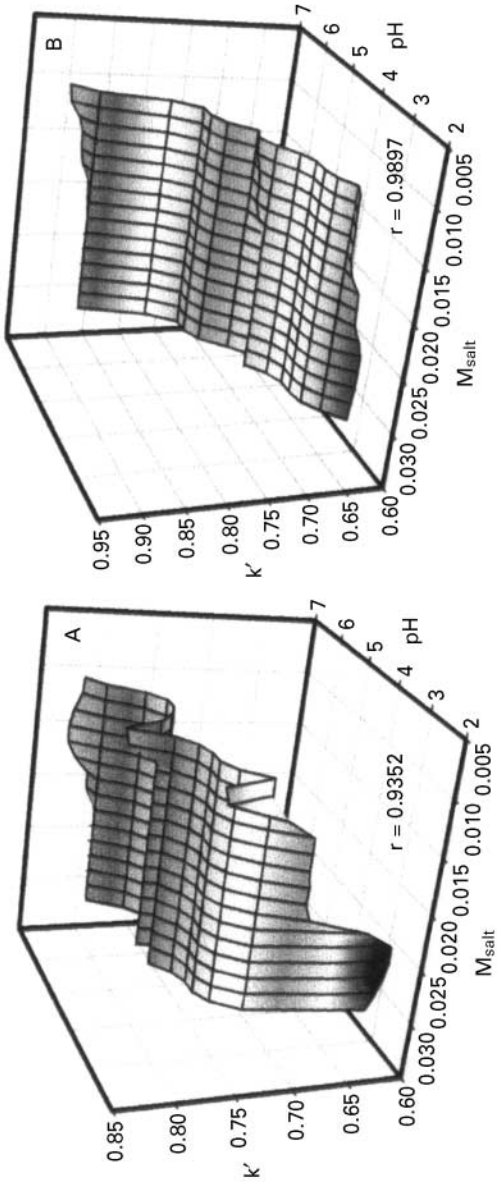

Figure 2. Predicted retention behavior of clobutinol hydrochloride based on response surface models as functions of pH and different concentrations of salt content (A) THBS and (B) THAS in mobile phase by employing non-linear regression analysis.

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THAS in the mobile phase were from 30×10^{-3} to -5×10^{-3} M. It should be clarified that the response surface model is valid (accurate enough) only for the experimental domain region where the work was carried out. Moreover, it was observed that smoother response surface model(s) reflect more stable chromatographic systems. On the basis of the chromatographic behavior of the analytes, optimum conditions were found with good peak symmetry and reasonable retention time (for clobutinol, column (a) t_R 8.75–9.07, column (b) t_R 13.88–14.16). Typical chromatograms of clobutinol and other analytes are illustrated in Fig. 3. Therefore, baseline separation among clobutinol and other analytes or biological compounds was achieved. For the peaks shown, R_s

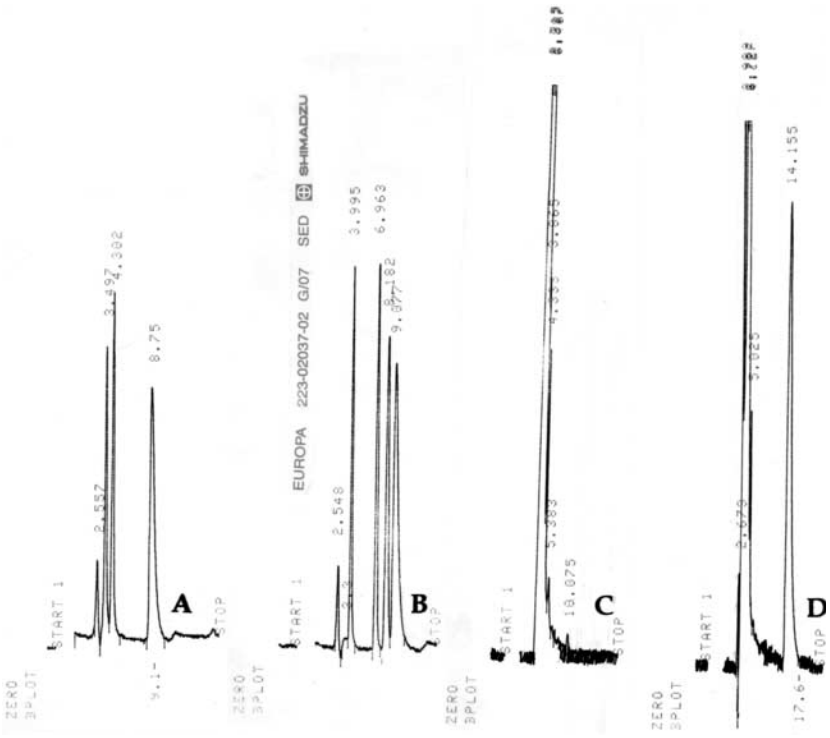

Figure 3. Typical chromatograms of (A) clobutinol (t_R 8.75, 137.89 μg mL^{-1}), meloxicam (t_R 3.49, 4.26 μg mL^{-1}), tolmetin (t_R 4.30, 1.76 μg mL^{-1}); (B) clobutinol (t_R 9.07, 137.89 μg mL^{-1}), nimesulide (t_R 6.96, 3.15 μg mL^{-1}), tolmetin (t_R 3.99, 1.76 μg mL^{-1}), diclofenac (t_R 8.18, 1.36 μg mL^{-1}) standards in mobile phase consisting of aqueous solution of THAS (0.015 M, pH 4.33)–CH_3CN–CH_3OH, 40 : 30 : 30 v/v/v, column (a) $\lambda = 258$ nm, flow rate 0.6 mL min^{-1}; (C) blank human urine extract; (D) human urine spiked with 250.55 μg mL^{-1} of clobutinol [column (b)].

values were >1 signifying complete separation. The chosen chromatographic conditions were methanol–acetonitrile–aqueous solution of THAS, 0.015 M, pH 4.33, 30 : 30 : 40 v/v/v, flow rate 0.6 mL min^{-1}, $\lambda = 258$ nm. The retention time(s) of clobutinol, together with other analytes (anti-inflammatory drugs), were found to be reproducible under the selected chromatographic conditions. Some chromatographic parameters, together with concentration range, linear regression, and correlation data of calibration curves of the compound are presented in Table 1. In addition, the primary objectives in the development of the extraction method were to minimize interfering endogenous sample components, while at the same time providing reasonable recoveries of the analytes. Liquid–liquid extraction, combined with several protein and other compounds precipitation techniques, were evaluated and proved adequate in the removal of endogenous sample components. From a series of extraction solvents, toluene afforded cleaner and higher yield extracts for the compounds investigated (~90%). Moreover, the intra- and inter-day accuracy and precision at low, medium, and high concentration(s) for the compounds were in the range %error 5.40–11.50 and %RSD 1.76–5.06, respectively.

CONCLUSION

A sensitive and efficient method for the extraction and simultaneous determination of clobutinol together with some anti-inflammatory drugs in urine, which are administered simultaneously according to medical prescriptions has been developed and validated. The described method yields high recoveries, good linearity, precision, and accuracy. The liquid–liquid extraction procedure proved an excellent means of sample clean-up. Thus, the method can be applicable for pharmacokinetic studies after single or multiple doses of each drug.

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